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International Plant Protection Convention
Protecting the world's plant resources from pests

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IPPC Secretariat

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1. Opening of the Session

1.1 FAO Opening

[1] The FAO Assistant Director-General and the FAO Regional Representative for Asia and the Pacific, Ms Kundhavi Kadiresan, welcomed delegates to the 12th Session of the Commission on Phytosanitary Measures (CPM) and conveyed appreciation to the government of the Republic of Korea for hosting the session of the CPM, noting that it was the first time it was held outside FAO headquarters. Ms Kundhavi Kadiresan noted the impact the movement of sea containers had on the spread of pests and the need for measures to prevent and respond to such disasters, highlighting the ongoing innovation and cooperation between Members and FAO in this field. She reiterated the importance of the work of the International Plant Protection Convention (IPPC) and its contribution to the UN Sustainable Development Goals (SDGs).

1.2 Republic of Korea Opening

[2] The Minister for Agriculture of the Republic of Korea welcomed participants to Korea and to the 12th Session of the CPM and congratulated the IPPC on the 65th Anniversary since its establishment. The Minister pledged his support to calls for increased efforts for plant protection in the ever-increasing agricultural trade leading to increased risk of migrating pests. He acknowledged the IPPC's contribution in the development and implementation of international standards aimed at facilitating trade and protecting plants and reiterated the Republic of Korea's commitment to supporting the work of the IPPC.

[3] The Mayor of Incheon Metropolitan City also welcomed Members and participants attending the CPM.

2. Keynote Address on Plant Health and Trade Facilitation

[4] Dr. Kunio Mikuriya, Secretary General of the World Customs Organization (WCO) delivered the keynote address on Plant Health and Trade Facilitation, outlining the role of Customs in facilitating global trade and inviting IPPC members to engage with WCO members, seeking synergies for collaboration at national entry points, with the possibility of assistance for support to Phytosanitary Services as needed.

3. Adoption of the Agenda

Agenda

[5] The CPM Chairperson, Ms Lois Ransom (Australia), gave special thanks to the Government of Korea for hosting the CPM for the first time outside of Rome, Italy. The Chairperson acknowledged the hard work that went into the preparation for the CPM, amongst others, the efforts by the Animal and Plant Quarantine Agency and its staff.

[6] The Chairperson detailed changes to the provisional agenda[1] and the order in which items would be addressed. The list of participants is presented in Appendix 03.

[7] The CPM:

(1) *Adopted* the Agenda without change and noted the List of Documents. (See Appendix 01 and 02)

3.1 EU Statement of Competence

[8] The CPM:

(1) *Noted* the Declaration of Competences and Voting Rights submitted by the European Union (EU) and its 28 member states[2].

[1] CPM 2017/02/Rev_01

[2] CPM 2017/INF/17

4. Election of the Rapporteur

[9] The CPM:

(1) *Elected* Ms. Jane Chard (United Kingdom) as rapporteur.

5. Establishment of the Credentials Committee

[10] The CPM:

(1) *Appointed* a Credentials Committee composed of seven members, one per FAO region and one CPM Bureau member, in conformity with FAO rules.

(2) *Elected* Ms Reem Barakat (Canada) as its Chairperson. The Credentials Committee endorsed a list of 113 valid credentials and set the quorum for the Commission at 92.

6. Report by the Chairperson of the Commission on Phytosanitary Measures

[11] The CPM took note of the Report, presented by the Chairperson[3] and noted the need for the CPM to take decisions that enabled and supported the IPPC Secretariat's core functions and planned activities as outlined in the report. The CPM further noted that the IPPC Secretariat's annual themes had significantly raised the profile and awareness of the IPPC globally. The Commission took note of the Chairperson's appreciation to the IPPC Secretariat for its commitment and dedication throughout the year, and noted the unprecedented number of Standards being presented to the CPM for adoption.

7. Report by the IPPC Secretariat

[12] The CPM took note of the 2016 Annual Report of the IPPC Secretariat, presented by the Secretary, Mr Jingyuan Xia[4], outlining the ten major highlights achieved by the Secretariat over the past year and the challenges and goals going forward (Appendix 04). The CPM took note of the appreciation to the IPPC Governing Bodies, including Regional Plant Protection Organisations (RPPOs) and National Plant Protection Organisations (NPPOs), and to all partners and collaborators globally for their support and collaboration.

8. Governance

8.1 Summary of the Strategic Planning Group report

[13] The CPM took note of the report[5] presented by the Chairperson of the Strategic Planning Group (SPG), Mr Javier Trujillo (Mexico), highlighting that the meeting marked the official launch of the development of the IPPC Strategic Plan 2020 - 2030, and that significant steps were taken at this meeting towards building momentum towards the proposed International Year of Plant Health (IYPH) in 2020. Support was emphasized for strong linkages between IPPC programmes and IPPC related topics. He further noted that five priority initiatives were identified as part of its strategic objectives and that there were several important issues, including the need to develop a sustainable funding mechanism for the IPPC Secretariat to deal with plant health emergencies.

[14] The CPM:

(1) *Took note of* the report.

8.2 Strategic Framework for 2020-2030

[15] The draft Strategic Framework (SF) for 2020-2030, drafted by Mr Peter Thompson (New Zealand). and Mr Ralf Lopian (Finland) was presented by one of the authors[6]. The CPM discussed the document during the

[3] CPM2017/40

[4] CPM 2017/33

[5] CPM 2017/39

[6] CPM 2017/24

meeting and agreed to take some more time to discuss this topic and to have an evening session to allow for a more detailed exchange of views on the various parts of the framework.

[16] During the evening session, it was generally agreed that the objectives of the SF should be closely tied to the UN SDGs. Issues raised included, the scope (a framework for the global plant health community), taking into account that the operational landscape will be different in 2030, and capturing more explicitly issues such as climate change. Other aspects that were deemed necessary to refine for the SF included, the Mission and Vision, the intended audience, how to define and measure success, and accountability for implementing the plan.

[17] It was emphasized that the SF should have three levels of information: a one page summary for the general audience; a second, more detailed section; and thirdly, an operational plan.

[18] A new draft will be produced for discussion and presented at the next SPG meeting in October 2017 and at CPM-13.

[19] The Chairperson encouraged Contracting Parties (CPs) to continue to provide comments to the authors, particularly on the development agenda.

[20] The CPM:

(1) *Provided* comments to the proposed high level structure content of the SF 2020-2030, with particular emphasis on the Vision, Mission and Strategic Objectives;

(2) *Provided* comments to the proposed IPPC Development Agenda 2020-2030, as an integral part of the SF

8.3 Sustainable Funding

[21] The Secretariat introduced the paper on sustainable funding[7]. With regard to the funding proposal, in October 2016 the SPG gave support for two options of sustainable funding of the IPPC Secretariat and its core activities: a "Voluntary Assessed Contribution Agreement" (VACA) system and a "Pay-As-You-Go" system.

[22] Some CPs expressed concern that this would represent an additional cost burden to them, in addition to the resources already provided through national allocations to the FAO Regular Programme.

[23] Additional CPs requested the CPM Bureau and its Financial Committee, and the SPG, to undertake a more in-depth analysis of these options and develop detailed provisions and understanding for the CPM of the mechanisms and how they could be implemented.

[24] Other CPs believed that resources were not adequate to support the additional roles the Secretariat had been asked to take on in recent years, in addition to the traditional standard setting activities, noting that the implementation of the Convention has gained increased priority, while the resources available for those activities have primarily been received through competing for project funding.

[25] CPs generally agreed that the need for long-term sustainable and "predictable" funding was important for the IPPC Work Programme and welcomed the initiatives taken to secure same.

[26] The CPM:

(1) *Agreed* to seek further development of a mechanism to secure sustainable funding, including a possible "Voluntary Assessed Contribution Agreement" (VACA) system and a "Pay-As-You-Go" system as components of a proposal for sustainable funding to be made at CPM-15 in 2020;

(2) *Requested* the CPM Bureau and its Financial Committee, as well as the SPG, to develop detailed provisions for such a sustainable funding proposal during 2017;

(3) *Called* for a Progress Report on the sustainable funding proposal to be presented to CPM-13 (2018), and

[7] CPM 2017/26

(4) *Encouraged* CPs in the interim period to commit extra-budgetary resources to the IPPC Work Programme.

8.4 Emerging Issues

[27] The Secretariat introduced the paper[8] on emerging issues, noting that requests were regularly received for advice on pest outbreaks. The CPM noted the importance of responding promptly to such requests through mechanisms that can provide relevant information to immediately support emergency activities. The CPM further noted that it should establish mechanisms to deal with emerging issues within the short term, but that a principal decision on this matter should fall within the boundaries of the Strategic Framework 2020-2030 and the Ministerial Meeting of the CPM planned for 2020. In the short term, the IPPC Secretariat would assist actions on emerging issues through expanding information collection and sharing to assist CPs to plan, undertake and report actions and outcomes on more than surveillance.

[28] CPs indicated to the CPM that extra-budgetary funding models be put in place. CPs noted that RPPOs play a role in policy issues and coordination for such activities. CPs further highlighted that there was a need to ensure that there was no duplication with other FAO programmes and activities. The CPM also noted the suggestion that the SPG take up the issue based on the Bureau's discussion.

[29] The CPM:

(1) *supported* the proposed short term approach,

(2) *requested* the Bureau dedicate an appropriate portion of the June meeting to establish priority ranking, as well as criteria and/or rules for this effort in the budget and work plan for the Secretariat.

8.5 Strategic Partnerships

[30] The Secretariat introduced the paper on strategic partnerships[9], noting that private sector representatives with a significant interest in phytosanitary issues, especially with regard to protecting the world's plant resources from pests, could be an untapped and potentially significant resource. The paper outlined the possibilities for working with private sector representatives which were consistent with the objectives of the IPPC as set out in the SF, subject to pertinent criteria. The paper further outlined that the development of Public-Private Partnerships between the IPPC and relevant stakeholders to support global plant health efforts is consistent with discussions which have taken place during the past year in both the Bureau and SPG meetings, and is envisioned in the IPPC Strategic Plan 2020-2030. In this regard, it was proposed to hold a stakeholder workshop in 2020. One of the objectives of the proposed workshop would be to provide private sector representatives with the opportunity to discuss and evaluate the establishment of an IPPC Stakeholder Advisory Group.

[31] The proposed advisory group would be in addition to existing private sector involvement and engagement in initiatives including ePhyto, sea containers and the grains standard – where specific industry experience and expertise was sought to ensure initiative outcomes are compatible with global trading systems. The advisory group would be independent of the IPPC in all aspects, including funding.

[32] Some CPs noted that the participation of the private sector in issues that directly or indirectly affect them is useful and important, particularly considering the benefits outlined by the Secretary-General of the WCO in his opening address at the CPM-12, and the potential value of collaboration and interaction.

[33] Other CPs requested that guidelines on interactions with private sector representatives for NPPOs be developed and provided to members, as well as an outline of the desired impact and outcome envisioned by the IPPC in engaging with the private sector.

[8] CPM 2017/35

[9] CPM 2017/37

[34] Some CPs indicated that the stakeholder group should include relevant Non-Governmental Organizations (NGOs) and other relevant entities, and that the CPM should be clear on what it wants to achieve through this stakeholder group.

[35] The CPM noted that some RPPOs already engaged with private sector representatives and relevant stakeholders.

[36] The CPM:

(1) *agreed* to continue to increase and improve collaboration between the IPPC and relevant stakeholders;

(2) *approved* the organization of a Stakeholder Workshop in 2020;

(3) *encouraged* globally and regionally relevant stakeholders to explore the formation of an IPPC Stakeholder Advisory Group to broaden its engagement in and contribution to the protection of the world's plant resources from pests; and

(4) *requested* that the CPM Bureau and SPG, in consultation with relevant stakeholders, prepare draft Terms of Reference (ToR) and Rules of Procedure (RoP) for such an IPPC stakeholder advisory body, if appropriate, for agreement at the IPPC/Stakeholder Workshop in 2020, if not sooner.

8.6 Sea Containers Complementary Action Plan

[37] The CPM took note of the paper presented by the Secretariat.[10] A special topics session was held during CPM-11 (2016) on the issue of sea containers. Presentations by the NPPOs, relevant international organizations and relevant stakeholders involved in the movement of sea containers, outlined the complex logistics of the movement of sea containers and the potential risks of the spread of pests. CPM recognized the risk of pests and regulated articles, other than cargo, that can be moved with sea containers and that managing these risks was complex to achieve. The CPM requested the Bureau to consider the development of a "set of complementary actions", which, combined, could offer some value in assessing and managing the pest threats associated with sea containers, and to propose a possible programme of complementary actions to CPM-12 (2017). Further discussion took place both within the SPG, as well as the Capacity Development Committee.

[38] The Bureau proposed several actions, pending extra-budgetary resources, to be provided by CPs or the private sector. These actions will measure the impact of the IMO/ILO/UNECE Code of Practice for Packing of Cargo Transport Units (CTU Code) during the next five years, increased awareness of pest risks of sea containers and information to assist NPPOs better manage these risks, as well as to establish oversight and governance arrangements for their implementation. Furthermore, the Bureau recommended that oversight of these actions be provided by the Implementation and Capacity Development Committee (IC).

[39] CPs gave their support in principle to this initiative, including the establishment of a Sea Container Task Force (SCTF). Some CPs offered assistance in the form of providing experts to the Secretariat in this regard.

[40] Some CPs expressed their concern for the funding for this initiative and expressed the importance of using extra-budgetary funds.

[41] Some CPs believed that the SCTF should not be a permanent structure and possibly dissolved by 2020, as it should have a limited timeframe.

[42] A CP hoped that the Joint Industry Guidelines for Cleaning of Containers[11] presented by the World Shipping Council and the Container Owners Association,, would be distributed to shippers and shipping terminals of CP by industry groups so that inspection and cleaning of containers effectively reduces risk posed by pests.

[43] The Chairperson, and CPs, thanked the World Shipping Council (WSC) and the Container Owners for providing their industry guidelines that could be used by NPPOs for the handling of sea containers.

[44] The CPM:

[10] CPM 2017/34/Rev_01

[11] CPM 2017/INF/05

(1) *Endorsed* a Sea Containers Complementary Action Plan (Appendix 05).

(2) *Noted* the priority actions identified by the CDC in Appendix 06.

(3) *Requested* that the Sea Container Task Force (SCTF) be established in 2017, according to a project and funding plan agreed by the CPM Bureau for a five-year period

(4) *Requested* that the Bureau invite nominations from CPs, Standards Committee (SC), and RPPOs reflecting the composition as outlined in the document "Establishing and operating the Task Force on Sea Containers (Appendix 07)

(5) *Requested* that the CDC/IC and the SCTF develop Rules of Procedure and Terms of Reference to facilitate the efficient implementation the Complementary Action Plan.

(6) *Encouraged* Contracting Parties to provide extra-budgetary resources to support the SCTF and commence implementation activities, including any significant in-kind contribution (following the ePhyto project manager model) to manage the implementation activities.

(7) *Encouraged* NPPOs to share information during CPM meetings and on the International Phytosanitary Portal (IPP) on actions taken at the country level to support the CPM Recommendation on Sea Containers.

(8) *Requested* NPPOs to contact their country representatives to the International Maritime Organization (IMO) and encourage them to support the adoption of the Joint Industry Guidelines for Cleaning of Containers by the Maritime Safety Committee in 2017

8.7 CPM Recommendations

[45] The Secretariat presented its paper on CPM Recommendations[12] stating that the IPPC Secretariat had reviewed and revised the CPM Recommendations with a view to updating them to ensure consistency and clarity, and further noted some CPM Recommendations had been superseded. During the review, the Secretariat found that the changes proposed to the CPM Recommendations may be considered as ink amendments. The main changes that will be implemented in all the CPM Recommendations have been agreed to by the CPM Bureau and will be published according to FAO/IPPC standards.

[46] Some CPs suggested minor modifications to the proposed criteria.

[47] The CPM:

(1) *revoked* the CPM Recommendations concerning 1) Information Exchange and 2) the Role of IPPC Contact Points as they have been superseded by CPM-10 (2015) decisions.

(2) *requested* the IPPC Secretariat to incorporate the approved ink amendments into the CPM Recommendations, post the CPM Recommendations in all languages on the IPP, and revoke previous versions of the CPM Recommendations.

(3) *supported* the revised format of CPM Recommendations and requested the IPPC Secretariat to post them on the IPP, revoking the previous version.

(4) *agreed* to the CPM Recommendations criteria as outlined in Appendix 08 and requested the IPPC Secretariat to annex them to the CPM Recommendation procedure and post them on the IPP.

8.8 Adjustments to the TC-RPPOs Roles and Functions

[48] The Secretariat presented an updated Roles and Functions of the TC-RPPOs outlining the relationship and areas of cooperation between the IPPC Secretariat and the RPPOs[13].

[49] Several CPs thanked the Secretariat for the adjustments which highlight the important role of the RPPOs in the IPPC family.

[12] CPM 2017/15/Rev_01

[13] CPM 2017/11/Rev_01

[50] The Caribbean CPs thanked the IPPC Secretariat, FAO Legal Council and other RPPOs for their guidance and recommendations on the way forward for the Caribbean RPPO.

[51] The CPM:

(1) *Requested* that the IPPC Secretariat, SPG, Capacity Development Committee (CDC) and CPM subsidiary bodies continue to collaborate with RPPOs as envisaged in this updated version of the RPPOs' roles and functions;

(2) *Encouraged* RPPOs to continue to collaborate and strengthen their partnerships with each other and with the IPPC Secretariat as envisaged in this updated version of the RPPOs' roles and functions and in the 2015 IPPC Secretariat enhancement review;

(3) *Encouraged* the active role of the Technical Consultation among RPPOs as a mechanism to facilitate this collaboration and to provide strategic input to the CPM Bureau and the CPM;

(4) *Recognized* that nothing in these Roles and functions of the RPPOs limits or replaces the rights or obligations of contracting parties under the IPPC;

(5) *Recognized* that nothing in these Roles and functions of the RPPOs affects the role of RPPOs or limit the activities that RPPOs may undertake;

(6) *Adopted* the revised version of the RPPOs' roles and functions in relation to the Commission on Phytosanitary Measures (Appendix 09).

8.9 Framework for Standards and Implementation

[52] The Secretariat presented its paper on the Framework for Standards and Implementation[14]. Based on the CPM-11 decision which endorsed the use of the Framework for Standards and Implementation[15] to record the standards and other tools for implementation that support and enable the implementation of the Convention and International Standards for Phytosanitary Measures (ISPMs) to facilitate harmonization, the SC met in May 2016 and the CDC in June 2016 to review and update the Framework for Standards and Implementation. The SPG, during its October 2016 meeting, reviewed the updated Framework for Standards and Implementation making no changes or comments.

[53] A CP thanked the SC, CDC and the IPPC Secretariat and expressed his hope that the Framework will be referred to by other CPs when considering new topics or tools.

[54] The CPM:

(1) *endorsed* the Framework for Standards and Implementation.

8.10 Proposal for a new Implementation Oversight Body

[55] The Secretariat introduced a paper[16] on the proposal for a new Implementation Oversight Body which was based on the outcomes of a focus group which met in July, 2016, as well as SPG and Bureau consideration. Based on the results of these discussions, the CPM was asked to consider a proposal that the new committee be known as the IPPC Implementation and Capacity Development Committee, abbreviated to IC. This reflects the two key elements in the committee's purpose (i) implementation of the IPPC, including the ISPMs and (ii) strengthening the phytosanitary capacity of contracting parties.

[56] Amendments were proposed by some members and submitted[17] to the IPPC Secretariat. A meeting was held to find a resolution, and a revised proposal was produced[18]. The Chairperson of the meeting highlighted that the main issues raised and agreed upon were: to increase membership of the body from 11 to 12; 1

[14] CPM 2017/36

[15] https://www.ippc.int/static/media/files/publication/en/2016/05/FrameworkForStandardsAndImplementation_2016-04-08.pdf

[16] CPM 2017/08

[17] CPM 2017/INF/10 and CPM 2017/INF/12

[18] CPM 2017/CRP/08

representative from the SC and 1 from RPPOs; selection of the members; ensure that there is a good balance based on expertise in capacity development and/or implementation, and regional representation. The responsibility for ensuring a good balance was left with the CPM Bureau. Renewal of membership would not be automatic, but left to the Bureau to decide after 3 years.

[57] In relation to delaying the Call for Topics, the CPM agreed that the SC and IC should develop the criteria for the SC/IC joint call for topics and issues during 2017 and present them to CPM-13 (2018) for approval. The Joint call would then be possible in 2018.

[58] Following discussion, the CPM:

(1) *Considered* the report and recommendations of the Implementation Focus Group
(2) *agreed* that the Implementation and Capacity Development Committee be established under the adopted Terms of Reference and Rules of Procedure (Appendix 10)
(3) *agreed* that the usual abbreviation for the Committee be IC
(4) *agreed* that the IC should start operations in the second half of 2017
(5) *agreed* that the National Reporting Obligations Advisory Group (NROAG), Triennial Review Group (TRG) and Subsidiary Body on Dispute Settlement (SBDS) be dissolved at the same time as the IC is established and the functions and procedures of these committees be transferred to the IC
(6) *agreed* that the call for topics be delayed so that a joint SC/IC call for topics for standards and issues for implementation can be held
(7) *agreed* that a priority task for the IC will be to develop criteria for the joint SC/IC call for topics and issues, in collaboration with SC
(8) *agreed* that until its dissolution, CDC starts work on these priority tasks of the IC
(9) *agreed* that the CDC also works to complete its programme as far as possible to ensure a smooth transition to the new Committee.

9. Standard Setting

9.1 Report on the activities of the Standards Committee

[59] As the Chairperson of the Standards Committee had vacated the position, the Vice Chairperson, Ms Shaza Omar (Egypt), presented the report[19]. She highlighted that 2016 was the busiest year on record for the SC, with 12 ISPMs adopted and 28 ISPMs were recommended for adoption. The SC has worked consistently to ensure delivery against its core mandate of ensuring that ISPMs are technically sound and of the highest quality possible. Support provided by CPs to SC members to facilitate their participation was acknowledged, noting that a large number of standards are expected in 2017.

[60] She thanked the previous SC Chairperson, Mr Jan Bart Rossel (Australia), for his dedicated service.

[61] One CP noted the proposed funding cuts for the SC May 2017 meeting and thanked Canada for providing additional resources and stressed that cuts to SC meetings should not be considered in the future.

[62] Another CP stressed the need for capacity building in the face of the increased number of standards being developed.

[63] The CPM:

(1) *noted* the Report on the activities of the Standards Committee in 2016.

[19] CPM 2017/22/Rev_01

9.2 Adoption of International Standards for Phytosanitary Measures

[64] The Secretariat introduced the full list of papers[20] for this agenda point. The paper presented standards for adoption as well as diagnostic protocols that have been adopted by the SC on behalf of the CPM. The Secretariat informed the CPM that two objections were received three weeks prior to the CPM-12 (2017).

[65] The Secretariat noted that the IPPC, through FAO, currently has eight co-publishing agreements with Brazil, Germany, Japan, Republic of Korea, Thailand, Turkey, Vietnam, and most recently with the North American Plant Protection Organization (NAPPO). The Secretariat indicated that such agreements could also be done for other documents.

[66] An objection from some CPs on the international movement of used vehicles, machinery and equipment (2006-004) was resolved through minor modifications[21] to the draft standard clarifying that it only applied to used vehicles, machinery and equipment. Although not covered by the standard, a note on the risk of new vehicles contamination was included in the background. The CPM Chairperson clarified that this was not a redrafting of the text but a clarification of the concept, which required minor changes, and that this did not set a precedent of redrafting standards at the CPM.

[67] One CP also provided an objection on heat treatment of wood using dielectric heating (2007-114), noting they had further research that questioned the efficacy of the treatment and agreed to provide their findings to the Secretariat two weeks prior to the May SC meeting.

[68] Some CPs expressed concerns on the international movement of growing media in association with plants for planting (2005-004) as growing media in association with plants for planting and growing media in international trade was not clearly differentiated and could cause problems for implementation.

[69] It was noted that the SC encouraged CPs to share experiences on arrangements for verification of compliance of consignments by the importing country in the exporting country (2005-003).

[70] Some CPs provided proposals for minor technical changes to some of the draft standards which were not discussed. The Secretariat noted, however, these suggestions will be retained and considered when the standard is next revised.

[71] Some CPs noted differences in advice given for the application of Sulphuryl Fluoride treatments in draft ISPM 15 and ISPM 28 and recommended to align them in the future.

[72] One CP expressed concern that some of the proposed treatments had multiple schedules and felt this to be confusing for the implementation.

[73] One CP expressed concern on using only laboratory results as the basis for decisions in phytosanitary treatments (PT) and, in addition, requested the development of technical manuals for temperature treatments and encouraged other CPs to share their manuals.

[74] The Chairperson reminded the CPM that a call for PTs was currently open and encouraged CPs and RPPOs to respond to this call.

[75] One CP expressed concerns about the limited access to technical documents used by the technical panels as the basis for standards and technical recommendations. The Chairperson noted this concern and informed the CPM that this will be discussed at the June 2017 Bureau meeting.

[76] The CPM:

(1) *adopted* ISPM 38 on the *International movement of seeds* (2009-003) contained in Appendix 17.

(2) *adopted* annex 1 *Arrangements for verification of compliance of consignments by the importing country in the exporting country* (2005-003) to ISPM 20 (*Guidelines for a phytosanitary import regulatory system*) contained in Appendix 17.

[20] CPM 2017/03 (Attachments 01 – 16) CPM 2017 INF/10, INF/12, INF/19, and INF/20 and CRP 01

[21] CPM 2017/CRP/09

(3) *adopted* ISPM 39 on the *International movement of wood* (2006-029) contained in CPM 2017/03_04.

(4) *adopted* ISPM 40 on the *International movement of growing media in association with plants for planting* (2005-004) contained in Appendix 17.

(5) *adopted* ISPM 41 on the *International movement of used vehicles, machinery and equipment* (2006-004) contained in Appendix 17.

(6) *adopted* the PT 22 Sulfuryl Fluoride fumigation treatment for insects in debarked wood (2007-101A) as Annex 22 to ISPM 28, contained in contained in Appendix 17.

(7) *adopted* the PT 23 as Sulfuryl Fluoride fumigation treatment for nematodes and insects in debarked wood (2007-101B) as Annex 23 to ISPM 28, contained in Appendix 17.

(8) *adopted* the PT 24 Cold treatment for *Ceratitis capitata* on *Citrus sinensis* (2007-206A) as Annex 24 to ISPM 28, contained in Appendix 17.

(9) *adopted* the PT 25 Cold treatment for *Ceratitis capitata* on *Citrus reticulata x C. sinensis* (2007-206B) as Annex 25 to ISPM 28, contained in Appendix 17.

(10) *adopted* the PT 26 Cold treatment for *Ceratitis capitata* on *Citrus limon* (2007-206C) as Annex 26 to ISPM 28, contained in Appendix 17.

(11) *adopted* the PT 27 Cold treatment for *Ceratitis capitata* on *Citrus paradisi* (2007-210) as Annex 27 to ISPM 28, contained in Appendix 17.

(12) *adopted* the PT 28 Cold treatment for *Ceratitis capitata* on *Citrus reticulata* (2007-212) as Annex 28 to ISPM 28, contained in Appendix 17.

(13) *adopted* the PT 29 Cold treatment for *Ceratitis capitata* on *Citrus clementina* (2010-102) as Annex 29 to ISPM 28, contained in Appendix 17.

(14) *adopted* the PT 30 Vapour heat treatment for *Ceratitis capitata* on *Mangifera indica* (2010-106) as Annex 30 to ISPM 28, contained in Appendix 17.

(15) *adopted* the PT 31 Vapour heat treatment for *Bactrocera tryoni* on *Mangifera indica* (2010-107) as Annex 31 to ISPM 28, contained in Appendix 17.

(16) *noted* that the SC adopted on behalf of CPM the following ten diagnostic protocols (DPs) as Annexes to ISPM 27:

- DP 13: *Erwinia amylovora*
- DP 14: *Xanthomonas fragariae*
- DP 15: *Citrus tristeza virus*
- DP 16: *Genus Liriomyza* Mik
- DP 17: *Aphelenchoides besseyi, A. ritzemabosi and A. fragariae*
- DP 18: *Anguina* spp. (2013-003)
- DP 19: *Sorghum halepense* (2006-027)
- DP 20: *Dendroctonus ponderosae* (2006-019)
- DP 21: *Candidatus* Liberibacter solanacearum (2013-001)
- DP 22: *Fusarium circinatum* (2006-021)

(17) *acknowledged* the contributions of Contracting Parties, RPPOs and organizations who hosted or helped organize standard setting meetings in 2016: Australia (EWG Grain), Canada (TPFQ), Japan (TPPT), Jamaica (TPDP) and the IAEA/FAO Joint Division (TPFF).

(18) *acknowledged* the contributions of the members of the Standards Committee (SC), in particular those who have left the SC in 2016:

- Algeria, Ms Nadia HADJERES

- Canada, Ms Marie-Claude FOREST
- Costa Rica, Mr Guillermo SIBAJA CHINCHILLA
- Ghana, Ms Ruth WOODE
- Iran, Ms Maryam Jalili MOGHADAM
- New Zealand, Mr John HEDLEY
- Norway, Ms Hilde Kristin PAULSEN
- Papua New Guinea, Mr Pere KOKOA
- Poland, Mr Piotr WLODARCZYK
- Sudan, Mr Kamaleldin Abdelmahmoud Amein BAKR
- Yemen (Republic of), Mr Gamil Anwar Mohammed RAMADHAN

(19) *acknowledged* the contributions of the members of the Technical Panel on Forest Quarantine (TPFQ) who have left in 2016:

- Brazil, Mr Edson Tadeu IEDE
- Chile, Marcos Beéche CISTERNAS
- Germany, Mr. Thomas SCHRÖDER
- Norway, Mr. Sven Christer MAGNUSSON

(20) *acknowledged* the contributions of individual experts for their efforts (specific roles are noted) in the development of the ISPMs adopted at CPM-12 (2017) as presented in Appendix 11.

[77] The Chairperson introduced the paper[22] regarding the Reorganisation, harmonisation and minor technical updates of the fruit fly ISPMs. It was noted that agreement could not be reached on the reorganization as proposed. COSAVE volunteered to lead a virtual working group which will also include Australia, Europe, and Japan to review the CPM papers. This working group is to provide a revised proposal to the IPPC Secretariat by 30 September 2017 for the SC to discuss and review at their November 2017 meeting aiming at presenting a revised proposal to CPM-13 (2018)consideration. If the proposal needs to be reviewed by the Technical Panel on Pest Free Areas and Systems Approaches for Fruit Flies (TPFF), then extra-budgetary resources will be required.

[78] The IPPC Secretariat presented its paper[23] on ink amendments to adopted ISPMs.

[79] The CPM:

(1) noted the ink amendments to ISPM 3 (*Guidelines for the export, shipment, import and release of biological control agents and other beneficial organism*), ISPM 4 (*Requirements for the establishment of pest free areas*), ISPM 5 (*Glossary of phytosanitary ter*ms), ISPM 8 (*Determination of pest status in an area*), ISPM 9 (*Guidelines for pest eradication programmes*), ISPM 11 (*Pest risk analysis for quarantine pests*), ISPM 14 (*The use of integrated measures in a systems approach for pest risk management*), ISPM 15 (*Regulation of wood packaging material in international trade*), ISPM 17 (*Pest reporting*), ISPM 24 (*Guidelines for the determination and recognition of equivalence of phytosanitary measures*), ISPM 29 (*Recognition of pest free areas and areas of low pest prevalence*) and ISPM 30 (*Establishment of areas of low pest prevalence for fruit flies (Tephritidae)*).

[22] CPM 2017/19

[23] CPM 2017/20

(2) *noted* that the ink amendments, translated into the FAO official languages, will be implemented into the language versions of the concerned standards as resources permit.

(3) *agreed* that, once the IPPC Secretariat has applied ink amendments, the previous versions of the standards are revoked and replaced by the newly noted versions.

9.3 Topics for IPPC standards - New topics and adjustments to the *List of topics for IPPC standards*

[80] The Secretariat introduced the document[24] summarizing proposed adjustments to the CPM adopted List of Topics for IPPC standards[25] (LOT) which can be viewed on the International Phytosanitary Portal (IPP).

[81] Some CPs did not agree to add the topic *Phytosanitary measures for commodities*, as outlined in their paper[26]. There were discussions, in particular the relationship of this topic with ISPM 32 and 11, categorization of commodities, and the scope and content of specific commodities standards. Consensus to add this topic was not reached. The CP which submitted the topic will continue discussions to revise it for resubmission at the next call for topics.

[82] One CP proposed that topics related to pest risks presented through passengers and the movement of goods and parcels, through mail and similar services, should be a high priority topic. The Chairperson noted that this could be a proposal in the next call for topics.

[83] Some CPs suggested that the proposed topic, *Use of systems approaches in managing risks associated with the movement of wood commodities* (2015-004), is too broad and needs to include specific requirements. Interested CPs met in the margins of the CPM and determined that these issues should be addressed in the development of the specification.

[84] Some CPs expressed disappointment with the inconsistent approach taken by the SC at their meeting in November 2016 when reviewing the proposals for the three commodity standards and suggested that the criteria for topics should be reviewed by the SC, in collaboration with the IC, prior to the next call for topics and tools.

[85] The CPM:

(1) *added* the following topic, with the indicated priorities and IPPC Strategic Objectives, to the *List of Topics for IPPC standards*:

- 2015-004: *Use of systems approaches in managing risks associated with the movement of wood commodities* (priority 3, strategic objectives B and C)

(2) *adopted* the *List of topics for IPPC standards*, with the above adjustment

(3) *requested* the Secretariat to incorporate this change into the List of Topics for IPPC standards and to post it on the IPP

9.4 Noting translation adjustments to International Standards for Phytosanitary Measures adopted at CPM-11

[86] CPM-5 (2010) adopted a Language Review Group (LRG) procedure to correct editorial errors in language versions of adopted ISPMs. The Secretariat has received the ISPMs adopted at CPM-11 (2016) with proposed modifications from the Arabic, Chinese and Spanish LRGs. The Secretariat submitted these to the FAO Translation Services, who reviewed the proposed changes. The proposed changes were then included in the revised ISPMs and were presented to CPM-12 (2017) in track changes.

[24] CPM 2017/17

[25] *List of topics for IPPC standards: https://www.ippc.int/en/core-activities/standards-setting/list-topics-ippc-standards/*

[26] CPM 2017/INF/10

[87] The Secretariat informed the CPM that an LRG Coordinator for Russian had recently been appointed.

[88] The CPM is invited to:

(1) *noted* that the following have been reviewed by the Arabic, Chinese and Spanish LRGs and FAO Translation services:

- Amendments to ISPM 5 (*Glossary of phytosanitary terms*)
- ISPM 37 (*Determination of host status of fruit to fruit flies (Tephritidae)*)
- PT 20 (Irradiation treatment for *Ostrinia nubilalis*) as annex to ISPM 28 (*Phytosanitary treatments for regulated pests*)
- PT 21 (Vapour heat treatment for *Bactrocera melanotus* and *Bactrocera xanthodes* on *Carica papaya*) as annex to ISPM 28
- DP 7 (*Potato spindle tuber viroid*) as annex to ISPM 27 (*Diagnostic protocols for regulated pests*)
- DP 8 (*Ditylenchus dipsaci* and *Ditylenchus destructor*) as annex to ISPM 27
- DP 9 (Genus *Anastrepha* Schiner) as annex to ISPM 27.

(2) *agreed* that once the Secretariat has applied the changes as indicated in track changes in the Attachments 1–7 (of this paper in the language versions concerned), the previous versions of the ISPMs are revoked and replaced by the newly noted versions.

(3) *Thanked* CPs and RPPOs involved in the LRGs, as well as FAO Translation Services for their efforts and hard work to improve the language versions of the ISPMs.

9.5 Adjustments to the language review process

[89] The Secretariat introduced the papers on Adjustments to the LRG Process[27]. The Secretariat noted that it was the first time the CPM discussed this issue. It was further noted that standards that had been through the LRG process are only of concern to the CPs who use that specific language. This means that contrary to other CPM agenda items where all CPs provide their input, issues related to translation adjustments are not of relevance to CPs who do not use that language. The IPPC Secretariat suggested that the LRG process be revised to reduce the onerous work involved in presenting standards for noting and to allow CPM to focus on issues where all CPs participate. Adjusted translations will no longer be presented to the CPM for noting, but instead CPs will be informed via an email once the LRG-adjusted standards have been published. The CPM will continue to note that the LRGs have provided adjustments to the translations of specific standards, but the actual translations will no longer be attached to the CPM paper.

[90] The CPM:

(1) *approved* the modified LRG process (Appendix 12) and agreed that the modified process take effect immediately.

10. Implementation Facilitation

10.1 Report on activities for IFU

[91] The Secretariat introduced the report on the activities of the Implementation Facilitation Unit (IFU) for 2016[28]. The Secretariat highlighted that the reduction in contributions from donors to the Special IPPC Multi-Donor Trust Fund (MTF) in 2016, severely impacted the operation of the IFU. Nevertheless, the IFU facilitated two

[27] CPM 2017/23, CPM 2017/INF/12

[28] CPM 2017/06

meetings of the CDC, organized seven side sessions at CPM-11 (2016), facilitated seven IPPC Regional Workshops and managed a number of projects. In addition, the IFU convened a focus group that developed a proposal for a new subsidiary body on implementation and capacity development. The Secretariat organized five workshops lasting two weeks each to train Phytosanitary Capacity Evaluation facilitators, of which ten were attending the CPM.

[92] CPs congratulated the IFU for a very productive year and highlighted the need for extra-budgetary resources.

[93] The CPM:

(1) *Noted* the report of the Implementation Facilitation Unit (IFU) for 2016.

10.2 Implementation pilot on surveillance

[94] The IPPC Secretariat presented its report[29] on the pilot project on surveillance noting that it aims to draw together pest surveillance managers and experts to exchange experiences, discuss challenges, showcase best practices and coordinate the development of pest surveillance products that are globally relevant and valuable. The Secretariat reported advances made during 2016, including an initiative at CPM-11 (2016) with three example pests for which information would be collated through a call for technical resources. The three pests were:

- *Xylella fastidiosa*
- *Bactrocera dorsalis* complex
- Invasive ants

[95] Subsequently, an Informal Working Group was held in Bangkok, Thailand, from 11-12 June 2016, with the support of APPPC and the Republic of Korea to work on the three selected pests. The Secretariat informed that technical resources aggregated on the 3 pests are being reviewed by the CDC and a factsheet on *Xylella fastidiosa* was available and distributed to the CPM. The pilot project on surveillance is intended to capitalize on existing resources and events related to surveillance, and to work in collaboration with NPPOs, RPPOs and partner institutions.

[96] The Secretariat reported that the results of the 2015 questionnaires on country level surveillance activities were presented during the 2016 IPPC regional workshops.

[97] CPs expressed their satisfaction with the work done and encouraged further contribution of resources to continue to build phytosanitary capacity. The Secretariat responded to one CP's request for clarity on the operation of the pilot project.

[98] The CPM:

(1) *Noted* the advances of the implementation pilot on surveillance.

(2) *Noted* the factsheets on the three example pests and *agreed* to promote them and the new webpages on www.phytosanitary.info.

(3) *Encouraged* contracting Parties to contribute financial resources to the implementation pilot on surveillance.

10.3 Implementation, Review and Support System (IRSS)

[99] The Secretariat introduced the report of the IRSS[30] that describes the integrated work activities of both the implementation pilot project on surveillance and the work programme of the IPPC Secretariat.

[29] CPM 2017/05

[30] https://www.ippc.int/static/media/files/publication/en/2017/02/07_CPM_April_Implementation_Review_and_Support_System_IRSS-2017-02-06.pdf

[100] The Secretariat highlighted the achievements of 2016 and advised that all expected activities and outputs had been completed in time for the end of the second project cycle on 31 March 2017. The Secretariat confirmed that it intends to commence a third project cycle in 2017 for a further three-year period and will seek contributions from its previous donor and other contracting parties and organizations to continue the project.

[101] Some CPs thanked the Secretariat for the report and requested other CPs to make contributions.

[102] The CPM:

(1) *Noted* the 2016 IRSS work activities that will contribute to the success of the IPPC work programme and the implementation pilot project on surveillance.

(2) *Noted* the intention of the IPPC Secretariat to continue the work of the IRSS and seek funding for a third project cycle.

(3) *Urged* contracting parties to contribute resources and motivate others to contribute resources to ensure that the IRSS project continues.

10.4 Report on National Reporting Obligations (NRO)

[103] The IPPC Secretariat presented its report on National Reporting Obligations (NROs)[31], the overview of the NRO programme[32] and a summary of statistical data from 2005 to 2016[33].

[104] The Secretariat reported that the NRO Programme contributed to the increase in numbers of new NRO reports by the countries on the International Phytosanitary Portal (IPP) in 2015 and 2016. Activities undertaken in 2016 included: publishing of the series of advocacy and awareness raising materials, launch of the IPP NRO reminders system, preparing scripts for 5 modules of NRO e-learning and conducting NRO Workshop for countries in the Asian region.

[105] In addition, 2016 was the NRO Year of Pest Reporting and the IPPC Secretary sent a letter to all Official Contact Points reminding about the importance of pest reporting. This year's NRO Year is the NRO Year of Phytosanitary Legislation.

[106] Several CPs expressed their appreciation and support of activities done by the Secretariat. They found the reminder system, NRO UPDATE and planned e-learning useful and these help them build their capacity to report.

[107] The CPM:

(1) *noted* the update on activities related to National Reporting Obligations (NRO).

10.5 Status of ISPM 15 Symbol Registration

[108] The Secretariat provided a report[34] on the status of ISPM 15 Symbol Registration. In 2016, the IPPC Secretariat initiated new registrations for 17 countries. In addition, the Secretariat noted that the work plan for 2017 included a fourth round of registrations, which when completed, will have finished the five-year agreed work plan and budget of USD 350,000.

[109] One CP indicated that registration of ISPM 15 allowed them to harmonize the wood packaging treatments and wanted the IPPC Secretariat to continue with the registration of ISPM 15 symbol. They, however, indicated that they are experiencing problems regarding implementation thereof, highlighting unqualified use of the symbol and evidence of counterfeit symbols being used.

[31] CPM 2017/04

[32] CPM 2017/INF/09

[33] CPM 2017/INF/06

[34] CPM 2017/28

[110] The CPM:

(1) *noted* the progress made in 2016 and the work plan for 2017 with regard to the registration of the ISPM 15 symbol.

(2) *encouraged* CPs to continuously support the process of registration of the ISPM 15 symbol, including renewals of registrations that are due to expire.

(3) *encouraged* CPs to reimburse the IPPC Secretariat for registration and registration renewal costs as soon as practically possible.

10.6 Report on ePhyto

[111] The Secretariat reported[35] that activity had commenced on the ePhyto project thanks to generous contributions from the Republic of Korea and the United States of America, in addition to human and financial resources support provided by Canada. These resources were used to establish a work agreement with the UNICC to commence the development of technical specifications for the hub and Generic National System (GeNS). The Secretariat further reported that sufficient funding (including the STDF project funding) has been received to build and test the ePhyto Solution and complete the pilot. A significant component of the project is the development of a fair and robust business model which supports the long-term operation of the Solution. The final establishment of the model to support operation is anticipated to occur after project funding lapses leaving a funding gap in the operation of the system. The Chairperson encouraged the CPs to contribute resources in order to close this funding gap.

[112] A CP reported that it intends to make a contribution to the project in 2017, and other CPs noted that further harmonization is needed and that they wished to be further involved. Several CPs called for assistance to implement ePhyto. The Chairperson noted that the project does contain elements of capacity building, but does not include funding for infrastructure development by CPs. The Chairperson also noted that there are several organizations interested in ePhyto and it was important for CPs to seek resources from those organizations to support infrastructure development. The IPPC Secretariat would not be in a position to help with this.

[113] Several CPs expressed their disappointment regarding the lack of progress in developing the Solution and encouraged a greater commitment to the timelines for achieving the project objectives.

[114] The CPM:

(1) *noted* the work of the IPPC Secretariat and the ePhyto Steering Group (ESG) in advancing the development of ePhyto;

(2) *supported* the continued work of the IPPC Secretariat and the ESG under the supervision of the CPM Bureau;

(3) *acknowledged* the support provided by the United States of America, Canada and the other member countries of the ESG (Australia, the Netherlands, Argentina, the People's Republic of China and Kenya) who have provided significant contributions to advancing the ePhyto Solution through funding and technical support;

(4) *acknowledged* the contributions of the proposed pilot countries as this will require resource contributions in support of the set-up, operation and evaluation of the pilot;

(5) *supported* the continued progress in implementing the ePhyto project and in particular to urge countries to financially support the project through donations to operate the hub and generic system following the pilot;

(6) *requested* that the Secretariat report back to CPM-13 on progress in implementing the ePhyto project.

[35] CPM 2017/32

11. Communications and Advocacy

11.1 Main activities on Communication and Advocacy of the IPPC Secretariat for 2016

[115] The Secretariat presented an update on the IPPC Secretariat Communication and Advocacy activities in 2016[36]. The creation of a Task Force for Communication and Advocacy (TFCA) had aided the Secretariat's efforts in communication, advocacy and information management. The TFCA work has been instrumental to ensure effective coordination and provision of positive outputs contributing towards the IPPC 2016 Theme: Plant Health & Food Security, which included a CPM-11 keynote address, a IPPC Seminar and a CFS-43 side-event, as well as the organization of two other IPPC Seminars and supporting the IPPC Steering Committee of International Year of Plant Heath (IYPH) and a side-event on IYPH. Additionally, 177 news items and 23 announcements were produced.

[116] The CPM:

(1) *noted* the report on Communication and Advocacy activities of the IPPC Secretariat in 2016.

11.2 Work plan on Communication and Advocacy of IPPC the Secretariat for 2017

[117] The Secretariat presented its report[37] on the communication and advocacy activities planned by the Secretariat for 2017, noting that the TFCA would continue to coordinate internal and external communication, advocacy and information management initiatives. The Secretariat noted that 2017 is the 65th anniversary of the IPPC, and that the ratification of the Convention would be celebrated with a series of communication activities. It was further noted that contribution for the annual theme: Plant Health & Trade Facilitation, continued support to the IPPC Steering Committee of the IYPH and timely production of headline news and announcements would be a priority in 2017.

[118] CPs expressed their appreciations for the Secretariat's efforts in communication and advocacy, which they found useful and relevant.

[119] CPs suggested possible improvements going forward, such as, lessons learned from each theme year (to help with the planning for the IYPH), directing social media activities to the general public and liaising with communication departments of other organizations, including RPPOs, to ensure common messages.

[120] The CPM:

(1) *noted* the communication and advocacy activities planned by the IPPC Secretariat for 2017.

(2) *agreed to consider* ways to effectively support communication and advocacy efforts by the IPPC Secretariat, also in view of an increasing involvement in the proposed IYPH activities.

12. Reports on the IPPC Network

12.1 Report on the IPPC Regional Workshops for 2016

[121] The Secretariat reported that seven annual IPPC Regional Workshops were organized in 2016[38]. A total of 212 persons from 114 countries benefited from these workshops. The participants recommended improvements, and these have been considered for the 2017 series of workshops. The IPPC Regional Workshops have been restructured to strengthen collaboration among CPs, RPPOs, FAO regional offices, cooperating institutions and the IPPC Secretariat. The Secretariat emphasized the critical financial situation for the organization of the 2017 IPPC Regional Workshops.

[122] CPs voiced strong support for the IPPC Regional Workshops and encouraged their continuation emphasizing the how informative and useful they are and their importance in building capacity.

[36] CPM 2017/12

[37] CPM 2017/29

[38] CPM 2017/09

[123] The CPM:

(1) *Noted* the organization and the new developments of the 2016 IPPC Regional Workshops.
(2) *Noted* the improvements suggested for the organization of the 2017 IPPC Regional Workshops.
(3) *Encouraged* contracting parties to actively participate in the 2017 IPPC Regional Workshops.
(4) *Encouraged* contracting parties and other institutions to provide financial resources to increase attendance to 2017 IPPC Regional Workshops.

12.2 Report on the 28th Technical Consultation (TC) among Regional Plant Protection Organizations (RPPOs)

[124] As the 2016 host, the Executive Director of the Near East Plant Protection Organization presented the report[39] of the TC-RPPOs to the CPM.

[125] The Secretary noted that, for the first time, all RPPOs plus the potential future RPPO of the Caribbean region were together.

[126] The next Technical Consultation will be held during the period of 30 October to 03 November 2017, in Paris, France.

[127] The CPM:

(1) *noted* the report.

13. International Year of Plant Health in 2020 (IYPH 2020)

[128] A report from the IPPC IYPH Steering Committee (StC) was presented to the CPM[40]. In addition, the CPM was informed of important milestones for this initiative. Two important meetings took place with FAO bodies to present the IYPH 2020 initiative and put forward for adoption. In September 2016, the 25th meeting of the FAO Committee on Agriculture (COAG) approved the proposal by the Government of Finland to establish IYPH 2020 in the UN system and endorsed the Draft Conference Resolution as proposed to COAG. The first meeting of the IYPH StC was held from 9-11 November 2016.

[129] There was overwhelming support from CPs and RPPOs who commended the work done thus far by the IPPC Secretariat and StC and the progress made.

[130] Some CPs reminded the CPM that the defining scope of the IYPH was adopted at CPM-11 and that it was important to take this scope into account when developing programmes and events. They also suggested that the IPPC Secretariat create a task force dedicated to preparations for the IYPH, which would amongst others, determine staff needs for the year.

[131] One CP encouraged CPs to liaise with their governments to endorse the IYPH so that national preparations for it could commence. The CP also stressed the importance of developing communications material at an early stage to allow for internal lobbying of the relevant authorities.

[132] There were various suggestions made by CPs and RPPOs on ways to mobilize resources and promote the initiative, including raising awareness with the public.

[133] The Chairperson reiterated that the regional members of the StC are the focal point for NPPOs to provide inputs and suggestions on IYPH programme events in their countries and regionally.

[134] The CPM:

(1) *Noted* the report of the 1st meeting of the IYPH StC.

[39] CPM 2017/INF/02
[40] CPM 2017/31

(2) *Adopted* the envisaged outputs and outcomes for the IYPH as laid down in Appendix 13.

(3) *Encouraged* CPs to provide extra-budgetary contributions to enable promotional activities to support the IYPH proclamation process.

(4) *Considered* how the IPPC Secretariat should be provided with staff resources enabling it to provide assistance to the planning and execution of IYPH 2020.

(5) *Urged* CPs to support the proposal for an IYPH in 2020 at the upcoming 40th Session of the FAO Conference (3-8 July 2017).

(6) *Invited* CPs to propose potential IYPH programme events and activities to their regional representatives in the IYPH StC.

14. International Cooperation

[135] The IPPC Secretariat presented its report[41] on the highlights of its activities and cooperation with various international organizations, including Codex and others as contained in the paper.

[136] The CPM expressed its appreciation for the cooperation with these organizations.

14.1 Oral reports from selected international organizations

[137] Oral reports were presented by the following international and regional organizations:

- World Trade Organization (WTO)[42] – The WTO continues to develop capacity for contracting parties to implement the Convention and ISPMs. They also indicated that the Trade Facilitation Agreement entered into force in February 2017 and would contribute significantly to trade facilitation;
- Standards and Trade Development Facility (STDF)[43] – STDF continues to engage with the IPPC Secretariat as a member of the STDF Working Group;
- Convention on Biological Diversity (CBD)[44] – reported on the outcome of their UN Biodiversity Conference (December 2016) noting decisions on invasive alien species, as well as linkages and synergies with the IPPC as a biodiversity related convention. The IPPC Secretariat urged CPs to contact their CBD and GEF focal points in order to further enhance the implementation of biodiversity related phytosanitary measures at national level.. It was further noted that there were several requests to the members of the Biodiversity Liaison Group (BLG) of which the IPPC is a member. Some CPs requested information on how the CBD decision, COP-13/24 would have impact on the resources of the IPPC Secretariat and if this would necessitate CPM decisions.. The Chairperson indicated that this issue would be discussed at the June Bureau meeting;
- Report from the Joint Food and Agriculture Organization / International Atomic Energy Agency Division of Nuclear Techniques in Food and Agriculture (FAO/IAEA)[45] – The FAO/IAEA continued to support the implementation of standards, in particular, Fruit Fly standards, and will support the 2017 meeting of the TPPT.

[138] The CPM:

(1) *noted* the reports.

14.2 Written reports from relevant international organizations

[139] Written reports or statements were presented by the following international and regional organizations:

[41] CPM 2017/30

[42] CPM 2017/INF/15

[43] CPM 2017/INF/14

[44] CPM 2017/CRP/03

[45] CPM 2017/INF/07_Rev_01

- International Seed Federation[46] - welcomed the adoption of the standard, offered assistance for the development of training material to help implement the standard, and informed the CPM that they will be holding a workshop for their members;
- International Forestry Quarantine Research Group[47] - continued to conduct and coordinate research for the development of forestry related standards. Some CPs encouraged the group to ensure its regional inclusiveness and noted that they aimed to become more involved in the group;
- Phytosanitary Measures Research Group[48] - PMRG coordinate and conduct research to support the development of phytosanitary treatments. CPs are encouraged to participate in the efforts of this group to ensure appropriate PTs can be adopted.

[140] The CPM:

(1) *noted* the written reports.

15. Financial Report and Budget

15.1 Financial report of the IPPC Secretariat for 2016

[141] The Secretariat presented the report containing financial statements for resources available in 2016 from FAO's Regular Programme (RP) budget and the Extra-Budgetary (EB) Trust Fund sources that were administered by the IPPC Secretariat during the reporting period[49].

[142] CPs expressed their appreciation for the improved financial reporting, in particular regarding the transparency of the Finance Committee, and the Chairperson of the Committee stressed that they would seek to improve their planning and reporting.

[143] The CPM acknowledged the contributions from Australia, France, New Zealand, Ireland, Republic of Korea and the United States / NAPPO to the IPPC Multi-Donor Trust Fund in 2016. The CPM acknowledged contributions to IPPC projects from European Union, STDF and China.

[144] The CPM encouraged other CPs to establish sustainable funding for the IPPC in their own countries.

[145] Two CPs mentioned that there was under-reporting of their contributions.

[146] The CPM acknowledged the contribution of USD 150,000 from the Republic of Korea from the regular government budget to the Multi-Donor Trust Fund for 2017, enabling sustainable funding to the IPPC Secretariat. Canada also informed the CPM that it is providing a contribution of USD 202,000 to the Multi-Donor Trust Fund in 2017.

[147] The CPM:

(1) *noted* the Financial Report for 2016 of the IPPC Secretariat

(2) *adopted* the Financial report for 2016 of the IPPC Multi-Donor Trust Fund (Special Trust Fund of the IPPC) (Appendix 14)

(3) *encouraged* contracting parties to contribute to the IPPC Multi-Donor Trust Fund (Special Trust Fund of the IPPC) and IPPC Projects, preferably on an ongoing basis

(4) *thanked* contracting parties which contributed to the IPPC Secretariat's programme of work in 2016.

[46] CPM 2017/INF/08

[47] CPM 2017/CRP/04

[48] CPM 2017/CRP/05

[49] CPM 2017/27

15.2 Work plan and budget of the IPPC Secretariat for 2017

[148] The Secretariat presented the work plan and budget[50].

[149] CPs were encouraged by the CPM to lobby their FAO representatives to highlight the importance of the IPPC and its work at the FAO Conference and request additional financial support.

[150] The CPM stressed the importance of sustainable funding in order to enable long term planning for the Secretariat and its work.

[151] The CPM:

(1) *Approved* the IPPC Secretariat Work Plan and IPPC Multi-Donor Trust Fund budget for 2017 (Appendix 16)

(2) *Noted* the IPPC Secretariat Regular programme budget for 2017 (Appendix 16).

15.3 Resource mobilization of the IPPC Secretariat for 2016

[152] The Secretariat presented the report51 on resource mobilization. Among other things, the Secretariat noted that as a result of an in-depth analysis of the situation concerning finance and resource mobilization challenges, the IPPC Secretariat urgently needs short and long-term financial support in order to be able to carry out the tasks assigned to it by the CPM. Regarding sustainable funding, 2016 was a significant year during which advances in proposed mechanisms and long-term funding models were made.

[153] The CPM:

(1) *Noted* the work on resource mobilization which has been done by the IPPC Secretariat in 2016 and planned for in 2017

(2) *Agreed* to continue the strategic discussion on sustainable funding such as: sustained contributions; contributions from industry; and contributions generated by articulating the "added value" of IPPC at the SPG and Bureau meetings, and report back to CPM-13 in 2018.

16. Conceptual Challenges in Standards Development in Terms of Implementation

[154] The Secretariat noted that the SC discussed the concept of compliance certification schemes and the use of a certificate of compliance by NPPOs and situations where such a concept could apply (e.g. as an alternative to a phytosanitary certificate (PC))[52].

[155] A small group was formed to discuss the issue and in reporting back, it was noted that some CPs were concerned that the introduction of an additional certification system could create confusion and problems in trade[53]. Furthermore, a new system of certification could add complexity to newly developed national systems and also cause difficulties for ePhyto.

[156] The Chairperson indicated that although the CPM did not consider that this certification system should be developed now, it might be considered at a later stage and may be used when agreed bilaterally.

[157] The CPM:

(1) *Decided* not to approve further work on the concept on the use of certificates of compliance in ISPMs.

[50] CPM 2017/38

[51] CPM 2017/25

[52] CPM 2017/18

[53] CPM 2017/INF/10

17. Successes and Challenges of Implementation of the Convention

[158] CPs were invited to share their successes and challenges in implementing the IPPC and ISPMs.[54]

[159] Presentations were made by China, Japan, European Union, COSAVE, and New Zealand[55].

[160] Before opening the Special Topics session, the Chairperson reflected on the loss of members of the Phytosanitary community, and held a minute silence in memory of their passing.

18. Special Topics Session: e-Commerce

[161] A special topics session was held on the issue of e-commerce. Presentations[56] were given by representatives of NPPOs, relevant international organizations and stakeholders involved in e-Commerce. They included: Marième Fall (WTO); Michele Medina (WCO); Junko Shimura (CBD); Sarah Brunel (IPPC Secretariat); Carlos Grau Tanner (Global Express Association); Mike Carlson (eBay Regulatory Policy Group); Kim Ritman (Australia); and Hong-Sook Park (Republic of Korea). Following discussion, a number of proposals developed by international organizations, NPPOs, and the express delivery companies were presented. These included suggestions related to business-to-consumer, and business-to-government actions, in addition to awareness raising measures.

[162] The CPM:

(1) *Requested* the Bureau to develop a way forward in the June 2017 meeting, including resource considerations.

19. Confirmation of Membership and Potential Replacement Members for CPM Subsidiary Bodies

19.1 CPM Bureau members and potential replacement members

[163] The Secretariat provided the CPM with the list of Bureau members and potential replacement members[57] as adjusted during CPM[58].

[164] The representative of Sudan requested that the CPM record his objection to the membership of the incumbent Near-East Representative in the Bureau.

[165] The CPM:

(1) *Noted* the current membership of Bureau members and potential replacement members (Appendix 15)

(2) *Elected* a replacement member from the Europe region to the CPM Bureau.

19.2 SC members and potential replacement members

[166] The Secretariat provided the CPM with the list of SC members and potential replacement members[59] as adjusted during CPM[60].

[167] The CPM:

[54] CPM 2017/16

[55] CPM 2017/INF/16

[56] CPM 2017/10

[57] CPM 2017/14

[58] CPM 2017/CRP/10

[59] CPM 2017/13

[60] CPM 2017/CRP/10

(1) *Noted* the current membership of the Standards Committee and the potential replacements for the Standards Committee.

(2) *Confirmed* new members and potential replacements (Appendix 15).

(3) *Confirmed* the order in which potential replacements will be called upon for each region.

19.3 SBDS members and potential replacement members

[168] The Secretariat provided the CPM with the list of SC members and potential replacement members[61] as adjusted during CPM[62].

[169] The CPM:

1) *Noted* the current membership of the Subsidiary Body on Dispute Settlement[63] (Appendix 15).
2) *Confirmed* new members and potential replacements.

20. Any Other Business

[170] The CPM acknowledged the warm welcome and excellent organization of CPM-12 by the Republic of Korea. It also sincerely appreciated the enormous financial contribution from the Republic of Korea for the conference.

[171] There were numerous interventions from members thanking the Republic of Korea for hosting CPM-12 and for making it a successful and fruitful conference, and its role in raising awareness of its function and role. One CP requested that the IPPC Secretariat investigate how other CPs could host the CPM.

21. Date and Venue of the Next Session

[172] CPM-13 (2018) was scheduled for 16-20 April 2018 in FAO Headquarters, Rome Italy.

22. Adoption of the Report

[173] The report was adopted.

[61] CPM 2017/13

[62] CPM 2017/CRP/10

[63] CPM 2017/13

Appendix 01 – Agenda

1. Opening of the Session

1.1 FAO Opening

1.2 Republic of Korea Opening

2. Keynote Address on Plant Health and Trade Facilitation

3. Adoption of the Agenda

3.1 EU Statement of Competence

4. Election of the Rapporteur

5. Establishment of the Credentials Committee

6. Report from the CPM Chairperson

7. Report from the IPPC Secretariat

8. Governance

8.1 Summary of the Strategic Planning Group report

8.2 Strategic Framework for 2020-2030

8.3 Sustainable funding

8.4 Emerging issues

8.5 Strategic partnerships

8.6 Sea containers - Complementary Action Plan

8.7 Ink amendments for CPM recommendations

8.8 Adjustments to the TC-RPPO rules of procedure

8.9 Framework for standards and implementation

8.10 Proposal for a new implementation oversight body

9. Standards Setting

9.1 Report of the activities of the Standards Committee

9.2 Adoption of International Standards for Phytosanitary Measures

9.3 Topics for IPPC Standards - New topics and adjustments to the *List of topics for IPPC standards*

9.4 Noting translation adjustment to International Standards for Phytosanitary Measures adopted at CPM-11

9.5 Adjustment to the language review process

10. Implementation Facilitation

10.1 Report on activities for IFU

10.2 Implementation pilot surveillance

10.3 Implementation Review and Support System (IRSS)

10.4 Report on National Reporting Obligations (NRO)

10.5 Status of ISPM 15 Symbol Registration

10.6 Report on ePhyto

11. Communication and Advocacy

11.1 Main activities on Communication and Advocacy of the IPPC Secretariat for 2016

11.2 Work plan on Communication and Advocacy of the IPPC Secretariat for 2017

12. Reports on IPPC Network

12.1 Report on the IPPC Regional Workshops for 2016

12.2 Report on the 28th Technical Consultation (TC) among Regional Plant Protection Organizations (RPPOs)

13. International Year of Plant Health in 2020 (IYPH 2020)

14. International Cooperation

14.1 Oral reports from selected international organizations

14.2 Written reports from relevant international organizations

15. Financial Report and Budget

15.1 Financial report of the IPPC Secretariat for 2016

15.2 Work plan and budget of the IPPC Secretariat for 2017

15.3 Resource mobilization of the IPPC Secretariat for 2016

16. Conceptual Challenges in Standards Development in Terms of Implementation

17. Successes and Challenges of Implementation of the Convention

18. Special Topics Session: e-Commerce

19. Confirmation of Membership and Potential Replacements members for CPM Subsidiary Bodies

19.1 CPM Bureau members and potential replacement members

19.2 SC members and potential replacement members

19.3 SBDS members and potential replacement members

20. Any Other Business

21. Date and Venue of the Next Session

22. Adoption of the Report

Appendix 02 – List of Documents

Document number	Agenda item	Document Title	Available Languages
CPM 2017/01	03	Provisional Agenda	EN/FR/ES/RU/AR/
CPM 2017/02/Rev_01	03	Detailed Agenda	EN/FR/ES/RU/AR/ZH
CPM 2017/03	09.2	Adoption of International Standards for Phytosanitary Measures	EN/FR/ES/RU/AR/ZH
CPM 2017/04	10.4	Report on National Reporting Obligations (NRO)	EN/FR/ES/RU/AR/ZH
CPM 2017/05	10.2	Implementation pilot surveillance	EN/FR/ES/RU/AR/ZH
CPM 2017/06	10.1	Report on activities for IFU	EN/FR/ES/RU/AR/ZH
CPM 2017/07	10.3	Implementation, Review and Support System (IRSS)	EN/FR/ES/RU/AR/ZH
CPM 2017/08	08.10	Proposal for a new implementation oversight body - Outcomes of the Focus Group and SPG and Bureau consideration	EN/FR/ES/RU/AR/ZH
CPM 2017/09	12.1	Report on the IPPC Regional Workshops for 2016	EN/FR/ES/RU/AR/ZH
CPM 2017/10	18	Special Topics Session: e-Commerce	EN/FR/ES/RU/AR/ZH
CPM 2017/11/Rev_01	08.8	Adjustments to the TC-RPPO rules of procedure - Roles and functions of Regional Plant Protection Organizations (RPPOS) in their relationship with The Commission on Phytosanitary Measures (CPM)	EN/FR/ES/RU/AR/ZH
CPM 2017/12	11.1	Main activities on Communication and Advocacy of the IPPC Secretariat for 2016	EN/FR/ES/RU/AR/ZH
CPM 2017/13	19.2; 19.3	SC members and potential replacement members - SBDS members and potential replacement members	EN/FR/ES/RU/AR/ZH
CPM 2017/14	19.1	CPM Bureau members and potential replacement members	EN/FR/ES/RU/AR/ZH
CPM 2017/15/Rev_01	08.7	Ink amendments for CPM Recommendations	EN/FR/ES/RU/AR/ZH
CPM 2017/16	17	Successes and Challenges of Implementation of the Convention	EN/FR/ES/RU/AR/ZH
CPM 2017/17	09.3	Topics for IPPC Standards - New topics and adjustments to the List of topics for IPPC standards	EN/FR/ES/RU/AR/ZH
CPM 2017/18	16	Conceptual Challenges in Standards Development in Terms of Implementation -	EN/FR/ES/RU/AR/ZH

Document number	Agenda item	Document Title	Available Languages
		Discussion paper on the Use of a Certificate of compliance	
CPM 2017/19	09.2	Adoption of International Standards for Phytosanitary Measures - Reorganization, Harmonization And Minor Technical Updates Of The Fruit Fly ISPMs	EN/FR/ES/RU/AR/ZH
CPM 2017/20	09.2	Adoption of International Standards for Phytosanitary Measures - Ink Amendments to Adopted ISPMs	EN/FR/ES/RU/AR/ZH
CPM 2017/21	09.4	Noting translation adjustment to International Standards for Phytosanitary Measures adopted at CPM-11 (2016)	EN/FR/ES/RU/AR/ZH
CPM 2017/22/Rev_01	09.1	Report of the activities of the Standards Committee	EN/FR/ES/RU/AR/ZH
CPM 2017/23	09.5	Adjustments to the Language Review Group Process	EN/FR/ES/RU/AR/ZH
CPM 2017/24	08.2	Strategic Framework for 2020-2030	EN/FR/ES/RU/AR/ZH
CPM 2017/25	15.3	Resource mobilization of the IPPC Secretariat for 2016	EN/FR/ES/RU/AR/ZH
CPM 2017/26	08.3	Sustainable funding - Sustainable funding mechanisms for the IPPC Secretariat Work Programme	EN/FR/ES/RU/AR/ZH
CPM 2017/27	15.1	Financial report of the IPPC Secretariat for 2016	EN/FR/ES/RU/AR/ZH
CPM 2017/28	10.5	Status of ISPM 15 Symbol Registration	EN/FR/ES/RU/AR/ZH
CPM 2017/29	11.1	Work plan on Communication and Advocacy of the IPPC Secretariat for 2017 - Summary of communication and advocacy activities planned by the IPPC Secretariat for 2017	EN/FR/ES/RU/AR/ZH
CPM 2017/30	14	International Cooperation - IPPC Secretariat cooperation with relevant organizations	EN/FR/ES/RU/AR/ZH
CPM 2017/31	13	International Year of Plant Health in 2020 (IYPH 2020) - Report on the activities relating to the International Year of Plant Health in 2020	EN/FR/ES/RU/AR/ZH
CPM 2017/32	10.6	Report on ePhyto - ePhyto Update	EN/FR/ES/RU/AR/ZH
CPM 2017/33	07	Report from the IPPC Secretariat - Report for 2016	EN/FR/ES/RU/AR/ZH

Document number	Agenda item	Document Title	Available Languages
CPM 2017/34	08.6	Sea containers - Complementary Action Plan	EN/FR/ES/RU/AR/ZH
CPM 2017/35	08.4	Emerging Issues	EN/FR/ES/RU/AR/ZH
CPM 2017/36	08.9	Framework for standards and implementation - Endorsement of the Framework for Standards and Implementation	EN/FR/ES/RU/AR/ZH
CPM 2017/37	08.5	Strategic partnerships	EN/FR/ES/RU/AR/ZH
CPM 2017/38	15.2	Work plan and budget of the IPPC Secretariat for 2017	EN/FR/ES/RU/AR/ZH
CPM 2017/39	08.1	Summary of the Strategic Planning Group report	EN/FR/ES/RU/AR/ZH
CPM 2017/40	06	Report from the CPM Chairperson	EN/FR/ES/RU/AR/ZH

Information Papers (INF)

Document number	Agenda item	Document Title	Available Languages
CPM 2017/INF/01	03	Local Information	EN only
CPM 2017/INF/02	12.2	Summary Report of the Twenty-eighth Technical Consultation among Regional Plant Protection Organizations	EN only
CPM 2017/INF/03	20	Any Other Business - Due dates for the CPM-12	EN only
CPM 2017/INF/04	20	Any Other Business - Exhibition Prospectus	EN only
CPM 2017/INF/05	08.6	Sea containers - Complementary Action Plan - Joint Industry Container Cleanliness Guidelines	EN only
CPM 2017/INF/06	10.4	Report on National Reporting Obligations (NRO) - National Reporting Statistical Data	EN only
CPM 2017/INF/07	14.2	Written reports from international organizations - Report from the Joint Food and Agriculture Organization / International Atomic Energy Agency Division of Nuclear Techniques in Food and Agriculture	EN only

Document number	Agenda item	Document Title	Available Languages
CPM 2017/INF/08	14.2	Written reports from relevant international organizations - Report by the International Seed Federation	EN only
CPM 2017/INF/09	10.4	Report on National Reporting Obligations (NRO) Overview of NRO Programme	
CPM 2017/INF/10	08.10; 09.2; 09.3; 16	Statements from COSAVE and its member countries regarding various CPM agenda items	EN only
CPM 2017/INF/11	09.2	Adoption of ISPMs - EU written statement on reorganization, harmonization and minor technical updates of the fruit fly ISPMs	EN only
CPM 2017/INF/12	8.3; 8.5; 8.7; 8.10; 9.5	EU written statements on various agenda items	EN only
CPM 2017/INF/13	08.2	IPPC Draft Strategic Framework 2020-2030 - Aligning IPPC's Future Work to its Core Competency	EN only
CPM 2017/INF/14	14.2	Written reports from relevant international organizations - Standards and Trade Development Facility (STDF) Overview	EN only
CPM 2017/INF/15	14.2	Written reports from relevant international organizations - WTO Report 2016	EN only
CPM 2017/INF/16	17	Successes and Challenges of Implementation of the Convention	EN only
CPM 2017/INF/17	03.1	EU statement of competence	EN only
CPM 2017/INF/18	20	Any Other Business - CPM-12 side sessions	EN only
CPM 2017/INF/19	09.2	Adoption of International Standards for Phytosanitary Measures - Objections to draft ISPMs presented for adoption by CPM-12 (2017)	EN only
CPM 2017/INF/20	09.2	Adoption of International Standards for Phytosanitary Measures - China's comments to draft ISPMs presented for adoption by CPM-12 (2017)	EN only

Conference room papers (CRP)

Document number	Agenda item	Document Title	Available Languages
CPM 2017/CRP/01	03	List of documents	EN only
CPM 2017/CRP/02	08.6	Sea containers - Complementary Action Plan - Positive Action to Address Potential Risks of the Spread of Pests Associated with Shipping Containers	EN only
CPM 2017/CRP/03	14.2	Written reports from relevant international organizations - Report from the Secretariat of the Convention on Biological Diversity	EN only
CPM 2017/CRP/04	14.2	Written reports from relevant international organizations - International Forestry Quarantine Research Group Report	EN only
CPM 2017/CRP/05	14.2	Written reports from international organizations - Report from the Phytosanitary Measures Research Group (PMRG) activities for 2016	EN only
CPM 2017/CRP/06	09.3	Topics for IPPC Standards - New topics and adjustments to the List of topics for IPPC standards - Key IPPC terms in need of TPG review and attention	EN only
CPM 2017/CRP/07	18	Special Topics Session: e-Commerce - Internet Trade (e-commerce) of plants	EN only
CPM 2017/CRP/08	08.10	Proposal for a new implementation oversight body - Outcomes of the Focus Group and SPG and Bureau consideration	EN only
CPM 2017/CRP/09	09.2	Adoption of International Standards for Phytosanitary Measures	EN only
CPM 2017/CRP/10	19; 19.1; 19.2; 19.3	Confirmation of Membership and Potential Replacements members for CPM Subsidiary Bodies - CPM Bureau members and potential replacement members - SC members and potential replacement members - SBDS members and potential replacement members	EN only

Appendix 03 – List of Participants

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Appendix 04 – 2016 Report from the IPPC Secretariat

[174] The year 2016 has been a milestone for the IPPC, as it was the first year for the Convention to implement the IPPC annual themes towards 2020. This year has been extraordinary for the IPPC community, as we collectively made remarkable achievements even with a substantial reduction in human resources. There are ten major achievements highlighted in this report:

- the IPPC 2016 annual theme,
- IPPC governance and strategic activities,
- coordination of standards,
- implementation of standards,
- enhancing communications and advocacy,
- developing the International Year of Plant Health,
- strengthening the IPPC network,
- strengthening the IPPC network,
- enhancing international cooperation,
- improving resource mobilization, and,
- strengthening the internal management of the Secretariat.

[175] The first achievement was to disseminate the IPPC annual theme for 2016 — Plant Health and Food Security. The IPPC Secretariat organized a keynote address at CPM 11 on the IPPC annual theme for the first time in its history, which was delivered by Professor Rudy Rabbinge from Wageningen University of the Netherlands. We also organized a series of activities to showcase this IPPC annual theme, including two IPPC seminars, one Committee on Food Security (CFS) side-event, and one video message from the IPPC Secretary to the 2016 IPPC Regional Workshops.

[176] The second achievement was to organize IPPC governance and strategic activities. The IPPC Secretariat provided strong support in the organization of all meetings of the IPPC governing bodies. We also made a close follow-up with implementation of all important CPM decisions, such as setting-up a Focus Group on the establishment of an oversight body for implementation, working on a sustainable funding mechanism for IPPC Secretariat work programmes, and initializing IPPC strategic planning for 2020-2030.

[177] The third achievement was to coordinate a record numbers of standards. Over 40 standards were progressed, with 12 Standards adopted (2 Regular Standards, 2 Phytosanitary Treatments, and 8 Diagnostic Protocols) and 28 Standards presented for adoption (5 Regular Standards, 11 PTs, and 12 DPs). This was the highest number of standards covered for a single year in the IPPC's history.

[178] The fourth achievement was to promote the implementation of standards. Five Training Workshops on PCE were organized with the participation of 40 phytosanitary experts from 36 countries, and 21 lawyers from 13 countries plus FAO staff. Sixteen projects were implemented with six completed and an additional ten were active, covering over 15 CPs. One focus group was established on a surveillance pilot project, dealing with three potential pests.

[179] The fifth achievement was to enhance communication and advocacy. The new IPP home page and new OCS were released. Over 170 items of headline and brief news were issued, an increase of 70% in comparison with 2015. The 2015 IPPC Annual Report was published, with distribution of 1,000 copies.

[180] The sixth achievement was to promote the development of the International Year of Plant Health (IYPH) 2020. The Steering Committee for IYPH 2020 was established, and its first meeting was organized in FAO-HQ in Rome, Italy. One side-event on IYPH 2020 was held during the 25th session of COAG of FAO. The resolution for IYPH 2020 was endorsed at the 25th session of COAG of FAO, and then endorsed at the 150th FAO Council.

[181] The seventh achievement was to strengthen the IPPC network. An IPPC Workshop on National Reporting Obligations (NROs) in Asia was organized for the first time in the several years. Seven IPPC Regional Workshops were held, with 212 participants from 144 CPs. The annual TC-RPPOs was

conducted in Rabat, Morocco, with the participation of all nine RPPOs and one region (Caribbean) for the first time in many years.

[182] The eighth achievement was promoting international cooperation. In particular, cooperation with the International Atomic Energy Administration (IAEA) was deepened on standard setting. Cooperation with the World Customs Organization (WCO) was initiated on ePhyto. Cooperation with UNEP was also started on biodiversity-related issues.

[183] The ninth achievement was to strengthen resource mobilization. The initiative of sustainable funding for the IPPC work programme was proposed, and strongly supported by the CPM Finance Committee, CPM Bureau, and SPG. The IPPC Multi-donor trust fund reached USD 6.65 million (a 42% increase compared to 2015), mainly from Australia, France, Korea, New Zealand and the USA. New IPPC Projects amounted to USD 4.07 million (highest in the history of the Convention), mainly from China (USD 2 million), STDF (USD 1.12 million) and EU (Euro 0.9 million). IPPC In-kind contributions were valued at over USD 0.7 million, mainly from Canada, China, Costa Rica, France, Republic of Korea, New Zealand and USA, as well as the International Centre for Advanced Mediterranean Agronomic Studies (CIHEAM), FAO Regional Office for Europe and Central Asia (FAO-REA) and Inter-American Institute for Cooperation on Agriculture (IICA). The complete list of IPPC in-kind contributions is presented in the Finance Report of the IPPC Secretariat for 2016.

[184] The tenth achievement was strengthening internal management. The action plan for the Enhancement Evaluation of the IPPC Secretariat was implemented, mainly for reshaping the IPPC Secretariat's structure with establishment two professional Units (Standard Setting and Implementation Facilitation) and one supporting Team (Integration Support). Quality management as well as standardization of documents and information materials were strengthened by setting up several relevant Standard Operating Procedures (SOPs). Team and culture building were promoted by organizing a Retreat Workshop, and the Monitoring & Evaluation Training Workshop, as well as by enhancing the work of task forces for Resource Mobilization (TFRM), and for Communication and Advocacy (TFCA).

[185] While summarizing the main activities and outcomes of the IPPC Secretariat for 2016, we feel that there are four important experiences for learning and continuation:

- First, more attention should be paid to Innovation, such as innovative thinking for strategic planning of the IPPC towards the 2030 UN Sustainable Development Goals, and innovative management for renewing the IPPC Secretariat on the basis of the Enhancement Evaluation.
- Second, we should insist on Prioritization by emphasizing three pillars, standard setting, implementation facilitation, and communication and partnerships.
- Third, we should make better Coordination among the IPPC governing bodies, the IPPC community, and FAO Senior Management.
- Last, we should promote Teamwork by shared learning through workshops/training, and by team performance through task forces.

[186] 2017 will be another important year for the IPPC, as it will be the year for implementation of the next IPPC annual theme — 'Plant Health and Trade Facilitation', as well as a year for celebrating the 65th Anniversary of the IPPC. We are confident that 2017 will be an even better year for the IPPC with your continued support and dedication in strengthening delivery of the IPPC work programme.

[187] Among many the tasks and activities for 2017, five are key:

a) *advocate* the IPPC annual theme "Plant health and trade facilitation" for 2017, and promote IYPH 2020 to be endorsed in FAO conference;
b) *organize* the CPM 12 in Republic of Korea, and establish a new oversight body for implementation;
c) *complete* the projects on STDF-401 and EC-IRSS, and implement the new projects on IPPC Implementation from EC, ePhyto from STDF, and Capacity Development from the FAO-China SSC Programme;

d) *strengthen* the IPPC network at regional and national levels, and enhance international cooperation with relevant technical, trade, environment and industry organizations; and
e) *continue* efforts to mobilize additional resources and restructure the IPPC Secretariat, and celebrate the 65th Anniversary of the IPPC.

[188] The IPPC Secretariat would like to take this opportunity to express our sincere gratitude and appreciation to all IPPC Bodies for their excellent governance, to all NPPOs and RPPOs for their strong support, and to all partners and collaborators for their usual cooperation.

[189] The CPM is invited to:

1) *note* the highlights presented in this report.

Appendix 05 – Complementary Action Plan for assessing and managing the pest threats associated with sea containers

[1] The CPM Bureau proposes a number of actions to reduce the pest risks associated with sea containers, pending extra-budgetary resources provided by contracting parties (CPs) or industry. These actions will measure the impact of the IMO/ILO/UNECE Code of Practice for Packing of Cargo Transport Units (CTU Code) during the next five years, increase awareness of pest risks of sea containers and information to assist NPPOs better manage these risks, and establish oversight and governance arrangements for their implementation.

[2] The Bureau encourages CPs or industry to provide resources to the IPPC Secretariat to facilitate this work, and suggested that the funding model of the ePhyto project could be applied to progress it.

(i) Measuring the impact of the CTU shipping code through:

- The development of a joint IPPC/IMO/industry protocol for the collection of data related to contamination of sea containers to be completed by CPM-16 (2021);
- Monitoring the uptake and implementation of the IMO/ILO/UNECE Code of Practice for Packing of Cargo Transport Units (CTU Code) through:
    - industry reporting
    - NPPO monitoring;
- Verifying the efficacy of the CTU Code in ensuring the arrival of clean sea container through:
    - monitoring for pest contamination and freedom of soil by NPPOs;
- Assisting NPPOs manage pest risks associated with sea containers,

(ii) Increasing awareness of pest risks of sea container through

- publication of the data of the Expert Working Group (EWG) by the IPPC Secretariat;
- a request by the IPPC Secretariat for countries having data on contamination of sea containers to make it publically available;
- calling for and publication of pest risk management guidance material for sea containers;
- encouraging NPPOs to inform industry on the risks and possible international actions to manage pest risks associated with sea containers;
- ensuring that any regulations on sea containers that are developed and implemented by NPPOs are based on pest risk analysis and consistent with Recommendation CPM 10/2015_01 on Sea Containers.

Oversight and governance

[3] Establishment of a Task Force that will operate under the oversight of the CDC/IC, to supervise the above actions and complement them with any other actions through:

- providing information on pest risks of sea containers and their management;
- coordinating with CPs, RPPOs, industry and other international organizations;
- establishing a mechanism for CPs to report to CPM on their progress and achievements;
- providing advice on how the CTU Code or any other instrument could be updated;
- providing, through the CDC/IC, updates on its activities to be presented annually to the CPM, as well as a final report for presentation to CPM-16 (2021).

[4] The Bureau will select members and invited experts to take part in the task force. Members of the task force should be nominated by contracting parties or RPPOs and have expertise in IPPC matters and sea container logistics. At least one member of the task force should be an EWG member on sea containers. In addition, industry experts and representatives of relevant international organizations could also be part of the task force as invited experts.

[5] The Task Force should have members from CPs knowledgeable in IPPC matters and sea container logistics. It should have industry experts and other relevant international organizations. The Task Force may consult experts on sea containers, such as ex EWG members, as required.

Appendix 06 – Priority actions to implement the Sea Container Complementary Action Plan

1. In December 2016, the CDC proposed a number of high priority and feasible activities for the Sea Container Task Force to undertake that will progress implementation of the Complementary Action Plan. These are as follows:

2. **Among the first administrative tasks to be undertaken:**

- Task Force nominees to be invited for the first face-to-face meeting (Priority 1/Feasible)
- The Secretariat collects and provides the Task Force with all available materials on sea containers (Priority 1/Feasible)
- The Task Force develops a work plan based on the Terms of Reference elaborated by the Bureau (Priority 1/Feasible).

3. **Activities of the Task Force will include:**

- A baseline study is performed by the Task Force (needs assessment) (Priority 1/Feasible).
- A call is issued for resources needed to fill gaps, including for pest risk management (Priority 1/Feasible, except that contributors might be willing to submit resources and a lot of follow up is needed to assess these resources).
- The Task Force would establish linkages with international organizations such as WCO and IMO and other stakeholders involved in sea container issues (Priority 1/Feasible).
- The Task Force establishes a list of stakeholders involved in sea containers (The EWG might already have this list) (Priority 1/Feasible).
- Monitoring of the uptake and implementation of the CTU shipping code:

  - Procedures set to monitor uptake and implementation of the CTU shipping code (to establish a baseline during the first year, to monitor implementation of the CTU up to 2021):

    - Establishing monitoring procedures (Priority 1/Feasible)
    - Surveys (Priority 1/Feasible, although collection of responses is somewhat difficult)
    - Call for pilot countries with broad participation and reflecting the situations.
    - Country assessment (Priority 2/Feasible/Costly)
    - Establishing national Committees (Customs, NPPO staff, IPPC contact points, industry)

  - Reporting framework by:

    - Industry (self-monitoring) (Priority 1/Feasible/hard to collect responses and coordinate reporting)
    - NPPO (Priority 1/Feasible/hard to collect responses and coordinate reporting)
    - RPPO (Priority 1/Feasible/hard to collect responses and coordinate reporting)
    - WCO or other relevant international organizations (Priority 1/Feasible/hard to collect responses and coordinate reporting)

  - Analyse data and report to the IC. The IC reports to the CPM –(Priority 1/Feasible/Costly) (staff and database are needed)

- Providing information on pest risks and management of sea containers. The task force should within one year:
  - Collect and analyse global information regarding pests known to be introduced in sea containers and soil for a period of 2 years. Pests should be categorized.
  - Establishing an industry advisory committee.
  - Available measures used.
  - Database/data-modelling.

    - Identify gaps.
- Awareness programme (Priority 1/Feasible/Costly, consultant is needed, costs of publication, see point 2.3):
    - Notifications developed to Industry on pest risk
    - A range of possible management actions communicated to NPPOs
    - Outreach spread to all stakeholders based on the list established
    - Means: fliers, videos, emails, Phytosanitary Resources page, media, social media, conferences

Legal instrument, if appropriate, for sea containers:

- Develop a model legal instrument for CTU code adoption for NPPOs (Priority 1/Feasible/Costly)
- Communicate to NPPOs model legal instrument (Priority 1/Feasible/Costly)
- Monitor consistency with CPM decisions of national legal framework on sea containers if in place up to 2021 (Priority 1/Feasible/Costly).

Appendix 07 – Establishing and operating the Task Force on Sea Containers

I Governance

1. The Sea Container Task Force (SCTF) is convened as a body of experts under the umbrella of the IC. It reports annually to the December meeting of the IC. The IC incorporates a report on progress against the prioritised sea container Complementary Action Plan in its annual report to the CPM.

II Operations

2. The SCFT could be activated by May 2017, subject to available funding. It would cease operations and be dissolved in 2021 by the CPM.

3. The SCFT operates mainly through virtual meetings and on-line communications. Periodic face to face meetings may be convened, as needed.

4. A meeting record and communique is prepared after each meeting and posted on the IPP.

III Establishing the SCTF

A Composition

5. The task force should be composed of representatives of Contracting Parties (CPs), Regional Plant Protection Organizations, international organizations and phytosanitary experts who already have an experience relevant to the pest risks on sea containers and their management.

6. This may be drawn from:

- Up to 3 representative of CPs
- 1 industry expert to be represented by COA
- 2 representatives of international organizations:
    - WCO (CTU-Code manager) - WCO will communicate with IMO
    - WSC
- 1 Expert on Sea Containers (EWG)
- 1 representative of RPPO

7. A fixed core membership of 6 to 8 experts may be supplemented by additional experts from NPPOs, the Convention on Biological Diversity and the OIE where expertise such as on risk management, implementation experience, economic and financial analysis, is needed to implement the action plan.

8. A member of the IC is appointed as a Steward of the SCTF to ensure appropriate linkage with the IC. The Steward is required to attend SCTF meetings and act as a liaison with the IC. An officer from the IPPC Secretariat would be assigned as a focal point to the topic and would ensure liaison and consistency across the different IPPC governing bodies.

B Nomination

9. The IPPC Secretariat nominates the focal point for the SCTF and the IC appoints a Steward.

10. Membership of the SCTF may be sought through a call, coordinated by the Secretariat on behalf of the IC. This may be for specific expertise or for a SCTF core member. Alternates may be sought for core membership. Where a call for experts was required, the IC will set criteria and recommend the expert(s) to the Bureau.

11. RPPOs may coordinate a call for membership and an alternate through the TC-RPPO forum or any other process they agree.

C Selection

12. The Bureau will select members and invited experts to take part in the task force.

Appendix 08 – Criteria for CPM Recommendations

1. The following are the main criteria to be considered when reviewing proposed topics for CPM Recommendations:

- In all cases, the proposed topic should address issues that fit within the legal framework of the Convention, its International Standards for Phytosanitary Measures (ISPMs), or strategic goals.
- And as much as possible, the proposed topic should:
    1. address important issues related to plant health, either to promote action on a specific phytosanitary issue or to address a more generalized issue;
    2. be relevant to the needs of the contracting parties, or at least a majority of the parties;
    3. cover issues or actions that contracting parties or national or regional plant protection organizations have some influence, authority or competence to address;
    4. offer "guidance" that is not possible or appropriate to offer, at the moment, in the form of a standard and
    5. provide practical guidance and support for improving the implementation of the convention, a specific ISPM or set of ISPMs.

Appendix 09 – Roles and Functions of Regional Plant Protection Organizations (RPPOs) in their relationship with the Commission on Phytosanitary Measures (CPM)

Areas of cooperation between RPPOs and the IPPC Secretariat in accordance with article IX.3 of the IPPC include the following:

1. Standard setting process

- participation in the development of standards, such as identifying topics for standards and providing comments during the consultation periods;
- identification of regional standards that should be proposed as the basis for future ISPMs;
- action as collaborators and assistance in hosting standard setting meetings, as appropriate;
- preparation of draft explanatory documents on ISPMs according to paragraph 111 of the Report of the Sixth Session of the ICPM under the auspices of the IPPC Secretariat;
- provision of technical and administrative support to Standards Committee members;
- participation of RPPO observers in the Standards Committee meetings.

2. Implementation Facilitation and Capacity Development [or their new name/form]

- [joint] organization of IPPC regional workshops in their respective region
- facilitation of implementation of the IPPC and its ISPMs and identification of implementation challenges
- reporting on IPPC and ISPM implementation successes and challenges to the Technical Consultation among RPPOs
- contribution to avoidance and resolution of disputes
- cooperation with the IPPC Secretariat in the delivery of capacity development activities
- participation of RPPO representative[s] in the CDC [or their new name/form]
- contribution to the global implementation of ePhyto.

3. Communications

- collaboration among RPPOs and with the IPPC Secretariat in the dissemination and exchange of information through, for example: annual reports, workshops, questionnaires, surveys, draft calendars and work plans, publications, websites, and technical resources.

4. Coordination and partnership amongst RPPOs and with the IPPC Secretariat

- attendance and active participation in the TC and CPM;
- may assist in the nominations for CPM, subsidiary and other bodies;
- ensuring RPPO representation in the IPPC Strategic Planning Group (SPG);
- nominating RPPO representatives as required to CPM bodies and groups;
- participation in global initiatives, such as IYPH and ePhyto;
- support to member countries in meeting IPPC obligations in appropriate areas such as pest reporting;
- assistance in the translation of IPPC documents;
- in-kind cooperation with RPPOs or potential RPPOs requesting support;
- provision of information on regional related activities (on standards, regulations, etc.);
- cooperation with other regions in the organization and active participation in IPPC regional workshops and other capacity development activities;

- provision of technical resources to the IPPC resources page, or appropriate links.

The CPM is invited to:

(1) *Recall* that RPPOs are established under Article IX of the IPPC as coordinating bodies, in their respective geographical areas ;

(2) *Recall* the role of the Technical Consultation (TC) among RPPOs in addressing phytosanitary issues leading to the 1997 revised text of the IPPC and the need for the establishment of the Interim Commission on Phytosanitary Measures (ICPM);

(3) *Recall* the key role of RPPOs in the development, update and implementation of the IPPC and ISPMs, as outlined in the Convention and the IPPC Strategic Framework 2012-2019;

(4) *Recall* that recommendations were adopted by ICPM in 2005 on the roles and functions of RPPOs;

(5) *Request* that the IPPC Secretariat, SPG, CDC [or their new name/form] and CPM subsidiary bodies continue to collaborate with RPPOs as envisaged in this updated version of the RPPOs' roles and functions;

(6) *Encourage* RPPOs to continue to collaborate and strengthen their partnerships with each other and with the IPPC Secretariat as envisaged in this updated version of the RPPOs' roles and functions and in the 2015 IPPC Secretariat enhancement review;

(7) *Encourage* the active role of the Technical Consultation among RPPOs as one mechanism to facilitate this collaboration and to provide strategic input to the CPM Bureau and the CPM;

(8) *Recognize* that nothing in this [Decision] limits or replaces the rights or obligations of contracting parties under the IPPC;

(9) *Recognize* that nothing in this [Decision] affects the role of RPPOs or limits the activities that RPPOs may undertake;

(10) *Adopt* the revised version of the RPPOs' roles and functions in their relationship to the Commission on Phytosanitary Measures

Appendix 10 – Terms of Reference of The IPPC Subsidiary Body Implementation and Capacity Development Committee – A Subsidiary Body of the CPM

Note on interpretation
References to implementation mean implementation of the International Plant Protection Convention (IPPC), including standards, guidelines and recommendations adopted by the Commission on Phytosanitary Measures (CPM).

1. Purpose
The IC develops, monitors and oversees an integrated programme to support the implementation of the IPPC and strengthen the phytosanitary capacity of contracting parties.

2. Scope of the IPPC Implementation and Capacity Development Committee (IC)
The IC, under the guidance of the CPM, provides technical oversight of activities to enhance the capacities of contracting parties to implement the IPPC and meet the strategic objectives agreed by CPM.
The IC:

- Identifies and reviews the baseline capacity and capability required by contracting parties to implement the IPPC.
- Analyses issues constraining the effective implementation of the IPPC and develops innovative ways to address impediments.
- Develops and facilitates delivery of an implementation support programme to enable contracting parties to meet and surpass the baseline capacity and capability.
- Monitors and evaluates the efficacy and impact of implementation activities and reports on progress which indicates the State of Plant Protection in the World.
- Oversees dispute avoidance and settlement processes.
- Oversees national reporting obligation processes.
- Works with the Secretariat, potential donors and the CPM to secure sustainable funding for its activities.

3. Composition

- The IC is composed of twelve experts with relevant skills and experience in implementation of phytosanitary-related instruments and/or capacity development. The Bureau, taking account of the balance of skills and experience required, and geographical representation, selects and appoints the members.
- In addition, one representative from the regional plant protection organizations (RPPOs) and one from the Standards Committee (SC).

4. Functions
The IC has the following functions:

i) Technical work programme

- Identify and keep under review baseline capacity and capability required by contracting parties to implement the IPPC.
- Identify and propose strategies for contracting parties to enhance their implementation of the IPPC, including national reporting obligations, taking into account their specific capacities and needs.
- Review the Secretariat's analyses of contracting parties' challenges associated with the implementation of the IPPC.
- Based on an analysis of outputs from the above activities, recommend priorities to CPM.

- Identify and assess new technologies which could enhance implementation.
- Monitor and evaluate actions under the IPPC Strategic Framework, other related strategies, frameworks and work plan(s).

ii) Effective and efficient management of the IC

- Develop, agree and maintain a work plan in alignment with CPM priorities.
- Develop procedures and criteria for the production, oversight and approval of technical resources for implementation.
- Establish, dissolve and provide oversight of sub-groups, undertaking specific activities and tasks.
- Seek advice and/or input on matters relevant to its work programme from technical panels (through the SC) and other groups or organisations that assist the IPPC.
- Periodically review its functions, procedures and outcomes.
- Monitor and evaluate the effectiveness of its activities and products.

iii) Working with the Secretariat

- Develop and manage projects that contribute to achieving the implementation priorities agreed by CPM.
- Provide guidance on implementation and capacity development activities for inclusion in the Secretariat's work plan.
- Assess and prioritize for inclusion in the International Phytosanitary Portal (IPP) or the Phytosanitary Resources website, as appropriate, technical resources that are relevant for developing capacity to implement the IPPC.
- Promote dispute avoidance as an outcome of effective implementation.
- Oversee the dispute settlement process as required.
- Contribute to the development and maintenance of links with donors, partners and other public and private organizations concerned with implementation and capacity development in the phytosanitary area.

iv) Working with other subsidiary bodies

- Work in close collaboration with the SC to make standards setting and implementation complementary and effective.
- Review the Framework for Standards and Implementation annually and recommend changes to the CPM through the SPG.
- Work with other subsidiary bodies and RPPOs regarding areas of mutual interest.

v) Actions directed by CPM

- Contribute to the delivery of the IPPC Communications Strategy.
- Provide oversight of bodies that have been established by CPM and entrusted to the IC.
- Undertake other functions as directed by the CPM.
- Report to the CPM on its activities.

5. Relationship with the IPPC Secretariat

- The Secretariat is responsible for coordinating the work of the IC and providing administrative, editorial operational and technical, support. The Secretariat advises the IC on the availability and use of financial and staff resources.

6. Relationship with the Standards Committee

The IC collaborates with the SC on the basis of aligned work plans for the implementation of the IPPC. This collaboration will take place at a number of levels (e.g. Secretariat, chairs, members, stewards and

sub-groups). The IC includes an SC representative and also selects a representative for participation in SC meetings. Subjects for collaboration will include at least:

- Alignment of work programmes
- Development of implementation plans for standards
- Analysis of responses to calls for topics and issues to be addressed
- Review of the Framework for Standards and Implementation
- Development and implementation of joint projects.

7. Relationship with the RPPOs

RPPOs provide a regional perspective on issues, challenges and the region operating context impacting contracting parties and their NPPOs. RPPOs provide support to contracting parties to enhance their phytosanitary capacities and capabilities. The IC includes an RPPO representative. Areas for collaboration include:

- Exchange of draft work programmes
- Sharing of technical resources and information
- Identification and provision of experts
- Coordination of activities and events, including IPPC Regional Workshops
- Development and implementation of joint projects.

Rules of Procedure of The IPPC Implementation and Capacity Development Committee (IC) – A Subsidiary Body of the CPM

Rule 1. Membership

The IC is composed of 12 members plus one representative from the regional plant protection organizations (RPPOs) and one from the Standards Committee (SC) of the International Plant Protection Convention (IPPC).

Members are selected on the basis of a balance of expertise with at least one from each Food and Agriculture Organization of the United Nations (FAO) region and representation from developing countries. Members should have experience of either implementation of phytosanitary related instruments and/or capacity development and will be selected and appointed by the Commission on Phytosanitary Measures (CPM) Bureau.

The Technical Consultation (TC) among RPPOs and the SC each appoints a representative to the IC through their own processes.

The members and representatives will serve with utmost integrity, impartiality, and independence and will prevent and disclose in advance possible conflicts of interest that may arise in the course of carrying out their duties. If they occur, the Bureau will resolve cases of a conflict of interest.

Rule 2. Qualification for membership

Nominations for members will include documented evidence of their experience in implementation and/or capacity development. This experience should include at least one of the following:

- Demonstrated experience in managing phytosanitary systems;
- Demonstrated experience in delivering phytosanitary capacity development activities;
- In depth knowledge of the IPPC and International Standards for Phytosanitary Measures;
- Experience in the implementation of phytosanitary regulations;
- Other specific knowledge, qualifications and/or experience, for example in developing and delivering training.

Nominees will also have a level of English which will allow them to actively participate in IC meetings and discussions.

Rule 3. Procedure for selection of members

The Secretariat will issue a call for members when vacancies arise. Member nominations, including supporting information and a letter of commitment as specified in the call, may be formally submitted by contracting parties or RPPOs.
The CPM Bureau will review nominations against the list of requirements outlined in Rule 2.
Members serve for a term of three years which may be renewed on acceptance of the CPM Bureau.

Rule 4. Alternate and replacement members At least one alternate for each FAO region should be appointed following the selection process detailed in Rule 3 and serves for a term of three years which may be renewed in accordance with that Rule.
An alternate may attend a meeting of the IC in place of a member who is unable to attend.
If a member resigns, no longer meets the qualifications for membership set forth in these Rules, or fails to attend two consecutive meetings of the IC, the member will be replaced. The replacement will be decided by the Bureau maintaining the balance of expertise, and the need to have at least one member from each FAO region. A replacement member will serve for a term of three years starting from the time of appointment.

Rule 5. Chairperson and Vice-Chairperson
The chairperson and vice-chairperson of the IC are elected by its members and serve for a term of three years with the possibility of re-election on acceptance of the CPM Bureau.

Rule 6. Meetings
The IC will hold two physical meetings a year. Additional meetings may be held when necessary, subject to available staff and financial resources. Meetings of the IC may also be held through electronic means, including by video and teleconference, as necessary.
A majority of members will constitute the quorum to hold meetings.

Rule 7. Observers and participation of invited experts to IC meetings
Subject to the provisions of the below paragraph, meetings of the IC will be open, in accordance with the applicable FAO and CPM rules and procedures.
The IC may determine that certain meetings, or part thereof, be conducted without observers, in consideration of the sensitivity or confidentiality of the subject.
With the prior agreement, or at the request, of the IC members, the Secretariat may invite individuals or representatives of organizations with specific expertise, to participate as observers in a specific meeting or part thereof.

Rule 8. Bodies established by CPM
A subsidiary body established by the CPM may be entrusted to the oversight of the IC. These bodies will have their own terms of reference and rules of procedure which will have been agreed by the CPM during their establishment.

Rule 9. IC Sub-groups
The IC may establish sub-groups to address specific implementation and capacity development issues subject to availability of financial resources. The IC will determine in their terms of reference the tasks, duration, membership and reporting duties of these sub-groups.
The IC may dissolve subgroups when they are no longer required.

Rule 10. Decision-making
The IC will endeavour to make decisions on the basis of consensus between members.
Situations where consensus is required but cannot be reached shall be described in the meeting reports detailing all positions maintained and presented to the CPM for discussion and appropriate action.

Rule 11. Reporting
The IC will report to the CPM.

Appendix 11 – Recognition related to Standard Setting activities

Gratitude is expressed to the experts of the drafting groups for their active contribution in the development of the following ISPMs, or Annexes to ISPMs, adopted in 2016/2017:

1. ISPM 38 on the *International movement of seeds* (2009-003)

Country	Expert	Role
Australia	Mr Bruce HANCOCKS	EWG member
Brazil	Mr Edson Tadeu IEDE	TPFQ member
Cameroon	Ms Alice Ntoboh Siben NDIKONTAR	EWG member
Canada	Mr Eric ALLEN	Member as Chair of IFQRG
Canada	Ms Marie-Claude FOREST	TPFQ member
Canada	Mr Shane SELA	TPFQ member
Chile	Mr Juan Pablo LÓPEZ	Host representative
Chile	Mr Marcos Beéche CISTERNAS	TPFQ member
China	Mr Lifeng WU	TPFQ member
China	Ms Wenxia ZHAO	Host representative
China	Mr Yuejin WANG	Organizer representative
France	Ms Valérie GRIMAULT	EWG member
Germany	Mr Thomas Schröeder	TPFQ member
Ghana	Mr Joseph Mireku ASOMANING	EWG member
Ghana	Mr Victor AGYEMAN	TPFQ member
Italy	Mr Lucio MONTECCHIO	TPFQ member
Japan	Mr Masahiro SAI	EWG member and TPFQ member
New Zealand	Mr Michael ORMSBY	TPFQ member
Norway	Mr Sven Christer MAGNUSSON	TPFQ member
Paraguay	Ms Ana Peralta	Organizer representative
Poland	Mr Krzysztof SUPRUNIUK	TPFQ member
Poland	Mr Piotr WLODARCZYK	TPFQ member
Republic of Korea	Ms Mi Chi YEA	EWG member
South Africa	Ms Phindile N.B. NGESI	EWG member
USA	Mr Edward PODLECKIS	EWG member
USA	Mr John Tyrone JONES	TPFQ member

Country	Expert	Role
USA	Ms Marina ZLOTINA	TPFQ member
The Netherlands	Mr Gerard MEIJERINK	Invited expert
The Netherlands	Mr Corné VAN ALPHEN	Organizer representative
The Netherlands	Mr Nico HORN	Host representative
Zambia	Mr Arundel SAKALA	Steward (2008-11)
Australia	Mr David PORRITT	Steward (2010-04) and Assistant Steward (2012-04)
Cameroon	Mr Marcel BAKAK	Assistant Steward (2011-05)
Chile	Ms Soledad CASTRO-DOROCHESSI	Steward (2012-04) and Assistant Steward (2013-11)
Japan	Mr Motoi SAKAMURA	Assistant Steward (2012-11)
USA	Ms Julie ALIAGA	Steward (2013-11) and Assistant Steward (2012-11)
Argentina	Mr Ezequiel FERRO	Assistant Steward (2014-11)
The Netherlands	Mr Nico HORN	Steward 2015-05

2. Annex 1 *Arrangements for verification of compliance of consignments by the importing country in the exporting country* (2005-003) to ISPM 20 (*Guidelines for a phytosanitary import regulatory system*)

Country	Expert	Role
Brazil	Mr Gilvio Westin COSENZA	EWG member
New Zealand	Mr Wayne HARTLEY	EWG member
Chile	Ms Sylvia Soledad FERRADA Chamorro	EWG member
Republic of Korea	Ms Kyu-Ock KIM	EWG member
France	Ms Clara PACHECO	EWG member
USA	Mr Paul Gerard MCGOWAN	EWG member
Zambia	Mr Kenneth MSISKA	Host representative
South Africa	Mr Mike Holtzhausen	Steward (2005-04) and Assistant Steward (2012-04)
Zambia	Mr Arundel SAKALA	Assistant Steward (2008-11)
Australia	Mr Bart ROSSEL	Assistant Steward (2012-04)
Chile	Ms Soledad Castro-Dorochessi	Assistant Steward (2012-04)
Canada	Ms Marie-Claude FOREST	Steward (2012-04)
New Zealand	Mr Stephen BUTCHER	Assistant Steward (2012-11)

Country	Expert	Role
Mexico	Ms Ana Lilia MONTEALEGRE	Assistant Steward (2012-11)
Argentina	Mr Ezequiel FERRO	Steward (2016-05)

3. ISPM 39 on the *International movement of wood* (2006-029)

Country	Expert	Role
Ghana	Mr Victor AGYEMAN	TPFQ member
Brasil	Mr Edson Tadeu IEDE	TPFQ member
Canada	Mr Eric ALLEN	TPFQ member
Chile	Mr Marcos Beéche CISTERNAS	TPFQ member
Japan	Mr Mamoru MATSUI	TPFQ member
Germany	Mr Thomas SCHRÖDER	TPFQ member
China	Mr Yuejin WANG	Organizer representative
China	Mr Wenxia ZHAO	Host representative
Chile	Mr Fuxiang WANG	TPFQ member
Paraguay	Mr Juan Pablo LOPEZ	Host representative
Canada	Mr Shane SELA	TPFQ member
Canada	Mr Greg WOLFF	Steward (2006-05) and Assistant Steward (2009-11)
China	Mr Yuejin WANG	Organizer representative
China	Ms Wenxia ZHAO	Host representative
China	Mr Fuxiang WANG	TPFQ member
Chile	Mr Juan Pablo LÓPEZ	Host representative
Paraguay	Ms Ana PERALTA	Organizer representative
Norway	Mr Christer MAGNUSSON	TPFQ member and Assistant Steward (2007-11)
Canada	Ms Marie-Claude FOREST	Steward (2009-11) and TPFQ Assistant steward (2014-11)
India	Mr D.D.K. SHARMA	Assistant Steward (2013-05)
Canada	Mr Rajesh RAMARATHAM	Steward (2016-05)
USA	Ms Marina ZLOTINA	TPFQ Steward (2016-05)
China	Mr Lifeng WU	TPFQ Assistant Steward (2016-05)

Country	Expert	Role
Poland	Mr Piotr WLODARCZYK	TPFQ Steward (2014-11) and Assistant Steward (2012-11)
USA	Ms Julie ALIAGA	TPFQ Steward (2012-04)

4. ISPM 40 on the *International movement of growing media in association with plants for planting* (2005-004)

Country	Expert	Role
Iran	Mr Mohammed Reza Asghari	EWG member
Chile	Ms Eliana Bobadilla	EWG member
Australia	Ms Barbara Hall	EWG member
USA	Ms Carissa Marasas	EWG member
Germany	Mr Bjoern Niere	EWG member
Canada	Ms Barbara Peterson	EWG member
Canada	Mr Dominique Pelletier	Host representative
Canada	Ms Rebecca Lee	Organizer representative
Jordan	Mr Mohammad KATBEH-BADER	Steward (2005-04)
Canada	Ms Marie-Claude FOREST	Steward (2008-11)
Norway	Ms Hilde PAULSEN	Steward (2012-11) and Assistant Steward (2016-05)
Indonesia	Mr Antarjo DIKIN	Assistant Steward (2012-11)
Mexico	Ms Ana Lilia MONTEALEGRE	Steward (2016-05) and Assistant Steward (2013-11)
Brazil	Mr Jesulindo DE SOUZA	Assistant Steward (2016-05)

5. ISPM 41 on the *International movement of used vehicles, machinery and equipment* (2006-004)

Country	Expert	Role
Australia	Mr Adam BROADLEY	EWG member
Republic of Korea	Mr Jae-Seung LEE	EWG member
Finland	Mr Ralf Lothar LOPIAN	EWG member
New Zealand	Ms Melanie Jane NEWFIELD	EWG member
USA	Mr Tim N. STEVENS	EWG member
Nigeria	Mr Gabriel ADEJARE	Steward (2007-05)

Country	Expert	Role
Uganda	Mr Robert KARYEIJA	Steward (2007-11)
Argentina	Mr Guillermo ROSSI	Steward (2009-05)
Cook Islands	Mr Ngatoko NGATOKO	Steward (2012-11)
Brazil	Mr Alexandre PALMA	Steward (2015-05) and Assistant Steward (2012-11)
Chile	Mr Álvaro SEPÚLVEDA LUQUE	Steward (2015-11) and Assistant Steward (2015-05)
Papua New Guinea	Mr Pere KOKOA	Assistant Steward 2015-11

ISPMs developed by the Technical Panel on Phytosanitary Treatments as annexes to ISPM 28 (*Phytosanitary treatments for regulated pests*)

TPPT Stewards:

Country	Steward
Indonesia	Mr Antarjo DIKIN
Australia	Mr Bart ROSSEL

6. PT 22 on Sulfuryl fluoride fumigation treatment for insects in debarked wood (2007-101A)

Country	Expert	Role
New Zealand	Mr Mike ORMSBY	Treatment lead

7. PT 23 on Sulfuryl fluoride fumigation treatment for nematodes and insects in debarked wood (2007-101B)

Country	Expert	Role
New Zealand	Mr Mike ORMSBY	Treatment lead

8. PT 24 for Cold treatment for *Ceratitis capitata* on *Citrus sinensis* (2007-206A)

Country	Expert	Role
South Africa	Ms Alice BAXTER	Treatment lead
Argentina	Mr Eduardo WILLINK	Treatment lead
USA	Mr Scott MYERS	Assistant Treatment Lead

9. PT 25 for Cold treatment for *Ceratitis capitata* on *Citrus reticulata x C. sinensis* (2007-206B)

Country	Expert	Role
USA	Mr Scott WOOD (US)	Treatment Lead
USA	Mr Patrick GOMES (US)	Treatment Lead
Argentina	Mr Eduardo WILLINK (AR)	Treatment Lead

New Zealand	Mr Mike ORMSBY	Assistant Treatment Lead

10. PT 26 for Cold treatment for *Ceratitis capitata* on *Citrus limon* (2007-206C)

Country	Expert	Role
China	Mr Yuejin WANG	Treatment lead
New Zealand	Mr Mike ORMSBY	Assistant Treatment lead

11. PT 27 for Cold treatment for *Ceratitis capitata* on *Citrus paradisi* (2007-210)

Country	Expert	Role
USA	Mr Scott WOOD (US)	Treatment lead
USA	Mr Patrick GOMES (US)	Treatment lead
China	Mr Daojian YU	Treatment lead
USA	Mr Scott MYERS	Assistant Treatment Lead

12. PT 28 for Cold treatment for *Ceratitis capitata* on *Citrus reticulata* (2007-212)

Country	Expert	Role
New Zealand	Mr Mike ORMSBY	Treatment lead

13. PT 29 for Cold treatment for *Ceratitis capitata* on *Citrus clementina* (2010-102)

Country	Expert	Role
UK	Mr Ray CANNON (UK)	Treatment lead
Australia	Mr Andrew JESSUP (AU)	Treatment lead
Argentina	Mr Eduardo WILLINK	Treatment lead
USA	Mr Guy HALLMAN	Assistant Treatment Lead

14. PT 30 for Vapour heat treatment for *Ceratitis capitata* on *Mangifera indica* (2010-106)

Country	Expert	Role
(US/IAEA)	Mr Guy HALLMAN	Treatment lead
Republic of Korea	Mr Min-Goo PARK (KR)	Treatment lead
USA	Mr Scott WOOD (US)	Treatment lead

15. PT 31 for Vapour heat treatment for *Bactrocera tryoni* on *Mangifera indica* (2010-107)

Country	Expert	Role
(US/IAEA)	Mr Guy HALLMAN	Treatment lead

ISPMs developed by the Technical Panel on Diagnostic Protocols as annexes to ISPM 27 (*Diagnostic protocols for regulated pests*)

TPDP Stewards:

Country	Steward
Germany	Mr Jens-Georg UNGER
UK	Ms Jane CHARD

16. DP 13 *Erwinia amylovora* (2004-009)

Country	Expert	Role
Spain	Ms Maria M. López GONZÁLEZ	Lead author
New Zealand	Mr Robert Taylor	Co-author
Australia	Mr Brendan RODONI	Discipline lead (TPDP member)
Canada	Mr Delano James	Referee (TPDP member)
Canada	Mr Solke H de Boer	Expert
Canada	Mr Won-Sik Kim	Expert
Germany	Mr Klaus Geider	Expert
Germany	Ms Annette Wensing	Expert
Spain	Mr J. Peñalver	Expert
Spain	Ms M.T. Gorris	Expert
Spain	Mr P. Llop	Expert
Spain	Mr Mariano Cambra	Expert
USA	Mr. Roberts	Expert
USA	Mr Larry Pusey	Expert
USA	Ms Virginia Stockwell	Expert

17. DP 14 *Xanthomonas fragariae* (2004-012)

Country	Expert	Role
USA	Mr Ed CIVEROLO	Lead author
Spain	Ms María M. López GONZÁLEZ	Co-author
UK	Mr John ELPHINSTONE	Co-author
New Zealand	Mr Robert TAYLOR	Discipline lead (TPDP member)
Netherlands	Mr Hans DE GRUYTER	Referee (TPDP member)
Canada	Mr Solke H. DE BOER	Expert
Canada	Mr Stephan BRIERE	Expert

18. DP 15 *Citrus tristeza virus* (2004-021)

Country	Expert	Role
Spain	Mr Mariano CAMBRA	Lead author
South Africa	Mr Stephanus Petrus	Co-author
USA	Ms Marta Isabel Mastalli	Co-author
USA	Ms Laurene LEVY	Co-author
Canada	Mr Delano JAMES	Discipline lead (TPDP member)
Australia	Mr Brendan RODONI	Referee (TPDP member)
Brazil	Mr Edson BERTOLINI	Expert
South Africa	Mr S.P.Fanie. van Vuuren	Expert
Uruguay	Ms M.I. Francis	Expert

19. DP 16 Genus *Liriomyza* (2006-017)

Country	Expert	Role
Australia	Mr Mallik MALIPATIL	Lead author
Australia	Mr Mark Blacket	Co-author
UK	Mr Dominique COLLINS	Co-author
Jamaica	Ms Juliet GOLDSMITH	Discipline lead (TPDP member)
USA	Mr Norman Barr	Referee (TPDP member)
Australia	Mr Anthony Rice	Expert
Japan	Mr Ren Iwaizumi	Expert
Latvia	Ms Ramona Vaitkevica	Expert
USA	Mr Stephen Gaimari	Expert

20. DP 17 *Aphelenchoides besseyi, A. ritzemabosi and A. fragariae* (2006-025)

Country	Expert	Role
USA	Mr Fengru ZHANG	Lead author
China	Mr Xie HUI	Co-author
South Africa	Mr Rinus KNOETZE	Co-author
UK	Ms Sue HOCKLAND	Co-author
France	Ms Géraldine ANTHOINE	Discipline lead (TPDP member)
Netherlands	Mr Hans DE GRUYTER	Referee (TPDP member)

21. DP 18 *Anguina* spp. (2013-003)

Country	Expert	Role
USA	Ms Andrea Skantar	Lead author
UK	Mr Thomas Prior	Co-author
UK	Mr Colin Fleming	Co-author
France	Ms Géraldine ANTHOINE	Discipline lead (TPDP member)
New Zealand	Mr Robert TAYLOR	Referee (TPDP member)
Kenya	Ms Pamela Kibwage	Expert
Poland	Mr Witold Karnkowski	Expert
Spain	Mr Juan Antonio Lezaun	Expert

22. DP 19 *Sorghum halepense* (2006-027)

Country	Expert	Role
China	Mr Qiang SHENG	Lead author
Turkey	Mr Ahmet ULUDAG	Co-author
USA	Mr Rodney YOUNG	Co-author
China	Ms Yin Linping	Discipline lead (TPDP member)
France	Ms Géraldine ANTHOINE	Referee (TPDP member)
Canada	Ms Cheryl DOLLARD	Expert
Canada	Ms Ruojing WANG	Expert
China	Mr Yonghong Zhou	Expert
China	Ms Jianqiu Zou	Expert
China	Ms Xiuling Shao	Expert
China	Mr Guoqi Chen	Expert
China	Mr Hongjie Xie	Expert
China	Mr Fuxiang WANG	Expert

23. DP 20 *Dendroctonus ponderosae* (2006-019)

Country	Expert	Role
Australia	Ms Linda Semeraro	Lead author
Brazil	Mr Edson Tadeu Iede	Co-author
Canada	Mr Hume Douglas	Co-author

Country	Expert	Role
France	Mr Jean-Francois Germain	Co-author
Netherlands	Ms Brigitta Wessels-Berk	Co-author
USA	Mr Norman BARR	Discipline lead (TPDP member)
France	Ms Géraldine ANTHOINE	Referee (TPDP member)

24. DP 21 *Candidatus* Liberibacter solanacearum (2013-001)

Country	Expert	Role
New Zealand	Ms Lia W. LIEFTING	Lead author
Spain	Ms María M. López GONZÁLEZ	Co-author
USA	Mr Joseph MUNYANEZA	Co-author
New Zealand	Mr Robert TAYLOR	Discipline lead (TPDP member)
Australia	Mr Brendan RODONI	Referee (TPDP member)

25. DP 22 *Fusarium circinatum* (2006-021)

Country	Expert	Role
UK	Ms Ana Pérez-Sierra	Lead author
France	Mr Renaud Ioos	Co-author
Kenya	Mr James Wanjohi MUTHOMI	Co-author
South Korea	Mr Ik-Hwa HYUN	Co-author
Netherlands	Mr Hans DE GRUYTER	Discipline lead (TPDP member)
New Zealand	Mr Robert Taylor	Referee (TPDP member)
Australia	Ms Jacqueline Edwards	Expert
Kenya	Mr William Muiru	Expert
Spain	Ms Mónica Berbegal Martínez	Expert

Appendix 12 – Procedure for Language Review Groups

Agreed by CPM-5 (2010); revised by CPM-6 (2011), CPM-8 (2013) and CPM-12 (2017)

Procedure to correct errors in International Standards for Phytosanitary Measures (ISPMs) in language versions other than English after adoption

1. Representatives from national plant protection organizations (NPPOs) and regional plant protection organizations (RPPOs) from each FAO language group, other than English, are invited to organize a Language Review Group (LRG) to consider the preferred use of terminology and to identify editing and formatting errors resulting from translation. Each LRG should identify a coordinator for communications with the Secretariat, describe how they will organize communications within the group (e.g. teleconference, exchange of documents etc.), explain its structure and respond to queries from members on how to join the LRG. Each LRG should invite a representative from the appropriate FAO language translation group and the respective TPG member(s) for that language to participate in order to ensure a clear understanding of the LRG issues.

2. Once established and recognized by the Secretariat, each LRG is invited to review adopted ISPMs and submit comments, in track changes, on terminology preferences, editorial and formatting mistakes to the Secretariat through their identified coordinator no later than three months after they have been advised that the adopted ISPMs are posted on the IPP (www.ippc.int); this time begins for the specified language once the ISPM has been posted on the IPP in that language.

3. FAO Translation services may participate as a member of the LRG but any official communication on proposed changes to the ISPMs should come from the LRG Coordinator to the IPPC Secretary (ippc@fao.org) in order to maintain version control of the standards.

4. If no comments are submitted, the version adopted at CPM would remain the final version.

5. If comments are submitted by the LRG coordinators through the above process, the Secretariat will forward the comments, in track changes, to the FAO Translation services.

6. The FAO Translation services will review the proposed changes. If all proposed changes are acceptable by the FAO translation services, the track change version of the ISPM produced by the LRG will be forwarded to the Secretariat. If FAO Translation services disagree with any of the LRG proposed changes, they will document the reasons and consult with the LRG to discuss and seek consensus. If consensus cannot be achieved, the FAO Translation service will make the final decision and provide explanations in writing and the Secretariat will make them available to IPPC contracting parties.

7. Comments regarding the translation of glossary terms will be transmitted to the Technical Panel for the Glossary (TPG) through the SC as they may result in consequential changes to numerous ISPMs. Formatting issues would be addressed by the Secretariat.

8. The Secretariat will post the modified ISPMs on the IPP and notify all contracting parties. The CPM agenda will include a standing item for noting that the specific standards were adjusted.

9. The CPM will note that the specific standards were adjusted and revoke previously adopted versions of the ISPMs.

Further information on LRG may be found on the IPP page:
https://www.ippc.int/en/core-activities/governance/standards-setting/ispms/language-review-groups/

Appendix 13 – Proposed ”Outputs” and ”Outcomes of the IYPH”

Objective	Output	Outcome
1. Raising the awareness of the public and political decision makers at the global, regional and national levels about plant health.	• More political and other decision makers know about plant health.	a. Increased compliance with IPPC and its standards b. Increased number of countries develop or update national legal plant health frameworks (through NRO) and is reflected in national agricultural policies c. Adoption of regional policies on the importance of plant health by Regional ministerial conferences.
	• Public is aware about plant health.	a. Public acts responsibly
	• Establishment of 6th December as an International Day of Plant Health.	a. Further increase of awareness about plant health
2. Promoting and strengthening of national, regional and global plant health efforts and their resources in light of increasing trade and new pest risks caused through climate change.	• Increased resources for plant health. • Strengthened capacity building activities. • Strengthened plant health disciplines.	a. A global strategic framework for plant health aligned with 2030 agenda for sustainable development is adopted. b. Increased number of countries with active participation of national experts in Regional Plant Protection meetings. c. All regions have a Regional Plant Protection Organization. d. Improved budgets for national, regional and global plant health services. e. Established sustainable financial mechanism for the IPPC. f. Better use of new plant pest management and control techniques. g. Increased availability of taxonomic and diagnostic expertise. h. Applied new technologies for trade facilitation (e.g. ePhyto).
3. Educating the public and increasing its knowledge about plant health.	• Public is educated about plant health.	a. Educational systems incorporate plant health matters. b. Increased reflection of plant health matters in academic curricula.
4. Enhancing dialogue and stakeholder involvement in plant health.	• Strengthened public/private partnerships on plant health at national, regional and global level.	a. More stakeholders are aware of the importance and benefits of plant health systems.
5. Increasing information about the state of plant protection in the world.	• Information on the state of plant protection in the world is available.	a. Adoption and publication of the “Review of the Status of Plant Protection in the World” (Article 11, 2 (a) of IPPC). b. Pest alert systems are improved and applied.
6. Facilitating the establishment of plant health partnerships on national, regional and global levels.	• Plant health partnerships are established on national, regional and global levels.	a. Better international networking structure on plant health. b. Increased linkage between plant health systems and organizations working on climate change, environmental protection, border control. c. Improved functional collaboration with research community.

Appendix 14 – Financial report of the IPPC Multi-Donor Trust Fund for 2016

Contributions	2004-2013*	2014	2015	2016
Australia		139,695	-	150,000
Canada		337,255	-	-
Ireland		-	27,352	-
France		-	-	25,000
Japan		28,500	40,000	-
Netherlands		50,000	-	-
New Zealand		-	100,000	38,929
Republic of Korea		100,000	162,597	311,126
South Africa		-	137,642	-
Sweden		70,000	-	-
USA/NAPPO		-	-	140,000
Other		3,381	2,619	1,343
Total	**2,938,606**	**728,831**	**470,210**	**666,398**

Expenditures by Cost type**	2004-2013*	2014	2015	2016
Professional and General service staff		240,328	630,182	237,082
Consultants		81,381	15	-
Travel		90,316	618	-
Contracts		92,626	89,400	-
Other		48,372	43,437	14,224
Total	**2,137,308**	**553,023**	**763,652**	**251,306**

Expenditures by Core activity**	2004-2013*	2014	2015	2016
IPPC Governance/Management/Strategy		279,453	168,389	-
Standard Setting		38,261	16,068	-
Implementation Facilitation		235,309	579,195	251,306
Total	**2,137,308**	**553,023**	**763,652**	**251,306**

Balance	**801,298**	**977,106**	**683,664**	**1,098,756**

* For easier reference, prior years (2004-2013) are grouped

** Total expenditures are the same, the difference is only in the presentation of the expenditure structure

Appendix 15 – Confirmed new members and potential replacements for CPM Bureau and Standards Committee and current membership of the SBDS

TABLE 01 – Membership of the Bureau of the CPM

Region	Country	Name	Nominated/ Re-nominated	Current term/duration	End of current term
Africa	Cote D'Ivoire	Mr Lucien KOUAME KONAN	CPM-7 (2012) CPM-9 (2014) CPM-11 (2016)	3rd Term/2 years	2018
Asia	Republic of Korea	Ms Kyu-Ock YIM	CPM-5 (2010) CPM-7 (2012) CPM-9 (2014) CPM-11 (2016)	4th term / 2 years	2018
Europe	Netherlands	Mr Cornelis Antonius Maria VAN ALPHEN	CPM-9 (2014) CPM-11 (2016)	2nd term / 2 years	2018
Latin America and Caribbean (Vice-Chairperson)	Mexico	Mr Francisco Javier TRUJILLO ARRIAGA	CPM-11 (2016)	1st term/ 2 years	2018
Near East	Sudan	Mr Kamal El Din Abdelmahmoud Amein BAKR	CPM-11 (2016)	1st term/ 2 years	2018
North America	Canada	Ms Marie-Claude FOREST	CPM-11 (2016)	1st term / 2 years	2018
Southwest Pacific (Chairperson)	Australia	Ms Lois RANSOM	CPM-7 (2012) CPM-11 (2016)	2nd term / 2 year	2018

TABLE 2 – Replacements of the Bureau of the CPM

Region	Country	Name	Nominated/ Renominated	Current term/duration	End of current term
Africa	Cameroon	Mr Edouard NYA	CPM-12 (2017)	Replacement for Mr Francis LEKU AZENAKU CPM-11 (2016)/1st term/ 2 years	2018
	Vacant, 2nd replacement optional				
Asia	1 China	Mr Wang FUXIANG	CPM-11 (2016)	1st term/ 2 years	2018
	2 Indonesia	Mr Antarjo DIKIN	CPM-11 (2016)	1st term/ 2 years	2018
Europe	1 Malta	Ms Marica GATT	CPM-12 (2017)	Replacement for Ms Emmanuelle SOUBEYRAN CPM-11 (2016)/1st term/ 2 years	2018
	2 United Kingdom	Mr Samuel BISHOP	CPM-12 (2017)	Replacement for VACANT position CPM-11 (2016)/1st term/ 2 years	2018
Latin America and Caribbean	Argentina	Mr Diego QUIROGA	CPM-11(2016)	1st term/ 2 years	2018
	Vacant, 2nd replacement optional				
Near East	Egypt	Mr Ibrahim Imbaby EL SHOBAKI	CPM-11 (2016)	1st term/ 2 years	2018
	Vacant, 2nd replacement optional				
North America	USA	Mr John GREIFER	CPM-11 (2016)	1st term/ 2 years	2018

	Vacant, 2nd replacement optional				
Southwest Pacific	Australia	Mr Kim RITMAN	CPM-11 (2016)	1st term/ 2 years	2018
	Vacant, 2nd replacement optional				

STANDARDS COMMITTEE MEMBERSHIP AND POTENTIAL REPLACEMENTS

TABLE 03 - Standards Committee Membership

FAO region	Country	Name	Nominated/ Re-nominated	Current term/duration	End of current term
	Algeria	Ms Alphonsine LOUHOUARI TOKOZABA	Replacement member for Ms Nadia HADJERES CPM-10 (2015)	Replacement	2018
	Kenya	Ms Esther KIMANI	CPM-9 (2014) CPM-12 (2017)	2nd term / 3 years	2020
Africa	Malawi	Mr David KAMANGIRA	CPM-11 (2016)	1st term / 3 years	2019
	Nigeria	Mr Moses Adegboyega ADEWUMI	Replacement member for Ms Alice Ntoboh Sibon NDIKONTAR CPM-10 (2015)	Replacement	2018
	Indonesia	Mr HERMAWAN	CPM-11 (2016)	1st term / 3 years	2019
Asia	Japan	Mr Masahiro SAI	Replacement member for Mr Lifeng WU CPM-10 (2015)	Replacement	2018
	Kingdom of Thailand	Ms Walaikorn RATTANADECHAKUL	CPM-10 (2015)	1st term / 3 years	2018
	Vietnam	Ms Thanh Huong HA	CPM-7(2012) CPM-10 (2015)	2nd term / 3 years	2018
Europe	France	Ms Laurence BOUHOT-DELDUC	CPM-10 (2015)	1st term / 3 years	2018
	Israel	Mr David OPATOWSKI[64]	CPM-1 (2006) CPM-4 (2009) CPM-12 (2017)	3rd term / 3 years	2020
	Netherlands	Mr Nicolaas Maria HORN	CPM-9 (2014) CPM-12 (2017)	2nd term / 3 years	2020
	United Kingdom	Mr Samuel BISHOP	Replacement member for Ms Hilde Kristin PAULSEN CPM-10 (2015)	Replacement	2018
	Argentina	Mr Ezequiel FERRO	CPM-8 (2013) CPM-11 (2016)	2nd term / 3 years	2019
	Brazil	Mr Jesulindo Nery DE SOUZA JUNIOR	CPM-11 (2016)	1st term / 3 years	2019

[64] *Under exceptional circumstances this SC membership takes effect immediately*

FAO region	Country	Name	Nominated/ Re-nominated	Current term/duration	End of current term
Latin America and Caribbean	Chile	Mr Álvaro SEPÚLVEDA LUQUE	CPM-10 (2015)	1st term / 3 years	2018
	Mexico	Ms Ana Lilia MONTEALEGRE LARA	CPM-7 (2012) CPM-10 (2015)	2nd term / 3 years	2018
Near East	Egypt	Ms Shaza OMAR	CPM-11 (2016)	1st term / 3 years	2019
	Jordan	Mr Nazir Al-BDOUR	CPM-11 (2016)	1st term / 3 years	2019
	Lebanon	Mr Youssef Al MASRI	CPM-11 (2016)	1st term / 3 years	2019
	Libya	Mr Ali Amin KAFU	Replacement member for Ms Maryam JALILI MOGHADAM CPM-11 (2016)	Replacement	2019
North America	Canada	Mr Rajesh RAMARATHNAM	CPM-11 (2016)	1st term / 3 years	2019
	USA	Ms Marina ZLOTINA	CPM-10 (2015)	1st term / 3 years	2018
Southwest Pacific	Australia	Mr Bruce HANCOCKS	CPM-12 (2017)	1st term / 3 years	2020
	New Zealand	Mr Stephen BUTCHER	Replacement member for Mr John HEDLEY CPM-11 (2016)	Replacement	2019
	Samoa	Mr Lupeomanu Pelenato FONOTI	CPM-12 (2017)	1st term / 3 years	2020

TABLE 04 - Standards Committee Potential Replacements

FAO region	Order	Country	Name	Nominated / Re-nominated	Current term/duration	End of current term
Africa	1	Guinea Bissau	Mr Lois Antonio TAVARES	CPM-12 (2017)	1st term / 3 years	2020
	2	Burundi	Mr Eliakim SAKAYOYA	CPM-11 (2016)	1st term / 3 years	2019
Asia	1	Philippines	Ms Merle Bautista PALACPAC	CPM-11 (2016)	1st term / 3 years	2019
	2	Sri Lanka	Ms. Jayani Wathukarage NIMANTHIKA	CPM-12 (2017)	1st term / 3 years	2020
Europe	1	Estonia	Ms Olga LAVRENTJEVA	CPM-12 (2017)	1st term / 3 years	2020
	2		VACANT			
Latin America and Caribbean	1	Panama	Ms Judith Ivette VARGAS AZCÁRRAGA	CPM-9 (2014) CPM-12 (2017)	2nd term / 3 years	2020
	2	Dominica	Mr Nelson LAVILLE	CPM-11 (2016)	1st term / 3 years	2019
Near East	1	Iraq	Mr Abbas ABDULQADER KHUDHAIR	CPM-12 (2017)	1st term / 3 years	2020
	2	Yemen	Mr Gamil Anwar Mohammed RAMADHAN	CPM-12 (2017)	1st term / 3 years	2020
North America	To replace Canada	Canada	Ms Marie-Claude FOREST	CPM-11 (2016)	1st term/ 3 years	2019
	To replace USA	USA	Ms Stephanie DUBON	CPM-11 (2016)	1st term / 3 years	2019
Southwest Pacific	1	To replace New Zealand or Australia	Ms Sophie Alexia PETERSON	CPM-12 (2017)	1st term / 3 years	2020
	2		VACANT			

SUBSIDIARY BODY ON DISPUTE SETTLEMENT: MEMBERSHIP AND POTENTIAL REPLACEMENTS

TABLE 05 - Subsidiary Body on Dispute Settlement Membership

FAO region	Country	Name	Nominated / Re-nominated	Current term / Duration	End of current term
Africa	Gabon	Ms Seraphine MINKO	CPM-10 (2015) CPM-12 (2017)	2nd term / 2 years	2019
Asia		VACANT			
Europe	France	Ms Clara PACHECO	CPM-12 (2017)	1st term / 2 years	2019
Latin America and Caribbean	Panama	Mr Luis BENAVIDES	CPM-8 (2013) CPM-10 (2015) CPM-12 (2017)	3rd term / 2 years	2019
Near East	Yemen	Mr Abdulah AL SAYANI	CPM-9 (2014) CPM-11 (2016)	2nd term / 2 years	2018
North America	Canada	Mr Steve CÔTÉ	CPM-7 (2012) CPM-9 (2014) CPM-11 (2016)	3rd term/ 2 years	2018
Southwest Pacific	Samoa	Mr Lupeomanu Pelenato FONOTI	CPM-11 (2016)	1st term / 2 years	2018

Appendix 16 - The IPPC Secretariat Work Plan and IPPC Multi-Donor Trust Fund budget for 2017 and the IPPC Secretariat Regular programme budget for 2017

Appendix 17 - Adoption of International Standards for Phytosanitary Measures

[1] The CPM adopted the following ISPMs and PTs (attached to this report):

- ISPM 38 on the *International movement of seeds* (2009-003)
- Annex 1 *Arrangements for verification of compliance of consignments by the importing country in the exporting country* (2005-003) to ISPM 20 (*Guidelines for a phytosanitary import regulatory system*)
- ISPM 39 on the *International movement of wood* (2006-029)
- ISPM 40 on the *International movement of growing media in association with plants for planting* (2005-004)
- ISPM 41 on the *International movement of used vehicles, machinery and equipment* (2006-004)

- ❖ PT 22 Sulfuryl Fluoride fumigation treatment for insects in debarked wood (2007-101A)
- ❖ PT 23 as Sulfuryl Fluoride fumigation treatment for nematodes and insects in debarked wood (2007-101B)
- ❖ PT 24 Cold treatment for *Ceratitis capitata* on *Citrus sinensis* (2007-206A)
- ❖ PT 25 Cold treatment for *Ceratitis capitata* on *Citrus reticulata x C. sinensis* (2007-206B)
- ❖ PT 26 Cold treatment for *Ceratitis capitata* on *Citrus limon* (2007-206C)
- ❖ PT 27 Cold treatment for *Ceratitis capitata* on *Citrus paradisi* (2007-210)
- ❖ PT 28 Cold treatment for *Ceratitis capitata* on *Citrus reticulata* (2007-212)
- ❖ PT 29 Cold treatment for *Ceratitis capitata* on *Citrus clementina* (2010-102)
- ❖ PT 30 Vapour heat treatment for *Ceratitis capitata* on *Mangifera indica* (2010-106)
- ❖ PT 31 Vapour heat treatment for *Bactrocera tryoni* on *Mangifera indica* (2010-107)

[2] The CPM noted that the SC adopted on behalf of CPM the following ten diagnostic protocols (DPs) as Annexes to ISPM 27 (attached to this report):

- DP 13: *Erwinia amylovora*
- DP 14: *Xanthomonas fragariae*
- DP 15: *Citrus tristeza virus*
- DP 16: *Genus Liriomyza* Mik
- DP 17: *Aphelenchoides besseyi, A. ritzemabosi and A. fragariae*
- DP 18: *Anguina* spp. (2013-003)
- DP 19: *Sorghum halepense* (2006-027)
- DP 20: *Dendroctonus ponderosae* (2006-019)
- DP 21: *Candidatus* Liberibacter solanacearum (2013-001)
- DP 22: *Fusarium circinatum* (2006-021)

INTERNATIONAL STANDARDS FOR
PHYTOSANITARY MEASURES

ISPM 38
International movement of seeds

Produced by the Secretariat of the
International Plant Protection Convention
Adopted 2017; published 2017

Publication history

This is not an official part of the standard

2009-11 SC introduced topic International movement of seed (2009-003).

2010-03 CPM-5 added topic.

2010-12 SC approved draft specification for member consultation via e-decision.

2011-02 Draft specification sent to consultation.

2011-05 SC revised and approved specification 54.

2013-07 EWG drafted ISPM.

2013-10 EWG participants reviewed draft ISPM.

2013-12 Steward reviewed draft ISPM.

2014-04 Steward consulted EWG and revised draft ISPM based on TPG comments on consistency.

2014-05 SC approved draft ISPM for consultation.

2014-07 First consultation.

2015-02 Steward reviewed comments and revised draft.

2015-05 SC-7 reviewed draft (not recommended for 2015 second consultation).

2016-01 Assistant steward and Steward reviewed comments

2016-05 SC-7 revised draft and approved for second consultation.

2016-06 TPFQ reviewed and suggested changes to cover the issue of forest tree seeds; Steward and SC-7 slightly adjusted proposed text.

2016-07 Second consultation.

2016-11 SC approved to send to CPM-12.

2017-04 CPM-12 adopted the standard.

ISPM 38. 2017. *International movement of seeds*. Rome, IPPC, FAO.

Publication history last updated: 2017-04

CONTENTS

Adoption

This standard was adopted by the Twelfth Session of the Commission on Phytosanitary Measures in April 2017.

INTRODUCTION

Scope

This standard provides guidance to assist national plant protection organizations (NPPOs) in identifying, assessing and managing the pest risk associated with the international movement of seeds (as a commodity class).

The standard also provides guidance on procedures to establish phytosanitary import requirements to facilitate the international movement of seeds; on inspection, sampling and testing of seeds; and on the phytosanitary certification of seeds for export and re-export.

Under ISPM 5 (*Glossary of phytosanitary terms*) seeds (as a commodity class) are intended for planting and not for consumption. Viable seeds, which are a sample of a seed lot, imported for laboratory testing or destructive analysis are also addressed by this standard.

This standard does not apply to grain or vegetative plant parts (e.g. tubers of potatoes).

References

The present standard refers to International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

Definitions

Definitions of phytosanitary terms used in this standard can be found in ISPM 5.

In addition to the definitions in ISPM 5, in this standard the following definitions apply.

Seed-borne pest	A pest carried by seeds externally or internally that may or may not be transmitted to plants growing from these seeds and cause their infestation
Seed-transmitted pest	A seed-borne pest that is transmitted via seeds directly to plants growing from these seeds and causes their infestation

Outline of Requirements

Seeds, as with other plants for planting, may present a pest risk because they may be introduced to an environment where pests associated with the seeds have a high probability of establishing and spreading.

Seeds are regularly moved internationally for commercial and research purposes. Therefore, when assessing the pest risk and determining appropriate phytosanitary measures, NPPOs should consider the intended use of the seeds (research, planting under restricted conditions or planting under natural conditions).

A pest risk analysis (PRA) should determine if the seeds are a pathway for the entry, establishment and spread of quarantine pests and their potential economic consequences in the PRA area, or if the seeds are a pest themselves or a pathway and the main source of infestation of regulated non-quarantine pests. The PRA should consider the purpose for which the seeds are imported (e.g. field planting, research, testing) and the potential for quarantine pests to be introduced and spread or for regulated non-quarantine pests to cause an economically unacceptable impact when present above a threshold.

Specific phytosanitary measures may be used to reduce the pest risk associated with the international movement of seeds, including phytosanitary measures that may be applied before planting, during growth, at seed harvest, post-harvest, during seed processing, storage and transportation, and on arrival in the importing country. Phytosanitary measures may be used either alone or in combination to manage the pest risk. Phytosanitary import requirements may be met by applying equivalent phytosanitary measures.

BACKGROUND

Seeds are moved internationally for many uses. They are planted for the production of food, forage, ornamental plants, biofuels and fibre as well as for forestry and for pharmacological uses. They also have pre-commercial uses (research, breeding and seed multiplication).

As with other plants for planting, seeds may present a pest risk when introduced to an environment where any pests associated with the seeds have a high probability of establishing and spreading (ISPM 32 (*Categorization of commodities according to their pest risk*)).

Seed companies may have breeding and multiplication programmes in several countries, and may distribute seeds from these countries to many other countries. Moreover, research and breeding are conducted internationally to develop new varieties that are adapted to a range of environments and conditions. The international movement of seeds may involve small or large quantities of seeds.

Contracting parties face challenges associated with the international movement of seeds that are distinct from the international movement of other types of plants for planting. For example, seeds produced in one country and exported to a second country for processing (e.g. pelleting and coating), testing and packing may then be re-exported to numerous other destinations (including the country of origin). At the time of production of the seeds, the destination countries and their phytosanitary import requirements may not be known, especially if a number of years pass between production and export to the final destinations.

IMPACTS ON BIODIVERSITY AND THE ENVIRONMENT

This standard may help manage the pest risk posed by seeds moved internationally, including the pest risk posed by invasive alien species (as defined in the Convention on Biological Diversity).

Harmonized international phytosanitary measures for seeds may help preserve biodiversity by increasing the potential for exchanging healthy seeds (free from pests).

REQUIREMENTS

1. Pest Risk Analysis

PRA for seeds performed in accordance with ISPM 2 (*Framework for pest risk analysis*), ISPM 11 (*Pest risk analysis for quarantine pests*) and ISPM 21 (*Pest risk analysis for regulated non-quarantine pests*) should identify the regulated pests potentially associated with seeds and seeds as pests. The PRA should consider the purpose for which seeds are imported (e.g. field planting, research, testing) and the probability of regulated pests establishing and spreading and in consequence causing economic impacts (ISPM 32).

1.1 Seeds as pests

PRA for seeds as pests should follow the guidance provided in Annex 4 of ISPM 11.

1.2 Seeds as pathways

In PRA for seeds as pathways, the ability of a pest to transfer to a suitable host and cause infestation needs specific consideration to identify pests that warrant regulation.

Some seed-borne pests associated with a suitable host upon entry may result in infestation of the host when the seed is planted while others may not.

Seed-borne pests include:

- seed-transmitted pests that are carried by the seed internally or externally and directly infest the host plant growing from the seed (category 1(a))
- non-seed-transmitted pests that are carried by the seed internally or externally and are transferred to the environment (e.g. water, soil) and then infest a host plant under natural conditions (category 1(b))
- pests carried by the seed, internally or externally, that do not transfer to a host plant under natural conditions (category 1(c)).

A further category of pests may be relevant even though the pests are not seed-borne. This is the category of contaminating pests present in a seed lot (including seeds of plants as pests) (category 2).

Pests in categories 1(a), 1(b) and 2 should be further assessed for establishment, spread and economic impacts. Pests in category 1(c) cannot establish because they are not transferred to a suitable host.

Examples of pests in each category are provided in Appendix 1.

The PRA should consider whether the transmission of pests has been observed or confirmed to occur under natural conditions or under experimental conditions (e.g. in a laboratory or a growth chamber). When the transmission of pests has been observed or confirmed under experimental conditions it is necessary to confirm that it can also occur under natural conditions.

Consideration of the biological and epidemiological characteristics of specific pest groups may help in determining the probability of a pest being introduced with seeds in an area. Guidance on the likelihood of pest groups being carried and introduced with seeds is provided in Appendix 2. The pests and host seeds should be assessed at the species level unless there is technical justification for using a higher or lower taxonomic level, in accordance with the requirements in ISPM 11.

1.3 Purpose of import

The production of seeds may involve several steps (e.g. breeding, multiplication, destructive analysis, restricted field planting), which may be performed in different countries. The purpose of import of seeds may impact the probability of establishment of quarantine pests and should be considered when conducting the PRA and determining phytosanitary measures (ISPM 32).

The purpose of import may be broadly ranked from lowest to highest pest risk as follows.

1.3.1 Seeds for laboratory testing or destructive analysis

Such seeds are not intended for planting or for release into the PRA area. PRA may not be necessary because these seeds will not be released into the environment.

Seeds imported for testing may be germinated to facilitate testing, but their purpose is not for planting. Requirements for laboratory testing or similar confinement and the destruction of the seeds and plants growing from these seeds should be sufficient as a phytosanitary measure.

The NPPO of the importing country may not require other phytosanitary measures for these seeds if the pest risk is considered low or negligible.

1.3.2 Seeds for planting under restricted conditions

Such seeds are imported for research and are grown in protected environments (e.g. glasshouses, growth chambers) or in isolated fields. These seeds should be planted under conditions that prevent the introduction of quarantine pests into the PRA area. Examples include seeds for evaluation, germplasm and seeds as breeding material.

For these seeds, NPPOs may require relevant phytosanitary measures, which should not be more stringent than needed to address the pest risk identified.

1.3.3 Seeds for field planting

Seeds intended for unrestricted release into the PRA area may present the highest pest risk for quarantine pests.

The NPPO of the importing country may require phytosanitary measures; any such measures should be proportionate to the assessed pest risk. Specific tolerance levels for regulated non-quarantine pests may be determined and published.

1.4 Mixing, blending and bulking of seeds

Mixing of seeds combines different species, varieties or cultivars into a single lot (e.g. lawn grass mixture, wildflower mixture). Blending of seeds combines different seed lots of the same variety into a single lot. Bulking combines seeds of the same variety from different fields immediately after harvest into a single lot.

Seeds from various origins and different harvest years may be mixed or blended. All seeds in a mixture, a blend or a bulk lot should meet the relevant phytosanitary import requirements.

In assessing the pest risk of mixed, blended or bulked seeds, all combinations of pests, hosts and origins should be considered. The impacts of the mixing, blending or bulking processes (e.g. dilution, increased handling) should also be considered in determining the overall pest risk of mixtures, blends and bulk lots of seeds.

Testing and inspection may be done either on the components or on the mixture or the blend to be certified.

All components of the mixture, blend or bulk lot should be traceable.

1.5 Pest management in seed production

Certain practices used in seed production may alone or in combination be sufficient to meet phytosanitary import requirements. Full documentation of phytosanitary measures applied to the seeds should be maintained to facilitate trace-back, as appropriate.

Phytosanitary measures may be included in integrated pest management and quality control protocols applied in seed production.

In the case of tree seeds, phytosanitary measures are often applied only at the time of harvest.

Production practices may vary between seed production sectors (e.g. field crops, forestry). Options that may be considered when determining pest risk management include:

Pre-planting:

- use of resistant plant varieties (section 1.5.2)
- use of healthy seeds (free from pests)
- seed treatment (section 1.5.3)
- crop management (e.g. rotation or mixed planting)
- field selection
- soil or growing medium treatment
- geographical or temporal isolation
- sanitation or disinfection of water

Pre-harvesting:

- hygiene measures (e.g. disinfection of workers' hands and shoes, farm equipment, machinery and tools)
- field inspection and, where appropriate, testing if symptoms are observed
- field sanitation (e.g. removal of symptomatic plants, removal of weeds)
- parent plant testing
- crop treatment
- protected environments (e.g. glasshouses, growth chambers)
- sanitation or disinfection of water

Harvesting and post-harvest handling:

- hygiene measures (e.g. disinfection of workers' hands and shoes, farm equipment, machinery and tools)
- timely harvest (e.g. just as seed matures, for tree seeds in mast years, from fruit at the pre-ripe stage)
- use of disinfectants during seed extraction
- seed cleaning, drying, conditioning and sorting
- seed testing
- seed storage
- seed treatment (section 1.5.3)
- sanitation (e.g. removing plant debris, soil or visibly infested plants and seeds)
- seed packaging and sealing
- mechanical treatment (e.g. separation of healthy seeds (free from pests))
- harvesting method (e.g. use of collection mats or tarpaulins for tree seeds).

1.5.1 Seed certification schemes

Certain elements of a seed certification scheme (a scheme to improve the quality of seeds) may have an effect on the pest risk of the seeds being certified. Some of these elements (e.g. inspection for the presence of pests, purity analysis to detect weed seeds) may be considered in pest risk management by NPPOs and assessed on a case-by-case basis.

Seed certification schemes should ensure seed traceability. Information on international seed certification schemes is provided in some of the sources in Appendix 3.

1.5.2 Resistant plant varieties

Modern breeding programmes may produce plant varieties that have a level of resistance to pests, which may include resistance to regulated pests. When confirmed resistance to a regulated pest is such that a resistant variety is not infested by the pest, the NPPO of the importing country may consider this resistance as an appropriate pest risk management option.

A plant variety's level of resistance to different regulated pests may vary depending on the resistance characteristics present in the plant. Resistance genes may be effective against all or some races, strains, biotypes or pathotypes of the targeted pest, but the emergence of new races, strains, biotypes or pathotypes may affect the level of resistance. The pest resistance should therefore be assessed on a case-by-case basis. The NPPO of the importing country may consider the use of resistant varieties as an appropriate phytosanitary measure in the framework of a systems approach.

A suggested bibliography on the use of resistant plant varieties is provided in Appendix 3.

1.5.3 Seed treatment

Seeds may be treated to eliminate an infestation by a pest; however, they may be treated even if not infested, either as a precaution by a general disinfection or to protect the seedlings growing from the seeds when exposed to pests in the environment. Seed treatments may also be unrelated to pests; for example, seeds may be treated with seedling growth enhancer.

Seed treatments include, but are not limited to:

- pesticides (fungicides, insecticides, nematicides and bactericides)
- disinfectants, which are generally used against bacteria and viruses; disinfection may take place during various steps in seed processing (e.g. seed extraction, seed priming[1]) or during a dedicated disinfection process
- physical treatments (e.g. dry heat, steam, hot water, irradiation by ultraviolet light, high pressure, deep-freezing)
- biological treatments based on different modes of action (e.g. antagonism, competition, induced resistance).

2. Phytosanitary Measures

In accordance with ISPM 11, phytosanitary measures proportionate to the assessed pest risk should be applied alone or in combination to prevent the introduction and spread of quarantine pests and to ensure that the tolerance levels of regulated non-quarantine pests are met, as identified through a PRA.

2.1 Consignment inspection and testing for pest freedom

Seed sampling, including sample size (the total number of seeds tested), should be appropriate for detecting regulated pests. Guidance on sample size is provided in ISPM 31 (*Methodologies for sampling of consignments*). Harvested seeds showing visible symptoms that suggest the presence of regulated pests may need to be tested to confirm the presence of the pests.

2.2 Field inspection for the presence of pests

Field inspection may be a phytosanitary measure to detect some regulated pests that produce visible symptoms.

2.3 Pest free areas, pest free places of production, pest free production sites and areas of low pest prevalence

Pest free areas, pest free places of production, pest free production sites and areas of low pest prevalence should be established, recognized and maintained in accordance with ISPM 4 (*Requirements for the establishment of pest free areas*), ISPM 10 (*Requirements for the establishment of pest free places of production and pest free production sites*) and ISPM 29 (*Recognition of pest free areas and areas of low pest prevalence*).

Areas of low pest prevalence in accordance with ISPM 22 (*Requirements for the establishment of areas of low pest prevalence*) may be used alone or in combination with other phytosanitary measures in a systems approach (ISPM 14 (*The use of integrated measures in a systems approach for pest risk management*)).

2.4 Treatments

2.4.1 Crop treatment

Pesticide application to parent plants may be used to prevent seed infestation.

[1]Seed priming is the pre-treatment of seeds by various methods in order to improve the percentage and uniformity of germination.

2.4.2 Seed treatment

Seed treatments may be used as phytosanitary measures (section 1.5.3).

Many tropical and some temperate tree species produce seeds that are sensitive to desiccation and particularly prone to latent pest development or pest infestation. Physical or chemical treatments may be applied to prevent latent pest development or pest infestation in seeds that need to be maintained at high moisture levels.

2.5 Systems approaches

Systems approaches provide the opportunity to consider both pre-harvest and post-harvest procedures that may contribute to effective pest risk management. Many pest management practices to reduce pest risk throughout the seed production process, from planting to harvesting, may be integrated in a systems approach. ISPM 14 provides guidelines for the development and evaluation of integrated measures in a systems approach as an option for pest risk management.

2.6 Post-entry quarantine

The NPPO of the importing country may require post-entry quarantine for seeds, including confinement in a quarantine station, in cases where a quarantine pest is difficult to detect, where symptom expression takes time, or where testing or treatment is required and no alternative phytosanitary measures are available. Guidance on post-entry quarantine stations is provided in ISPM 34 (*Design and operation of post-entry quarantine stations for plants*).

As part of post-entry quarantine, a representative sample of the seed lot may be sown and the plants growing from these seeds tested (this may be an option for small seed lots used for research).

The NPPO of the importing country may consider, based on the findings of a PRA, that the pest risk can be adequately managed by requiring the imported seeds to be planted in a designated planting area. The planting area should be isolated from other host plants, and weed control, sanitation, and hygiene measures for people, machinery and equipment may be required.

2.7 Prohibition

NPPOs may prohibit the importation of seeds of certain species or origins when a PRA determines that the seeds pose a high pest risk as a pathway for quarantine pests and no alternative phytosanitary measures are available. This includes situations where the seeds may pose a high risk of being a pathway for plants as pests (e.g. weeds, invasive alien species). Guidance on prohibition of importation can be found in ISPM 20 (*Guidelines for a phytosanitary import regulatory system*).

The NPPO of the importing country may allow – for research purposes and under an import authorization that indicates specific conditions to prevent the introduction and spread of quarantine pests – the entry of seeds that are normally prohibited.

3. Equivalence of Phytosanitary Measures

The equivalence of phytosanitary measures (ISPM 1 (*Phytosanitary principles for the protection of plants and the application of phytosanitary measures in international trade*)) is particularly important for the international movement of seeds as seed companies may have breeding and multiplication programmes in several countries and may export these seeds to other countries, and there may be frequent re-export from a single seed lot.

Determination of the equivalence of phytosanitary measures may be initiated by the exporting country making a request for equivalence to the importing country, as described in ISPM 24 (*Guidelines for the determination and recognition of equivalence of phytosanitary measures*). It may also be initiated by the importing country. NPPOs are encouraged to provide multiple options when setting phytosanitary import requirements.

Equivalent phytosanitary measures may provide NPPOs with options to achieve the required protection. An example of an equivalent phytosanitary measure is the substitution of a requirement for field inspection of the seed crop in the country of origin with appropriate seed testing or seed treatment for the regulated pest. ISPM 24 provides further guidance on the equivalence of phytosanitary measures.

For seeds (including organic seeds) requiring for import a specific chemical treatment, if the chemical is not permitted for use in the country of origin, export or re-export, the NPPO of the importing country should consider an equivalent phytosanitary measure, where possible, provided that the measure is technically feasible and reduces the assessed pest risk to an acceptable level. It is recommended that phytosanitary import requirements do not specify chemical products, active ingredients or exact protocols.

4. Specific Requirements

Specific requirements for inspection, sampling and testing of seeds for phytosanitary certification or verification are provided as follows.

4.1 Inspection

Inspection may be conducted on the seed consignment or as field inspection of the growing crop, or both, as required. ISPM 23 (*Guidelines for inspection*) and ISPM 31 provide further guidance on inspection and sampling.

4.1.1 Inspection of seed consignments

Seed consignments may be inspected for the presence of seeds of plants regulated as pests (i.e. weeds, invasive alien species), for signs or symptoms of regulated pests, for the presence of regulated articles (e.g. soil) or for the presence of contaminating pests. Inspection for pest symptoms may be effective where infested seeds are known to display characteristic symptoms such as discoloration or shrivelling. However, the presence of the pest should be confirmed by laboratory testing. Visual examination should be combined with testing if pest freedom or a specific tolerance level is required for asymptomatic or unreliably symptomatic regulated pests.

Inspection of seeds can be done with or without the help of devices that automatically sort seeds based on visible physical characteristics. Although inspection may be effective for the detection of insects and mites, the majority of seed-borne pests (i.e. bacteria, fungi, nematodes, viroids, viruses) are not detectable by inspection with the naked eye and require a more specialized examination (e.g. with a binocular microscope) or laboratory testing. Washing, sieving or breaking seeds may be necessary before inspection.

Inspection of seeds that are coated, pelleted or embedded in tape, mats or any other substrate may require removal of the covering material by washing it off the seeds or breaking it because such material may reduce the ability to see the seeds or symptoms of the pest on the seeds. In such cases, the NPPO of the importing country may require the NPPO of the exporting country to systematically sample the seeds before coating, pelleting or embedding them, and to test them. For monitoring at import, the NPPO of the importing country may request the NPPO of the exporting country to provide a sample of the seeds (of a size proportional to the seed lot) before coating, pelleting or treating them, for inspection and testing, or, alternatively, if agreed bilaterally, to collect an official sample and test the seeds without coating, pelleting or treating them and to provide the test results.

4.1.2 Field inspection

Inspection of the seed crop in the field by trained staff at an appropriate time may be useful to detect regulated pests known to cause visible symptoms. A pest observed in the field on the parent plant may not necessarily be present on or in the seeds produced by these plants (section 1.2). A laboratory test may be conducted on the harvested seeds to determine if they are infested.

4.2 Sampling of lots

Sampling of a seed lot may be done to inspect or test for the absence of a pest in the lot.

Inspection for pests is usually based on sampling. Sampling methodologies used by NPPOs will depend on the sampling objectives (e.g. sampling for testing or inspection) and may be solely statistically based or developed noting particular operational constraints.

Guidance on the sampling of consignments for inspection is given in ISPM 31.

4.2.1 Sampling of small lots

Testing of samples that are taken in accordance with ISPM 31 from a small lot may result in the destruction of a large proportion of the lot. In such cases, alternative sampling methodologies (e.g. clustering small samples of different lots for testing) or equivalent phytosanitary procedures should be considered by the NPPO of the importing country, as per the guidance in ISPM 24.

In cases where sampling from small lots is not possible, specific post-entry quarantine requirements may be determined by the NPPO of the importing country.

4.3 Testing

Inspection may not be sufficient to determine if a regulated pest is present and other forms of examination may be needed (e.g. laboratory testing). Some bacteria, fungi, insects, nematodes, viroids and viruses may not be detectable by inspection of seed consignments or plants during growth, but they may be detectable by specific laboratory tests that follow validated diagnostic protocols for regulated pests.

Molecular and serological diagnostic methods are considered indirect protocols to detect pests in seeds. These methods may give a positive result even when no viable pests are present. Consequently, when testing seeds with these methods, results should be interpreted carefully. Confirmatory tests or additional tests based on a different biological principle may be required to confirm the presence of a viable pest in a sample. NPPOs should ensure that internationally recognized or validated diagnostic protocols are used to avoid false positives or false negatives.

The purpose and use of diagnostic protocols are described in ISPM 27 (*Diagnostic protocols for regulated pests*) and adopted protocols are provided as annexes to ISPM 27. Information on a range of other protocols, some of which have been validated, can be found in the sources listed in Appendix 3.

4.3.1 Testing of treated seeds

Seed treatment may influence the sensitivity of testing. Ideally, a detection method that detects only viable pests should be used to determine treatment efficacy, so when the treatment has been successful the test result is negative. Examples of such detection methods are techniques for the detection of bacteria and fungi where the organism will grow on the substrate (i.e. media or blotters), and techniques for the detection of viruses where the seeds are sown and plants growing from the seeds are observed for symptoms. Most established seed testing methods have been developed and validated for use on untreated seeds. If treated seeds are to be tested, the testing method should be validated for treated seeds.

The test results of treated seeds should be interpreted carefully, as the following situations may be encountered:

- The treatment inactivates the pest but the detection method detects both viable and non-viable pests. This may be the case with some serological or molecular tests or when detection is based on morphological identification of pests or pest structures that may remain even after treatment (e.g. nematodes, spores). In such cases, determination of the efficacy of the treatment is conclusive only if a test validated for treated seeds is used.
- The treatment physically or chemically inhibits the detection method; for example, some detection methods for bacteria are affected by fungicide treatments.

- The treatment adversely affects the detection method; for example, a method detects only pests present externally and any pests remaining internally after the treatment cannot be detected. In these situations, other detection methods that are able to detect internal infection should be used.

5. Phytosanitary Certification

The global and temporal nature of the seed trade (i.e. re-export to many destinations, repeated re-export from the same seed lot, long-term storage) presents phytosanitary certification challenges distinct from those of the international movement of other commodities.

NPPOs are encouraged to exchange additional official phytosanitary information at the time of export certification with other NPPOs to enable certification for re-export of seeds, as described in ISPM 12 (*Phytosanitary certificates*). Additional official phytosanitary information, which is not required by the first country of import, may be included on the phytosanitary certificate issued by the country of origin when so requested by the exporter in order to facilitate future re-export to other countries (ISPM 12).

A country's phytosanitary import requirement for a field inspection may not be known at the time of production. Where appropriate, the NPPO of the importing country may consider equivalent phytosanitary measures (such as tests or treatments) to fulfil its phytosanitary import requirements for seeds already harvested, in accordance with ISPM 24. However, it is the responsibility of the exporting country to meet the phytosanitary import requirements.

On phytosanitary certificates, "place of origin" refers primarily to places where the seeds were grown. If seeds are repacked, stored or moved, the pest risk may change as a result of their new location through possible infestation or contamination by regulated pests. The pest risk may also change if a seed treatment or disinfection removes possible infestation or contamination. In such cases, each country or place, as necessary, should be declared with the initial place of origin in brackets, in accordance with ISPM 12. If the consignment has not been exposed to infestation in the country or place of re-export, this can be indicated on the phytosanitary certificate for re-export. If different lots within a consignment originate in different countries or places, or if lots are mixed, blended or bulked, all countries or places should be indicated.

6. Record Keeping

Because seeds may be stored for many years before being exported or re-exported, official phytosanitary information on the seed lot, including in the case of re-export the original phytosanitary certificate for export, when available, should be retained as long as the seeds are in storage.

This appendix is for reference purposes only and is not a prescriptive part of the standard

APPENDIX 1: Examples of seed-transmitted, seed-borne and contaminating pests

This appendix provides examples of pests in the categories presented in section 1.2 (Seeds as pathways) of the standard.

Category 1(a): Seed-transmitted pests that are carried by the seed internally or externally and directly infest the host plant growing from the seed

- *Acidovorax citrulli* in seeds of *Citrullus lanatus*
- *Clavibacter michiganensis* subsp. *michiganensis* in seeds of *Solanum lycopersicum*
- *Ditylenchus dipsaci* on or in seeds of *Vicia faba* and *Medicago sativa*
- *Fusarium circinatum* on or in seeds of *Pinus* spp. and *Pseudotsuga menziessii*
- *Pea seed-borne mosaic virus* in seeds of *Pisum sativum*
- *Squash mosaic virus* in seeds of *Cucumis melo*
- *Tomato mosaic virus* in seeds of *S. lycopersicum*

Category 1(b): Non-seed transmitted pests that are carried by the seed internally or externally and are transferred to the environment (e.g. water, soil) and then infest a host plant under natural conditions

- *D. dipsaci* on or in seeds of *V. faba* and *M. sativa*
- *Fusarium oxysporum* f.sp. *lycopersici* on seeds of *S. lycopersicum*
- *Gibberella avenaceae* on seeds of *Linum usitatissimum*
- *Megastigmus* spp. in seeds of *Abies* spp.

Category 1(c): Pests carried by the seed, internally or externally, that do not transfer to a host plant under natural conditions

- *Callosobruchus chinensis* and *C. maculatus* on seeds of Fabaceae
- *Rice yellow mottle virus* on seeds of *Oryza sativa*

Category 2: Contaminating pests

- *Cyperus iria* in seed lots of *Oryza sativa*
- *Mycosphaerella pini* in seed lots of *Pinus* spp. contaminated with needle debris
- *Sclerotium cepivorum*, sclerotia in seed lots of *Allium cepa*

This appendix is for reference purposes only and is not a prescriptive part of the standard

APPENDIX 2: Guidance on the likelihood of pest groups being carried and introduced with seeds

This appendix provides general guidance on assessing the probability of different pest groups being carried and introduced with seeds. In accordance with ISPM 11, pests and their hosts are recommended to be assessed at the species level unless there is technical justification for using a higher or lower taxonomic level. Guidance for assessing the probability of pests being associated with seeds or being present in consignments of seeds and their potential to establish and spread via this pathway is provided in section 1.2 of the standard and in ISPM 11.

There is limited, and at times conflicting, information available regarding the seed transmission of pests. In addition, a pest that has been proven to be seed-transmitted in one host is not necessarily seed-transmitted in all known hosts. Seed transmission in other hosts and the level of host infestation before seed formation should be considered.

NPPOs should consider in their determination of pest–host interaction that plants that may host certain pests under experimental conditions may not be hosts under natural conditions.

1. Arthropods

1.1 Pre-harvest pests

Arthropods in the field include pests that feed on and in seeds during the seed development period, before harvest.

Arthropods in the field that have a low probability of being present in seed consignments include:

- External feeders: arthropods that feed on external parts of seeds are often dislodged during harvesting and cleaning.
- Internal feeders that cause seed abortion: arthropods that feed on internal parts of seeds usually cause seeds to fall before maturity and harvest.

Arthropods that are internal feeders on the mature seed in the field have a high probability of being present in seed consignments because they are usually collected with seeds during harvest. Consideration during the pest risk management stage of the PRA is needed to determine whether these arthropods (e.g. *Bruchidae*) would be visible during quality grading or inspection and whether they would survive storage conditions.

1.2 Post-harvest pests

Stored product arthropods can infest seeds after harvest, particularly if the seeds are stored in poor conditions (e.g. in high moisture or with previously stored seeds). Good storage conditions, as generally applied for high value seeds, greatly decrease or remove the likelihood of arthropods feeding on stored seeds.

Stored product arthropods that are external feeders have a low probability of being present in seed consignments. Arthropods that feed on but are not attached to external parts of seeds may destroy the seeds and pose a risk as contaminating pests. Secondary pests (e.g. *Mycetophagus* spp., *Acarus* spp., *Liposcelis* spp.) may also be present when sanitation is poor or extraneous matter excessive.

Stored product arthropods that are internal feeders have a high probability of being present in seed consignments. Thus consideration should be given to the likelihood of infestation in poor storage conditions. Arthropods that feed on internal parts of seeds can infest seeds that are left exposed before packaging.

2. Fungi

Fungal and fungal-like organisms may be associated with seeds both externally and internally without causing disease in the plants growing from these seeds; however, many species cause seed rot, necrosis, reduced germination and infestation of seedlings. Seed fungal pathogens can be grouped as field pathogens and storage pathogens. Fungi may be present on the surface of seeds or mixed with seeds as contaminating pests, and may be introduced and spread to the host crop or to other crops (e.g. by contamination of the growing medium). Fungi may also be present in the integuments or in the internal part of the seed and can be introduced and spread to the host crop in this way.

3. Bacteria

Although not all bacteria are seed-transmitted, bacteria can be found on or within seeds as external or internal infections, respectively.

4. Viruses

Not all viruses are seed-transmitted. Viruses as a general rule are seed-transmitted only if the seed embryo is infected, although there are exceptions in the *Tobamovirus* genus. For seed-transmitted viruses, the percentage of infected seedlings is often lower than the percentage of infested seeds.

5. Viroids

Seed transmission has been demonstrated for many but not all viroids.

6. Phytoplasmas and Spiroplasmas

There is no substantial evidence of seed transmission for phytoplasmas and spiroplasmas under natural conditions.

7. Nematodes

The majority of plant-parasitic nematode species are recorded as internal or external root parasites; however, some species of nematodes are known to attack above-ground plant parts, including seeds (e.g. *Ditylenchus dipsaci*, *Anguina tritici* and *Anguina agrostis*). Nematodes identified as seed-transmitted pests generally are species that are known to be endoparasites (internal feeders). Some species that are ectoparasites (external feeders) have dormant stages in seeds, plant debris and soil (e.g. *Aphelenchoides besseyi*) or become endoparasitic, invading inflorescenses and developing seeds (e.g. *A. tritici*).

8. Plants as Pests

Seeds of plants as pests (e.g. weeds, parasitic plants) may be introduced into a country as contaminating pests in seed lots.

This appendix is for reference purposes only and is not a prescriptive part of the standard

APPENDIX 3: Bibliography

The references included in this appendix are generally recognized as authoritative. The list is neither comprehensive nor static.

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INTERNATIONAL STANDARDS FOR
PHYTOSANITARY MEASURES

ISPM 20

Guidelines for a phytosanitary import regulatory system

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2011-05 SC reviewed draft and returned to steward.

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2014-05 SC discussed concepts related to pre-clearance.

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CONTENTS

Adoption

This standard was adopted by the Sixth Session of the Interim Commission on Phytosanitary Measures in March–April 2004.

INTRODUCTION

Scope

This standard describes the structure and operation of a phytosanitary import regulatory system and the rights, obligations and responsibilities which should be considered in establishing, operating and revising the system.

References

The present standard refers to International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

IPPC. 1997. *International Plant Protection Convention*. Rome, IPPC, FAO.

WTO. 1994. *Agreement on the Application of Sanitary and Phytosanitary Measures*. Geneva, World Trade Organization.

Definitions

Definitions of phytosanitary terms used in the present standard can be found in ISPM 5 (*Glossary of phytosanitary terms*).

Outline of Requirements

The objective of a phytosanitary import regulatory system is to prevent the introduction of quarantine pests or limit the entry of regulated non-quarantine pests with imported commodities and other regulated articles. A phytosanitary import regulatory system should consist of two components: a regulatory framework of phytosanitary legislation, phytosanitary regulations and phytosanitary procedures; and an official service, the national plant protection organization (NPPO), responsible for operation or oversight of the system. The legal framework should include legal authority for the NPPO to carry out its duties; phytosanitary measures with which imported commodities should comply; other phytosanitary measures (including prohibitions) concerning imported commodities and other regulated articles; and phytosanitary actions that may be taken when incidents of non-compliance or incidents requiring emergency action are detected. It may include phytosanitary measures concerning consignments in transit.

In operating a phytosanitary import regulatory system, the NPPO has a number of responsibilities. These include the responsibilities identified in Article IV.2 of the IPPC relating to import including surveillance, inspection, disinfestation or disinfection, the conduct of pest risk analysis, and training and development of staff. These responsibilities involve related functions in areas such as administration; audit and compliance checking; action taken on non-compliance; emergency action; authorization of personnel; and settlement of disputes. In addition, contracting parties may assign to NPPOs other responsibilities, such as regulatory development and modification. NPPO resources are needed to carry out these responsibilities and functions. There are also requirements for international and national liaison, documentation, communication and review.

REQUIREMENTS

1. Objective

The objective of a phytosanitary import regulatory system is to prevent the introduction of quarantine pests or limit the entry of regulated non-quarantine pests (RNQPs) with imported commodities and other regulated articles.

2. Structure

The components of a phytosanitary import regulatory system are:

- a regulatory framework of phytosanitary legislation, phytosanitary regulations and phytosanitary procedures
- an NPPO that is responsible for the operation of the system.

Legal and administrative systems and structures differ among contracting parties. In particular, some legal systems require every aspect of the work of its officials to be detailed within a legal text whilst others provide a broad framework within which officials have the delegated authority to perform their functions through a largely administrative procedure. This standard accordingly provides general guidelines for the regulatory framework of a phytosanitary import regulatory system. This regulatory framework is further described in section 4.

The NPPO is the official service responsible for the operation or oversight (organization and management) of the phytosanitary import regulatory system. Other government services, such as the Customs service, may have a role (with defined separation of responsibilities and functions) in the control of imported commodities and liaison should be maintained. The NPPO often utilizes its own officers to operate the phytosanitary import regulatory system, but may authorize other appropriate government services, or non-governmental organizations, or persons to act on its behalf and under its control for defined functions. The operation of the system is described in section 5.

3. Rights, Obligations and Responsibilities

In establishing and operating its phytosanitary import regulatory system, the NPPO should take into account:

- rights, obligations and responsibilities arising from relevant international treaties, conventions or agreements
- rights, obligations and responsibilities arising from relevant international standards
- national legislation and policies
- administrative policies of the government, ministry or department, or NPPO.

3.1 International agreements, principles and standards

National governments have the sovereign right to regulate imports to achieve their appropriate level of protection, taking into account their international obligations. Rights, obligations and responsibilities associated with international agreements as well as the principles and standards resulting from international agreements, in particular the IPPC and the World Trade Organization Agreement on the Application of Sanitary and Phytosanitary Measures (WTO, 1994), affect the structure and implementation of phytosanitary import regulatory systems. These include effects on the drafting and adoption of phytosanitary import regulations, the application of phytosanitary regulations, and the operational activities arising from regulations.

The drafting, adoption and application of phytosanitary regulations require recognition of certain principles and concepts such as in ISPM 1 (*Phytosanitary principles for the protection of plants and the application of phytosanitary measures in international trade*), including:

- transparency

- sovereignty
- necessity
- non-discrimination
- minimal impact
- harmonization
- technical justification (such as through pest risk analysis (PRA))
- consistency
- managed risk
- modification
- emergency action and provisional measures
- equivalence
- recognition of pest free areas and areas of low pest prevalence.

In particular, the phytosanitary procedures and phytosanitary regulations should take into consideration the concept of minimal impact and issues of economic and operational feasibility in order to avoid unnecessary trade disruption.

3.2 Regional cooperation

Regional organizations, such as regional plant protection organizations (RPPOs) and regional agricultural development organizations, may encourage the harmonization of their members' phytosanitary import regulatory systems and may cooperate in the exchange of information for the benefit of members.

A regional economic integration organization recognized by FAO may have rules that apply to its members and may also have the authority to enact and enforce certain phytosanitary regulations on behalf of members of that organization.

4. Regulatory Framework

The issuing of regulations is a government (contracting party) responsibility (Article IV.3(c) of the IPPC). Consistent with this responsibility, contracting parties may provide the NPPO with the authority for the formulation of phytosanitary import regulations and the implementation of the import regulatory system. Contracting parties should have a regulatory framework to provide the following:

- the specification of the responsibilities and functions of the NPPO in relation to the import regulatory system
- legal authority to enable the NPPO to carry out its responsibilities and functions with respect to the import regulatory system
- authority and procedures, such as through PRA, to determine import phytosanitary measures
- phytosanitary measures that apply to imported commodities and other regulated articles
- import prohibitions that apply to imported commodities and other regulated articles
- legal authority for action with respect to non-compliance and for emergency action
- the specification of interactions between the NPPO and other government bodies
- transparent and defined procedures and time frames for implementation of regulations, including their entry into force.

Contracting parties have obligations to make their regulations available according to Article VII.2(b) of the IPPC; these procedures may require a regulatory basis.

4.1 Regulated articles

Imported commodities that may be regulated include articles that may be infested or contaminated with regulated pests. Regulated pests are either quarantine pests or regulated non-quarantine pests. All

commodities can be regulated for quarantine pests. Products for consumption or processing cannot be regulated for regulated non-quarantine pests. Regulated non-quarantine pests can only be regulated with respect to plants for planting. The following are examples of regulated articles:

- plants and plant products used for planting, consumption, processing, or any other purpose
- storage facilities
- packaging materials including dunnage
- conveyances and transport facilities
- soil, organic fertilizers and related materials
- organisms capable of harbouring or spreading pests
- potentially contaminated equipment (such as used agricultural, military and earthmoving equipment)
- research and other scientific materials
- travellers' personal effects moving internationally
- international mail including international courier services
- pests and biological control agents[1].

Lists of regulated articles should be made publically available.

4.2 Phytosanitary measures for regulated articles

Contracting parties should not apply phytosanitary measures to the entry of regulated articles such as prohibitions, restrictions or other phytosanitary import requirements unless such measures are made necessary by phytosanitary considerations and are technically justified. Contracting parties should take into account, as appropriate, international standards and other relevant requirements and considerations of the IPPC when applying phytosanitary measures.

4.2.1 Phytosanitary measures for consignments to be imported

The phytosanitary regulations should specify the phytosanitary measures with which imported consignments[2] of plants, plant products and other regulated articles should comply. These phytosanitary measures may be general, applying to all types of commodities, or the measures may be specific, applying to specified commodities from a particular origin. Phytosanitary measures may be required prior to entry, at entry or post entry. Systems approaches may also be used when appropriate (see ISPM 14 (*The use of integrated measures in a systems approach for pest risk management*)).

Phytosanitary measures required in the exporting country, which the NPPO of the exporting country may be required to certify (ISPM 7 (*Phytosanitary certification system*)) include:

- inspection prior to export
- testing prior to export
- treatment prior to export
- produced from plants of specified phytosanitary status (for example grown from virus-tested plants or under specified conditions)
- inspection or testing in the growing season prior to export

[1] Pests *per se* and biological control agents do not fall within the definition of "regulated articles" (Article II.1 of the IPPC). However, where there is technical justification, they may be subjected to phytosanitary measures (IPPC, Article VI with respect to regulated pests, and Article VII.1(c) and VII.1(d)) and for the purposes of this standard may be considered as regulated articles.

[2] For the purpose of this standard, import is considered to cover all consignments moving into the country (except in transit), including movement into free trade zones (including duty free areas and consignments in bond) and illegal consignments detained by other services.

- origin of the consignment to be a pest free place of production or pest free production site, area of low pest prevalence or pest free area
- accreditation procedures
- maintenance of consignment integrity.

Phytosanitary measures that may be required during shipment include:

- treatment (for example appropriate physical or chemical treatments)
- maintenance of consignment integrity.

Phytosanitary measures that may be required at the point of entry include:

- documentation checks
- verification of consignment integrity
- verification of treatment during shipment
- phytosanitary inspection
- testing
- treatment
- detention of consignments pending the results of testing or verification of the efficacy of treatment.

Phytosanitary measures that may be required after entry include:

- detention in quarantine (such as in a post-entry quarantine station) for inspection, testing or treatment
- detention at a designated place pending specified measures
- restrictions on the distribution or use of the consignment (for example for specified processing).

Other phytosanitary measures that may be required include:

- requirements for licences or permits
- limitations on the points of entry for specified commodities
- the requirement that importers notify in advance the arrival of specified consignments
- audit of procedures in the exporting country
- pre-clearance.

The phytosanitary import regulatory system should make provision for the evaluation and possible acceptance of alternative phytosanitary measures proposed by exporting contracting parties as being equivalent.

4.2.1.1 Provision for special imports

Contracting parties may make special provision for the import of pests, biological control agents (see also ISPM 3 (*Guidelines for the export, shipment, import and release of biological control agents and other beneficial organisms*)) or other regulated articles for scientific research, education or other purposes. Such imports may be authorized subject to the provision of adequate safeguards.

4.2.1.2 Pest free areas, pest free places of production, pest free production sites, areas of low pest prevalence and official control programmes

Importing contracting parties may designate pest free areas, areas of low pest prevalence (ISPM 4 (*Requirements for the establishment of pest free areas*), ISPM 22 (*Requirements for the establishment of areas of low pest prevalence*), ISPM 29 (*Recognition of pest free areas and areas of low pest prevalence*)) and official control programmes within their country. Phytosanitary regulations may be required to protect or sustain such designations within the importing country. However such phytosanitary measures should respect the principle of non-discrimination.

Phytosanitary import regulations should recognize the existence of such designations and those related to other official procedures (such as pest free places of production and pest free production sites) within the countries of exporting contracting parties including the facility to recognize these phytosanitary measures as equivalent where appropriate. It may be necessary to make provision within phytosanitary regulatory systems to evaluate and accept the designations by other NPPOs and to respond accordingly.

4.2.2 Import authorization

The authority to import may be provided as a general authorization or through specific authorization on a case-by-case basis.

General import authorization

General import authorizations may be used:

- when there are no specific phytosanitary import requirements
- where specific phytosanitary import requirements have been established permitting entry as set out in the regulations for a range of commodities.

General import authorizations should not require a licence or a permit but may be subject to checking at import.

Specific import authorization

Specific import authorizations, e.g. in the form of a licence or permit, may be required where official consent for import is necessary. These may be required for individual consignments or a series of consignments of a particular origin. Cases where this type of authorization may be required include:

- emergency or exceptional imports
- imports with specific, individual phytosanitary import requirements such as those with post-entry quarantine requirements or designated end use or research purposes
- imports where the NPPO requires the ability to trace the material over a period of time after entry.

It is noted that some countries may use permits to specify general import conditions. However, the development of general authorizations is encouraged wherever similar specific authorizations become routine.

4.2.3 Prohibitions

The prohibition of import may apply to specified commodities or other regulated articles of all origins or specifically to a particular commodity or other regulated article of a specified origin. The prohibition of import should be used when no alternatives for pest risk management exist. Prohibitions should be technically justified. NPPOs should make provision to assess equivalent, but less trade restrictive measures. Contracting parties, through their NPPOs where authorized, should modify their phytosanitary import regulations if such measures meet their appropriate level of protection. Prohibition applies to quarantine pests. Regulated non-quarantine pests should not be subject to prohibition but are subject to established pest tolerance levels.

Prohibited articles may be required for research or other purpose and provision may be required for their import under controlled conditions including appropriate safeguards through a system of licence or permit.

4.3 Consignments in transit

Consignments in transit are not imported. However, the phytosanitary import regulatory system may be extended to cover consignments in transit and to establish technically justified phytosanitary measures to prevent the introduction and/or spread of pests (Article VII.4 of the IPPC, ISPM 25 (*Consignments in transit*)). Measures may be required to track consignments, to verify their integrity or to confirm that they leave the country of transit. Countries may establish points of entry, routes within the country, conditions for transportation and time spans permitted within their territories.

4.4 Measures concerning non-compliance and emergency action

The phytosanitary import regulatory system should include provisions for phytosanitary action to be taken in the case of non-compliance or for emergency action (Article VII.2(f) of the IPPC; detailed information is contained in ISPM 13 (*Guidelines for the notification of non-compliance and emergency action*)), taking into consideration the principle of minimal impact.

Phytosanitary actions which may be taken when an imported consignment or other regulated articles does not comply with phytosanitary regulations and is initially refused entry include:

- treatment
- sorting or reconditioning
- disinfection of regulated articles (including equipment, premises, storage areas, means of transportation)
- direction to a particular end use such as processing
- reshipment
- destruction (such as incineration).

Detection of a non-compliance or an incident requiring emergency action may result in a revision of the phytosanitary import regulations, or in revocation or suspension of authorization to import.

4.5 Other elements that may require a regulatory framework

International agreements give rise to obligations which may require a legal base or may be implemented through administrative procedures. Arrangements that may require such procedures include:

- notification of non-compliance
- pest reporting
- designation of an official contact point
- publication and dissemination of regulatory information
- international cooperation
- revision of regulations and documentation
- recognition of equivalence
- specification of points of entry
- notification of official documentation.

4.6 Legal authority for the NPPO

In order that the NPPO can discharge its responsibilities (Article IV of the IPPC), legal authority (powers) should be provided to enable the officers of the NPPO and other authorized persons to:

- enter premises, conveyances, and other places where imported commodities, regulated pests or other regulated articles may be present
- inspect or test imported commodities and other regulated articles
- take and remove samples from imported commodities or other regulated articles, or from places where regulated pests may be present (including for analysis which may result in the destruction of the sample)
- detain imported consignments or other regulated articles
- treat or require treatment of imported consignments, or other regulated articles including conveyances, or places or commodities in which a regulated pest may be present
- refuse entry of consignments, order their reshipment or destruction
- take emergency action
- set and collect fees for import-related activities or associated with penalties (optional).

5. Operation of a Phytosanitary Import Regulatory System

The NPPO is responsible for the operation or oversight (organization and management) of the phytosanitary import regulatory system (see also section 2). This responsibility arises in particular from Article IV.2 of the IPPC.

5.1 Management and operational responsibilities of the NPPO

The NPPO should have a management system and resources adequate to carry out its functions.

5.1.1 Administration

The administration of the phytosanitary import regulatory system by the NPPO should ensure the effective and consistent application of phytosanitary legislation and regulations and compliance with international obligations. This may require operational coordination with other government services or government agencies involved with imports, e.g. Customs. Administration of the phytosanitary import regulatory system should be coordinated at national level but may be organized on a functional, regional or other structural basis.

5.1.2 Regulatory development and revision

The issuing of phytosanitary regulations is a contracting party responsibility (Article IV.3(c) of the IPPC). Consistent with this responsibility, contracting parties may make the development or revision of phytosanitary regulations the responsibility of their NPPO. This action may be under the initiative of the NPPO in consultation or cooperation with other authorities as appropriate. Appropriate regulations should be developed, maintained and reviewed as necessary and in compliance with applicable international agreements, through the normal legal and consultative processes of the country. Consultation and collaboration with relevant agencies as well as affected industries and appropriate private sector groups can be helpful in increasing the understanding and acceptance of regulatory decisions by the private sector and is often useful for the improvement of regulations.

5.1.3 Surveillance

The technical justification of phytosanitary measures is determined in part by the pest status of regulated pests within the regulating country. Pest status may change and this may necessitate revision of phytosanitary import regulations. Surveillance of cultivated and non-cultivated plants in the importing country is required to maintain adequate information on pest status (according to ISPM 6 (*Guidelines for surveillance*)), and may be required to support PRA and pest listing.

5.1.4 Pest risk analysis and pest listing

Technical justification such as through PRA is required to determine if pests should be regulated and the strength of phytosanitary measures to be taken against them (ISPM 11 (*Pest risk analysis for quarantine pests*); ISPM 21 (*Pest risk analysis for regulated non-quarantine pests*)). PRA may be done on a specific pest or on all the pests associated with a particular pathway (e.g. a commodity). A commodity may be classified by its level of processing or its intended use (see ISPM 32 (*Categorization of commodities according to their pest risk*)). Regulated pests should be listed (according to ISPM 19 (*Guidelines on lists of regulated pests*)) and lists of regulated pests should be made available (Article VII.2(i) of the IPPC). If appropriate international standards are available, measures should take account of such standards and should not be more stringent unless technically justified.

The administrative framework of the PRA process should be clearly documented, if possible with a time frame for the completion of individual PRAs and with clear guidance on prioritization.

5.1.5 Audit and compliance procedures

5.1.5.1 Audit of procedures in the exporting country

Phytosanitary import regulations often include specific requirements that should be done in the country of export, such as production procedures (usually during the growing period of the crop concerned) or

specialized treatment procedures. In certain circumstances, such as in the development of a new trade, the requirements may include, in cooperation with the NPPO of the exporting country, an audit in the exporting country by the NPPO of the importing country of elements such as:

- production systems
- treatments
- inspection procedures
- phytosanitary management
- accreditation procedures
- testing procedures
- surveillance.

An importing country should make known the scope of any audit. The arrangements for such audits are normally written into a bilateral agreement, arrangement or work programme associated with import facilitation. Such arrangements may extend to clearance of consignments within the exporting country for entry into the importing country which usually facilitates a minimum of procedures at entry to the importing country. These types of audit procedure should not be applied as a permanent measure and should be considered satisfied as soon as the procedures in the exporting country have been validated. This approach, in its limitation on the length of its application, may differ from ongoing pre-clearance inspections mentioned in section 5.1.5.2.1. The results of audits should be made available to the NPPO of the exporting country.

5.1.5.2 Compliance procedures at import

There are three basic elements to compliance checking:

- documentary checks
- verification of consignment integrity
- phytosanitary inspection, testing etc.

Verification of compliance for imported consignments and other regulated articles may be required:

- to determine their compliance with phytosanitary regulations
- to check that phytosanitary measures are effective in preventing the introduction of quarantine pests and limiting the entry of RNQPs
- to detect potential quarantine pests or quarantine pests whose entry with that commodity was not predicted.

Phytosanitary inspections should be carried out by, or under the authority of, the NPPO.

Compliance procedures should be undertaken promptly (Article VII.2(d) and VII.2(e) of the IPPC). Where possible, compliance procedures should be carried out in cooperation with other agencies involved with the regulation of imports, such as Customs, so as to minimize interference with the flow of trade and the impact on perishable products.

5.1.5.2.1 Inspection

Inspections may be done at the point of entry, at points of transhipment, at the point of destination or at other places where imported consignments can be identified, such as major markets, provided that their integrity is maintained and that appropriate phytosanitary procedures can be carried out. By bilateral agreement or arrangement, they may also be done in the country of origin as a part of a pre-clearance programme in cooperation with the NPPO of the exporting country.

Phytosanitary inspections, which should be technically justified, may be applied:

- to all consignments as a condition of entry
- as a part of an import monitoring programme where the level of monitoring (i.e. the number of consignments inspected) is established on the basis of predicted risk.

Inspection and sampling procedures may be based on general procedures or on specific procedures to achieve predetermined objectives.

5.1.5.2.2 Sampling

Samples may be taken from consignments for the purposes of inspection, or for subsequent laboratory testing, or for reference purposes (see ISPM 31 (*Methodologies for sampling of consignments*)).

5.1.5.2.3 Testing including laboratory testing

Testing may be required for:

- identification of a visually detected pest
- confirmation of a visually identified pest
- checking of compliance with requirements concerning infestations not detectable by inspection
- checking for latent infections
- audit or monitoring
- reference purposes particularly in cases of non-compliance
- verification of the declared product.

Testing should be performed by persons experienced in the appropriate procedures and, if possible, following internationally agreed protocols. Cooperation with appropriate academic and international experts or institutes is recommended when validation of test results is needed.

5.1.6 Non-compliance and emergency action

Detailed information about non-compliance and emergency action is contained in ISPM 13.

5.1.6.1 Action in case of non-compliance

Examples where phytosanitary action may be justified regarding non-compliance with phytosanitary import regulations include:

- the detection of a listed quarantine pest associated with consignments for which it is regulated
- the detection of a listed RNQP present in an imported consignment of plants for planting at a level which exceeds the required tolerance level for those plants
- evidence of failure to meet prescribed requirements (including bilateral agreements or arrangements, or import permit conditions) such as field inspection, laboratory tests, registration of producers or facilities, lack of pest monitoring or surveillance
- the interception of a consignment which does not otherwise comply with the import regulations, such as because of the detected presence of undeclared commodities, soil or some other prohibited article or evidence of failure of specified treatments
- phytosanitary certificate or other required documentation invalid or missing
- prohibited consignments or articles
- failure to meet “in-transit” measures.

The type of phytosanitary action will vary with the circumstances and should be the minimum necessary to counter the pest risk identified. Administrative errors such as incomplete phytosanitary certificates may be resolved through liaison with the NPPO of the exporting country. Other infringements may require action such as:

Detention. This may be used if further information is required, taking into account the need to avoid consignment damage as far as possible.

Sorting and reconfiguring. The affected products may be removed by sorting and reconfiguring the consignment including repackaging if appropriate.

Treatment. Used by the NPPO when an efficacious treatment is available.

Destruction. The consignment may be destroyed in cases where the NPPO considers the consignment cannot be otherwise handled.

Reshipment. The non-complying consignment may be removed from the country by reshipping.

In the case of non-compliance for an RNQP, action should be consistent with domestic measures and limited to bringing the pest incidence in the consignment, where feasible, into compliance with the required tolerance level, e.g. through treatment or by downgrading or reclassification where this is permitted for equivalent material produced or regulated domestically.

The NPPO is responsible for issuing the necessary instructions and for verifying their application. Enforcement is normally considered to be a function of the NPPO but other agencies may be authorized to assist.

An NPPO may decide not to apply phytosanitary action against a regulated pest or in other instances of non-compliance where phytosanitary actions are not technically justified in a particular situation, such as if there is no risk of establishment or spread (e.g. a change of intended use such as from consumption to processing or when a pest is in a stage of its life cycle which will not enable establishment or spread), or for some other reason.

5.1.6.2 Emergency action

Emergency action may be required in a new or unexpected phytosanitary situation, such as the detection of quarantine pests or potential quarantine pests:

- in consignments for which phytosanitary measures are not specified.
- in consignments or other regulated articles in which their presence is not anticipated and for which no phytosanitary measures have been specified.
- as contaminants of conveyances, storage places or other places involved with imported commodities.

Phytosanitary action similar to that required in cases of non-compliance may be appropriate. Such actions may lead to the modification of existing phytosanitary measures, or the adoption of provisional measures pending review and full technical justification.

Commonly encountered situations requiring emergency action include:

Pests not previously assessed. Non-listed organisms may require emergency phytosanitary actions because they may not have been previously assessed. At the time of interception, they may be categorized as regulated pests on a preliminary basis because the NPPO has a cause to believe they pose a pest risk. In such instances, it is the responsibility of the NPPO to be able to provide a sound technical basis. If provisional measures are established, the NPPO should actively pursue additional information, if appropriate with the participation of the NPPO of the exporting country, and complete a PRA to establish in a timely manner the regulated or non-regulated status of the pest.

Pests not regulated for a particular pathway. Emergency phytosanitary actions may be applied for pests that are not regulated with respect to particular pathways. Although regulated, these pests may not have been listed or otherwise specified because they were not anticipated for the origin, commodity, or circumstances for which the list or measure was developed. Such pests should be included on the appropriate list or within other measure if it is determined that the occurrence of the pest in the same and similar circumstances may be anticipated in the future.

Lack of adequate identification. In some instances, a pest may justify phytosanitary action because the pest cannot be adequately identified or is inadequately described taxonomically. This may be because the specimen has not been described (is taxonomically unknown), is in a condition which does not allow its identification, or the life stage being examined cannot be identified to the required taxonomic level. Where identification is not feasible, the NPPO should have a sound technical basis for the phytosanitary actions taken.

Where pests are routinely detected in a form that does not allow for adequate identification (e.g. eggs, early instar larvae, imperfect forms), every effort should be made to raise sufficient specimens to allow identification. Contact with the exporting country may assist with the identification or provide a presumed identification. Such pests in this state may be deemed temporarily to require phytosanitary measures. Once identification is achieved and if, on the basis of PRA, it is confirmed that such pests justify phytosanitary actions, NPPOs should add such pests to the relevant list of regulated pests, noting the identification problem and the basis for requiring phytosanitary actions. Interested contracting parties should be informed that future action will be based on a presumed identification if such forms are detected. However, such future phytosanitary action should only be taken with respect to origins where there is an identified pest risk and the possibility of the presence of quarantine pests in imported consignments cannot be excluded.

5.1.6.3 Reporting of non-compliance and emergency action

The reporting of interceptions, instances of non-compliance and emergency action is an obligation for contracting parties to the IPPC so that the NPPOs of the exporting countries understand the basis for phytosanitary actions taken against their products on import and to facilitate corrective action in export systems. Systems are needed for the collection and transmission of such information.

5.1.6.4 Withdrawal or modification of phytosanitary regulation

In the case of repeated non-compliance, or where a significant non-compliance or interception warranting emergency action occurs, the NPPO of the importing contracting party may withdraw the authorization (e.g. permit) allowing import, modify the phytosanitary regulation, or institute an emergency or provisional measure with modified entry procedures or a prohibition. The NPPO of the exporting country should be notified promptly of the change and rationale for this change.

5.1.7 Systems for authorization of non-NPPO personnel

NPPOs may authorize, under their control and responsibility, other government services, non-governmental organizations, agencies or persons to act on their behalf for certain defined functions. In order to ensure that the requirements of the NPPO are met, operational procedures are required. In addition, procedures should be developed for the demonstration of competency and for audits, corrective actions, system review and withdrawal of authorization.

5.1.8 International liaison

Contracting parties have international obligations (Articles VII and VIII of the IPPC) including the:

- provision of an official contact point
- notification of specified points of entry
- publication and transmission of lists of regulated pests, phytosanitary import requirements, and prohibitions
- notification of non-compliance and emergency action (ISPM 13)
- provision of the rationale for phytosanitary measures, on request
- provision of relevant information.

Administrative arrangements are required to ensure that these obligations are discharged efficiently and promptly.

5.1.9 Notification and dissemination of regulatory information

5.1.9.1 New or revised phytosanitary regulations

Proposals for new or revised phytosanitary regulations should be published and provided to interested parties on request, allowing reasonable time for comment and implementation.

5.1.9.2 Dissemination of established regulations

Established import regulations, or relevant sections of them, should be made available to interested and affected contracting parties as appropriate, to the IPPC Secretariat and to the RPPO(s) of which they are a member. Through appropriate procedures, they may also be made available to other interested parties (such as import and export industry organizations and their representatives). NPPOs are encouraged to make import regulatory information available by publication, whenever possible using electronic means including Internet websites and linkage to these via the IPPC International Phytosanitary Portal (IPP) (http://www.ippc.int).

5.1.10 National liaison

Procedures that facilitate cooperative action, information sharing and joint clearance activities within the country should be established with relevant government agencies or services as appropriate.

5.1.11 Settlement of disputes

The implementation of a phytosanitary import regulatory system may give rise to disputes with the authorities of other countries. The NPPO should establish procedures for consultation and exchange of information with other NPPOs, and for settlement of such disputes "shall consult among themselves as soon as possible" prior to considering calling on formal international dispute-settlement procedures (Article XIII.1 of the IPPC).

5.2 Resources of the NPPO

Contracting parties should provide to their NPPO appropriate resources to carry out its functions (Article IV.1 of the IPPC).

5.2.1 Staff, including training

The NPPO should:

- employ or authorize personnel who have appropriate qualifications and skills
- ensure that adequate and sustained training is provided to all personnel to ensure competency in the areas for which they have responsibility.

5.2.2 Information

The NPPO should, as far as possible, ensure that adequate information is available to personnel, in particular:

- guidance documents, procedures and work instructions as appropriate covering relevant aspects of the operation of the phytosanitary import regulatory system
- the phytosanitary import regulations of its country
- information on its regulated pests including biology, host range, pathways, global distribution, detection and identification methods, treatment methods.

The NPPO should have access to information on the presence of pests in its country (preferably as pest lists), to facilitate the categorization of pests during pest risk analysis. The NPPO should also maintain lists of all its regulated pests. Detailed information on lists of regulated pests is contained in ISPM 19.

Where a regulated pest is present in the country, information should be maintained on its distribution, pest free areas, official control and, in the case of an RNQP, official programmes for plants for planting. Contracting parties should distribute information within their territory regarding regulated pests and the means of their prevention and control, and may assign this responsibility to their NPPOs.

5.2.3 Equipment and facilities

The NPPO should ensure that adequate equipment and facilities are available for:

- inspection, sampling, testing, surveillance and consignment verification procedures

- communication and access to information (by electronic means as far as possible).

DOCUMENTATION, COMMUNICATION AND REVIEW

6. Documentation

6.1 Procedures

The NPPO should maintain guidance documents, procedures and work instructions covering all aspects of the operation of the phytosanitary import regulatory system. Procedures to be documented include:

- preparation of pest lists
- pest risk analysis
- where appropriate, establishment of pest free areas, areas of low pest prevalence, pest free places of production or production sites, and official control programmes
- inspection, sampling and testing methodology (including methods for maintaining sample integrity)
- action on non-compliance, including treatment
- notification of non-compliance
- notification of emergency action.

6.2 Records

Records should be kept of all actions, results and decisions concerning the regulation of imports, following the relevant sections of ISPMs where appropriate, including:

- documentation of pest risk analyses (in accordance with ISPM 11, and other relevant ISPMs)
- where established, documentation of pest free areas, areas of low pest prevalence, and official control programmes (including information on the distribution of the pests and the phytosanitary measures used to maintain the pest free area or area of low pest prevalence)
- records of inspection, sampling and testing
- non-compliance and emergency action (in accordance with ISPM 13).

If appropriate, records may be kept of imported consignments:

- with specified intended uses
- subject to post-entry quarantine or treatment procedures
- requiring follow up phytosanitary action (including trace-back), according to pest risk, or
- as necessary to manage the phytosanitary import regulatory system.

7. Communication

The NPPO should ensure that it has communication procedures to contact:

- importers and appropriate industry representatives
- NPPOs of exporting countries
- the Secretariat of the IPPC
- the secretariats of the RPPOs of which it is a member.

8. Review Mechanism

8.1 System review

The contracting party should periodically review its phytosanitary import regulatory system. This may involve monitoring the effectiveness of phytosanitary measures, auditing the activities of the NPPO and authorized organizations or persons, and modifying the phytosanitary legislation, regulations and procedures as required.

8.2 Incident review

The NPPO should have procedures in place to review cases of non-compliance and emergency action. Such a review may lead to the adoption or modification of phytosanitary measures.

This annex was adopted by the Twelfth Session of the Commission on Phytosanitary Measures in April 2017.

This annex is a prescriptive part of the standard.

ANNEX 1: Arrangements for the verification of compliance of consignments by the importing country in the exporting country (2017)

The NPPO of the importing country usually verifies compliance of consignments with phytosanitary import requirements on entry into the importing country. However, to facilitate trade logistics, contracting parties may in some cases bilaterally or multilaterally negotiate an arrangement that allows verification procedures to be performed by the NPPO of the importing country in the exporting country. Such arrangements are distinct from audits of procedures in exporting countries referred to in this standard (section 5.1.5.1).

NPPOs of the importing country and the exporting country should only establish and use a bilateral or multilateral arrangement (hereinafter referred to as an "arrangement") for verification procedures to be performed on consignments of specified commodities in the exporting country on a voluntary and case-by-case basis and for a time period agreed by both parties.

Arrangements described in this annex should not be established as a phytosanitary measure or as a condition to allow trade.

The establishment of an arrangement may be an option to facilitate trade logistics in the following situations:

- to expedite consignment release at the destination
- when measures associated with the refusal of a consignment at the point of entry are too costly or difficult to apply
- when inspection at the point of entry adversely affects commercial packaging (e.g. the commodity is individually wrapped and destructive sampling is required) or commodity quality (e.g. the commodity is highly perishable)
- when additional infrastructure is necessary to address instances of non-compliance.

The terms of the arrangement for a particular regulated article should be developed once the phytosanitary import requirements have been set based on a pest risk analysis.

The arrangement should only include procedures to verify compliance of consignments with established and published phytosanitary import requirements for the relevant commodities in accordance with this standard and where appropriate with ISPM 23 (*Guidelines for inspection*). Consignments verified under the arrangement should not be subject to the same verification procedures again at the point of entry. The NPPO of the importing country may, however, perform other verification procedures, such as document and identity checks, at the point of entry.

Irrespective of any arrangement between the NPPOs of the importing country and the exporting country, issuance of phytosanitary certificates remains the exclusive responsibility of the NPPO of the exporting country as stated in Articles I.2, IV.2(a), IV.2(b), IV.2(c), IV.2(d), IV.2(e), IV.2(g) and V.1 of the IPPC. Any actions undertaken by the NPPO of the importing country in the exporting country under an arrangement are subject to and must comply with the legislation of the exporting country.

The following sections provide options to be considered by NPPOs in relation to arrangements for the verification of compliance of consignments by the NPPO of the importing country in the exporting country.

1. General Requirements for an Arrangement

An arrangement should be developed jointly by the NPPOs of the importing country and the exporting country, in consultation with relevant stakeholders, when appropriate.

The financial aspects of the arrangement should be agreed on by the NPPOs of the importing country and the exporting country, in consultation with relevant stakeholders.

The arrangement should be subject to regular review and a mechanism may be put in place to deal with any changes that may arise. The conditions for reducing compliance verification activities and suspending or terminating the arrangement should be specified on a case-by-case basis.

2. Process for Establishing an Arrangement

The steps to establish an arrangement are outlined below.

2.1 Proposal

The NPPO of the importing or of the exporting country may initiate the request for an arrangement. The proposal may be a response to a need identified by the initiating NPPO or by relevant stakeholders. The proposal should specify the scope and objectives of as well as the reasons for the arrangement, and be agreed on by both NPPOs.

Factors that may be considered in the proposal include:

- timing and duration of the arrangement
- proposed verification levels and, when appropriate, sampling schemes for specified commodities and regulated pests
- criteria that could initiate review and evaluation of the arrangement
- criteria that could initiate suspension or termination of the arrangement
- availability of resources
- feasibility of programme implementation.

2.2 Evaluation

The NPPO receiving the proposal for an arrangement should undertake a timely review of the proposal and prepare a response. Evaluation of the proposal should encompass any effects of the arrangement on pest risk concerns, operational and economic feasibility, and regulatory aspects.

2.3 Elements

The NPPO proposing an arrangement has the primary responsibility for its development. However, on request of the proposing NPPO, the other NPPO is encouraged to assist in its development.

Elements of the arrangement that may need to be agreed between the NPPO of the importing country and the NPPO of the exporting country include:

- sampling and inspection of consignments
- adequacy of inspection facilities
- testing procedures
- verification of treatments
- verification of consignment integrity
- the time of and location for the different steps of the verification of compliance of consignments, when appropriate
- notification to the point of entry of the arrival of consignments
- whether a certificate is to accompany the phytosanitary certificate
- availability of qualified staff to implement provisions under the arrangement
- timing of the activities for the verification of compliance
- approval procedures and expense or estimated expense for growers and exporters participating in the arrangement

- accommodation, transport, work health and safety, security and other logistical aspects for the deployed officers.

The steps of the verification of compliance will be identified by the NPPOs entering into the arrangement.

2.4 Technical requirements

The technical requirements for an arrangement should be determined and developed on a case-by-case basis and should be described in the arrangement.

The arrangement may include specific information on:

- legal and regulatory authorities
- phytosanitary and other relevant legislation or regulations
- roles and responsibilities (including those of NPPOs, exporters, growers and other relevant stakeholders)
- timing and duration of the activities
- regulated articles
- all regulated pests and the relevant phytosanitary measures for these pests required by the NPPO of the importing country
- phytosanitary actions such as sampling, inspection, testing, verification of treatment and verification of consignment integrity
- infrastructure and equipment used for the verification of compliance of consignments
- documentation to be maintained and provided by the NPPO of the exporting country to the NPPO of the importing country
- financial aspects
- notification of non-compliance
- corrective actions on a consignment following non-compliance
- frequency and timing of reviews of the arrangement
- criteria that could result in review, evaluation, suspension or termination of the arrangement.

3. Implementation of an Arrangement

The verification of compliance described in an arrangement may be subject to implementation conditions; for example, verification may be for all exported consignments of a particular commodity or only a percentage thereof, for categories of regulated commodities or for a defined time period during the shipping season.

The activities for the verification of compliance to be implemented should be limited to those under the arrangement.

When an arrangement is in place, with verification of compliance being undertaken in the exporting country, the same verification upon import should not be required. However, other procedures undertaken in the importing country may be:

- checks of consignment documentation and identity
- inspection of consignments where packaging has been compromised and the consignments' phytosanitary integrity may have been compromised
- inspection of consignments for contaminating pests in containers
- inspection of consignments in response to an emerging pest risk that was not known at the time of inspection in the exporting country
- inspection of consignments where the arrangement allows for a phytosanitary measure after inspection in the exporting country (e.g. cold treatment for fruit flies during transport).

4. Review of an Arrangement

The effectiveness of an arrangement should be reviewed regularly to identify problems and allow their discussion and resolution in order to improve the arrangement or to determine if it could be downscaled or terminated. The frequency and timing of reviews should be described in the arrangement. Some elements of the arrangement may need to be reviewed more frequently than others.

Changes to the existing arrangement may be proposed by the NPPO of the importing country or the NPPO of the exporting country and require the agreement of both NPPOs before implementation.

5. Termination of an Arrangement

If the reasons for establishing an arrangement are no longer valid (e.g. because of changes in trade logistics between the two countries) or if the arrangement is no longer needed, the arrangement should be terminated.

Once an arrangement has been terminated, verification procedures will be conducted in the importing country.

INTERNATIONAL STANDARDS FOR
PHYTOSANITARY MEASURES

ISPM 39
International movement of wood

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CONTENTS

Adoption

This standard was adopted by the Twelfth Session of the Commission on Phytosanitary Measures in April 2017.

INTRODUCTION

Scope

This standard provides guidance for the assessment of the pest risk of wood and describes phytosanitary measures that may be used to reduce the risk of introduction and spread of quarantine pests associated with the international movement of wood, in particular those that infest trees.

This standard covers only raw wood commodities and material resulting from the mechanical processing of wood: (1) round wood and sawn wood (with or without bark); and (2) materials resulting from the mechanical processing of wood such as wood chips, sawdust, wood wool and wood residue (all with or without bark). This standard covers wood of gymnosperms and angiosperms (i.e. dicotyledons and some monocotyledons, such as palms), but not bamboo and rattan.

Wood packaging material is covered within the scope of ISPM 15 (*Regulation of wood packaging material in international trade*) and therefore is not covered in this standard.

Products manufactured from wood (such as furniture), processed wood material (e.g. pressure treated, glued or heated wood) and wooden handicrafts are not covered in this standard.

Wood may also carry contaminating pests; however, they are not covered in this standard.

References

The present standard refers to International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

FAO. 2009. *Global review of forest pests and diseases.* FAO Forestry Paper 156. Rome, FAO. 222 pp.

FAO. 2011. *Guide to the implementation of phytosanitary standards in forestry*. FAO Forestry Paper 164. Rome, FAO. 101 pp.

Definitions

Definitions of phytosanitary terms can be found in ISPM 5 (*Glossary of phytosanitary terms*).

Outline of Requirements

Pest risk varies among wood commodities such as round wood, sawn wood and wood material resulting from mechanical processing, depending on the level of processing that the wood has undergone.

National plant protection organizations (NPPOs) should use the pest risk analysis (PRA) to provide the technical justification for phytosanitary import requirements for quarantine pests associated with the international movement of wood.

Proportionate to the pest risk identified, phytosanitary measures for managing the pest risk related to wood, including bark removal, treatment, chipping and inspection, should be applied.

The NPPO of the importing country may require as a phytosanitary import requirement an individual phytosanitary measure or a combination of phytosanitary measures under a systems approach.

BACKGROUND

Wood produced from infested trees or woody plants may carry pests. These pests may then infest trees in the PRA area. This is the pest risk primarily dealt with in this standard.

Wood may also become infested by some pests after harvesting. The risk of such infestation is closely tied to the condition of the wood (e.g. the size, presence or absence of bark, moisture content) and exposure to pests after harvest.

Pests that have been shown historically to move with wood in international trade and establish in new areas include: insects that oviposit on bark, bark beetles, wood wasps, wood borers, wood-inhabiting nematodes, and certain fungi with dispersal stages that can be transported with wood. Therefore, wood (with or without bark) moved in international trade is a potential pathway for the introduction and spread of quarantine pests.

Wood is commonly moved as round wood, sawn wood and mechanically processed wood. The pest risk presented by a wood commodity depends on a range of characteristics, such as the commodity's type, the level of processing and the presence or absence of bark, and on factors such as the wood's origin, age, species and intended use and any treatment applied to the wood.

Wood is usually moved internationally to a specific destination and for a specific intended use. Given the frequency of association between key pest groups and key wood commodities, it is important to provide guidance on phytosanitary measures. This standard provides guidance for effectively assessing the risk of quarantine pests and for harmonizing the use of appropriate phytosanitary measures.

The FAO publication *Global review of forest pests and diseases* (2009) provides information on some of the major forest pests of the world. The FAO *Guide to the implementation of phytosanitary standards in forestry* (2011) provides information on best management practices that reduce pest risk during growing, harvesting and shipping of wood.

To differentiate wood from bark as used in this standard, a drawing and photographs of a cross-section of round wood and sawn wood are provided in Appendix 1.

IMPACT ON BIODIVERSITY AND THE ENVIRONMENT

Implementation of this standard is considered to reduce significantly the likelihood of introduction and spread of quarantine pests, thereby contributing to tree health and the protection of forest biodiversity. Certain treatments may have a negative impact on the environment and countries are encouraged to promote the use of phytosanitary measures that have a minimal negative impact on the environment.

REQUIREMENTS

1. Pest Risk Related to Wood Commodities

The pest risk of the commodities addressed in this standard varies depending on: the wood's origin and species; characteristics such as the level of processing and the treatment the wood has undergone and the presence or absence of bark; and the intended use.

This standard describes the general pest risk related to each wood commodity by indicating major pest groups associated with it. In addition to the risk factors listed above, the pest risk associated with a wood commodity may also depend on factors such as age, size, moisture content, pest status at origin and destination, and duration and mode of transport.

Phytosanitary measures should not be required without appropriate technical justification based on PRA (as described in ISPM 2 (*Framework for pest risk analysis*) and ISPM 11 (*Pest risk analysis for quarantine pests*)), taking into account:

- the pest status where the wood originated

- the degree of processing before export
- the ability of a pest to survive on or in the wood
- the intended use of the wood
- the likelihood of establishment of a pest in the PRA area, including the presence of a vector if needed for the dispersal of the pest.

Wood may be infested by pests present in the area of origin at the time of growing or harvesting. Several factors can influence a pest's ability to infest trees or wood. These factors can also affect pest survival on or in the harvested wood, and in turn impact the risk of pest association with the wood. Such factors are: outbreaks of pests in the area of origin, forestry management practices, conditions during transportation, storage time, place and conditions, and treatments applied to the harvested wood. These factors should be considered when evaluating the probability of introduction and spread of quarantine pests.

In general, the greater the level of processing or treatment of the wood after harvest, the greater the reduction in the pest risk. However, it should be noted that processing may change the nature of the pest risk. For example, the physical process of wood chipping is in itself lethal to some insect pests, particularly when a small chip size is produced, but the increase in surface area of the wood may facilitate its colonization by fungi. Chip size varies according to industry specifications and is usually related to the intended use of the chips. Pests that are associated with specific wood tissues (e.g. bark, outer sapwood) pose virtually no pest risk when the tissues that they inhabit are removed during processing. The pest risk associated with the removed material should be assessed separately if it is to be moved in trade as another commodity (e.g. cork, biofuel, bark mulch).

The pest groups identified in Table 1 are known to move with wood commodities and have shown the potential to establish in new areas.

Table 1. Pest groups that may be associated with the international movement of wood

Pest group	Examples within the pest group
Aphids and adelgids	Adelgidae, Aphididae
Bark beetles	Molytinae, Scolytinae
Non-wood-boring moths and wasps	Diprionidae, Lasiocampidae, Lymantriinae, Saturniidae, Tenthredinidae
Scales	Diaspididae
Termites and carpenter ants	Formicidae, Kalotermitidae, Rhinotermitidae, Termitidae
Wood-boring beetles	Anobiidae, Bostrichidae, Buprestidae, Cerambycidae, Curculionidae, Lyctidae, Oedemeridae, Platypodinae
Wood-boring moths	Cossidae, Hepialidae, Sesiidae
Wood flies	Pantophthalmidae
Wood wasps	Siricidae
Canker fungi	Cryphonectriaceae, Nectriaceae
Pathogenic decay fungi	*Heterobasidion* spp.
Pathogenic stain fungi	Ophiostomataceae
Rust fungi	Cronartiaceae, Pucciniaceae
Vascular wilt fungi	Ceratocystidaceae, Ophiostomataceae
Nematodes	*Bursaphelenchus cocophilus*, *B. xylophilus*

There are some pest groups among water moulds, bacteria, viruses and phytoplasmas that, even if known to be associated with wood, are unlikely to establish in new areas by transfer from imported wood to hosts.

1.1 Round wood

Most round wood, with or without bark, is moved internationally for subsequent processing at destination. The wood may be sawn for use as construction material (e.g. as timber framing) or it may be used to produce wood materials (e.g. wood chips, wood wool, bark chips, pulp, firewood, biofuels, manufactured wood products).

Removing bark from round wood reduces the probability of introduction and spread of some quarantine pests. The level of reduction depends on the degree to which the bark and underlying wood have been removed and on the pest group. For example, complete bark removal will greatly reduce the risk of infestation of most bark beetles in the wood. However, bark removal is unlikely to influence the incidence of deep wood borers, some species of fungi and wood-inhabiting nematodes.

The pest risk of round wood is greatly influenced by the total amount of remaining bark on the debarked wood, which in turn is greatly influenced by the shape of the round wood, the machinery used to remove the bark and, to a lesser extent, by the species of tree. In particular, the widened areas at the base of a tree, especially where large root buttresses are present, and around branch nodes are the preferred locations for beetle infestation and oviposition.

The pest groups likely to be associated with round wood are listed in Table 2.

Table 2. Likelihood of pest groups to be associated with round wood

Commodity	Likely	Less likely
Round wood with bark	Aphids and adelgids, bark beetles, non-wood-boring moths, scales, termites and carpenter ants, wood-boring beetles, wood-boring moths, wood flies, wood wasps; canker fungi, pathogenic decay fungi, pathogenic stain fungi, rust fungi, vascular wilt fungi; nematodes	
Round wood without bark	Termites and carpenter ants, wood-boring beetles, wood-boring moths, wood flies, wood wasps; canker fungi, pathogenic decay fungi, pathogenic stain fungi, vascular wilt fungi; nematodes	Aphids and adelgids, bark beetles†, non-wood-boring moths, scales; rust fungi

† Some bark beetles have life stages that are found in the wood below the surface of the bark and cambium and, therefore, may be present after debarking or complete bark removal.

1.2 Sawn wood

Most sawn wood, with or without bark, is moved internationally for use in building construction and furniture manufacturing and for the production of wood packaging material, wood lathing, wood stickers, wood spacers, railway sleepers (ties) and other constructed wood products. Sawn wood may include fully squared pieces of wood without bark or partially squared wood with one or more curved edges that may or may not include bark. The thickness of the piece of sawn wood may affect the pest risk.

Sawn wood from which some or all bark has been removed presents a much lower pest risk than sawn wood with bark. Reducing the size of pieces of bark remaining on wood reduces the pest risk.

The pest risk of bark-related organisms is also dependent on the moisture content of the wood. Wood from freshly harvested living trees has a high moisture content that decreases over time to ambient moisture conditions, which are less likely to allow bark-related organisms to survive. Further information on addressing pest risk through a combination of treatment and moisture reduction is provided in Appendix 2.

The pest groups likely to be associated with sawn wood are listed in Table 3.

Table 3. Likelihood of pest groups to be associated with sawn wood

Commodity	Likely	Less likely
Sawn wood with bark	Bark beetles, termites and carpenter ants, wood-boring beetles, wood-boring moths, wood flies, wood wasps; canker fungi, pathogenic decay fungi†, pathogenic stain fungi, rust fungi, vascular wilt fungi; nematodes	Aphids and adelgids, non-wood-boring moths, scales‡
Sawn wood without bark	Termites and carpenter ants, wood-boring beetles, wood-boring moths, wood flies, wood wasps; canker fungi, pathogenic decay fungi†, pathogenic stain fungi, vascular wilt fungi; nematodes	Aphids and adelgids, bark beetles, non-wood-boring moths, scales‡; rust fungi

† Although pathogenic decay fungi may be present in sawn wood, most present a low risk of establishment because of the intended use of the wood and the limited potential for the fungi to produce spores on the wood.

‡ Many scale species are removed during the squaring of wood, but remaining bark may present sufficient surface area for some species to survive after sawing.

1.3 Wood materials produced from mechanical processing of wood (excluding sawing)

Mechanical processes that reduce the size of wood pieces reduce the pest risk of some pests. However, for other pests, alternative pest risk management measures are necessary.

1.3.1 Wood chips

In addition to the pest risk factors mentioned in section 1 pertaining to wood in general, the pest risk of wood chips varies with their size and uniformity, and also with their storage conditions. The pest risk is reduced when bark is removed and the chip size is less than 3 cm in at least two dimensions (as described in Table 4 and section 2.3). The physical process of wood chipping is in itself lethal to some insect pests, particularly when a small chip size is produced. Chip size varies according to industry specifications and is usually related to the intended use of the chips (e.g. biofuel, paper production, horticulture, animal bedding). Some wood chips are produced in accordance with strict quality standards to minimize bark and fines (very small particles).

Depending on their size, insect pests normally found under the bark may be present in wood chips with bark. Many species of pathogenic decay fungi, canker fungi and nematodes may also be present in wood chips with or without bark. Spore dispersal of wood-inhabiting rust fungi would be very unlikely after the production of chips.

1.3.2 Wood residue

Wood residue is normally considered to present a high pest risk because it varies greatly in size and may or may not include bark. Wood residue is generally a waste by-product of wood being mechanically processed during production of a desired article; nevertheless, wood residue may be moved as a commodity.

The pest groups likely to be associated with wood chips and wood residue are listed in Table 4.

Table 4. Pest groups likely to be associated with wood chips and wood residue

Commodity	Likely	Less likely
Wood chips with bark and greater than 3 cm in at least two dimensions	Bark beetles, termites and carpenter ants, wood-boring beetles, wood-boring moths, wood flies, wood wasps; canker fungi pathogenic decay fungi†, pathogenic stain fungi, rust fungi†, vascular wilt fungi; nematodes	Aphids and adelgids, non-wood-boring moths, scales
Wood chips without bark and greater than 3 cm in at least two dimensions	Termites and carpenter ants, wood-boring beetles, wood-boring moths, wood flies, wood wasps; canker fungi, pathogenic decay fungi†, pathogenic stain fungi, vascular wilt fungi; nematodes	Aphids and adelgids, bark beetles, non-wood-boring moths, scales; rust fungi†
Wood chips with bark and less than 3 cm in at least two dimensions	Bark beetles, termites and carpenter ants; canker fungi, pathogenic decay fungi†, pathogenic stain fungi, rust fungi†, vascular wilt fungi; nematodes	Aphids and adelgids, non-wood-boring moths, scales, wood-boring beetles, wood-boring moths, wood flies, wood wasps
Wood chips without bark and less than 3 cm in at least two dimensions	Termites and carpenter ants; canker fungi, pathogenic decay fungi†, pathogenic stain fungi, vascular wilt fungi; nematodes	Aphids and adelgids, bark beetles, non-wood-boring moths, scales, wood-boring beetles, wood-boring moths, wood flies, wood wasps; rust fungi†
Wood residue with or without bark	Aphids and adelgids, bark beetles, non-wood-boring moths, scales, termites and carpenter ants, wood-boring beetles, wood-boring moths, wood flies, wood wasps; canker fungi, pathogenic decay fungi†, pathogenic stain fungi, rust fungi†, vascular wilt fungi; nematodes	

† Rust and pathogenic decay fungi may be present in consignments of wood chips or wood residue but are unlikely to establish or spread.

1.3.3 Sawdust and wood wool

Sawdust and wood wool present a lower pest risk than the commodities above. In certain cases, fungi and nematodes may be associated with sawdust. Wood wool is considered to present a similar pest risk as sawdust.

2. Phytosanitary Measures

The phytosanitary measures described in this standard should be required only if technically justified, based on PRA. A specific element to consider through PRA is how pest risk may be mitigated by the intended use of the commodity. Certain phytosanitary measures may be implemented to protect wood that has been produced in pest free areas but that may be at risk of infestation (e.g. during storage and transportation). Various methods to safeguard against infestation after the application of a phytosanitary measure should be considered; for example, covering wood with tarpaulin for storage or using an enclosed conveyance.

The NPPO of the importing country may require limitations on the time frame for import. The pest risk associated with wood moved in trade may be managed by the NPPO of the importing country specifying a certain time in which dispatch or import of a consignment may occur (e.g. during a time when a pest is inactive).

The NPPO of the importing country may require the application of specific methods of processing, handling and appropriate disposal of waste after import.

If necessary to comply with the phytosanitary import requirements, the NPPO of the exporting country should verify the application and the effectiveness of phytosanitary measures before export in accordance with ISPM 23 (*Guidelines for inspection*) and ISPM 31 (*Methodologies for sampling of consignments*).

Many pests associated with wood are specific to particular tree genera or species, and hence phytosanitary import requirements for wood are often genus or species specific. Therefore, the NPPO of the exporting country should verify that the genus or species of the wood in the consignment complies with phytosanitary import requirements, where such genus or species requirements exist.

The following sections describe commonly used options for phytosanitary measures.

2.1 Removal of bark

Some quarantine pests are commonly found in or just beneath the bark. To reduce the pest risk, the NPPO of the importing country may require the removal of bark (to produce bark-free or debarked wood) as a phytosanitary import requirement and, in the case of debarked wood, the NPPO may set tolerance levels for remaining bark. Where bark remains with wood, treatments may be used to reduce the pest risk associated with bark.

2.1.1 Bark-free wood

The complete removal of bark from round wood and other wood commodities physically removes a layer of material in which a large number of pests may develop, and eliminates large areas of uneven surface that provide concealment for other pests.

Bark removal eliminates pests found mostly on the surface of bark such as aphids, adelgids, scale insects, and non-wood-boring moths in some life stages. Moreover, bark removal eliminates most bark beetles and also prevents post-harvest infestation by other wood pests such as wood wasps and large wood borers (e.g. *Monochamus* spp.).

Where the NPPO of the importing country requires that the wood be bark-free, the commodity should meet the definition of bark-free wood stated in ISPM 5 (see Appendix 1 for illustration of ingrown bark and bark pockets). Bark completely surrounded by cambium presents a much lower pest risk as compared with that of surface bark. In many cases, the wood may have evidence of cambium, which may appear as a brown discoloured tissue on the surface of the wood, but this should not be considered as the presence of bark and does not pose a pest risk for pests associated with bark. Verification of bark-free wood should simply confirm that there is no evidence of the layer of tissue above the cambium.

2.1.2 Debarked wood

The mechanical process used in the commercial removal of bark from wood may not completely remove all bark and some pieces of bark may remain. The number and size of any remaining pieces of bark determines to what extent the risk of pests associated with bark (e.g. bark beetles, aphids, adelgids, scales) is reduced.

Some countries specify the tolerance levels for bark in imported wood in their regulations. Debarking to the tolerances indicated below reduces the risk of pests completing their life cycle in untreated wood.

When technically justified and prescribed as a phytosanitary import requirement by the NPPO of the importing country, the NPPO of the exporting country should ensure that the following requirements for debarked wood have been met.

For example, to mitigate the risk of presence of bark beetles, any number of visually separate and clearly distinct small pieces of bark may remain if they are:

- less than 3 cm in width (regardless of the length) or
- greater than 3 cm in width, with the total surface area of an individual piece of bark less than 50 cm^2.

2.2 Treatments

Treatments accepted internationally, found as annexes to ISPM 28 (*Phytosanitary treatments for regulated pests*), may be used as phytosanitary import requirements for some wood commodities.

The efficacy of all chemical treatments is affected by the penetration depth, which varies by treatment schedule (e.g. dosage, temperature), the wood species and moisture content, and the presence of bark. The removal of bark often improves chemical treatment penetration and may reduce the incidence of infestation of treated wood.

Treatments should be applied under the supervision or with the authorization of the NPPO of the exporting country to meet the phytosanitary import requirements. The NPPO of the exporting country should make arrangements to ensure that treatments are applied as prescribed and, where appropriate, should verify that wood is free of target pests by inspection or testing prior to phytosanitary certification. Specific tools (e.g. electronic thermometers, gas chromatographs, moisture meters connected to recording equipment) may be used to verify treatment application.

The presence of live quarantine pests should be considered as non-compliance of the consignment, with the exception of wood treated by irradiation, which may result in live but sterile pests. In addition, findings of suitable indicator organisms (or fresh frass) indicates treatment failure or non-compliance, depending on the treatment type.

Some treatment types may not be effective against all pests. Further guidance on treatments that may be used to mitigate the pest risk of wood is provided in Appendix 2.

2.3 Chipping

The mechanical action of chipping or grinding wood can be effective in destroying most wood-dwelling pests. Reduction of the chip size to a maximum of 3 cm in at least two dimensions may mitigate the pest risk posed by most insects. However, fungi, nematodes and small insects such as some Scolytinae, or small Buprestidae, Bostrichidae or Anobiidae may continue to present a pest risk.

2.4 Inspection and testing

Inspection or testing may be used for the detection of specific pests associated with wood. Depending on the wood commodity, inspection may be used to identify specific signs or symptoms of pests. For example, inspection may be used to detect the presence of bark beetles, wood borers and decay fungi on round wood and sawn wood. Inspection may also be carried out at various points along the production process to determine if phytosanitary measures applied have been effective.

Where undertaken, inspection methods should enable the detection of any signs or symptoms of quarantine pests. The detection of certain other organisms may indicate treatment failure. Signs may include the fresh frass of insects, galleries or tunnels of wood borers, staining on the surface of the wood caused by fungi, and voids or signs of wood decay. Signs of wood decay include bleeding cankers, long discontinuous brown streaks on outer sapwood and outer sapwood discoloration, soft areas in the wood, unexplained swelling, resin flow on logs, and cracks, girdling and wounds in sawn wood. Where bark is present it may be peeled back to look for signs of insect feeding and galleries, and for staining or streaking of the wood underneath, which may indicate the presence of pests. Acoustic, sensory and other

methods may also be used for detection. Further examination should be made to verify whether live quarantine pests or indicator organisms are present; for example, examination for living life stages of insects such as egg masses and pupae.

Testing may be used to verify the application or effect of other phytosanitary measures such as treatments. Testing is generally limited to the detection of fungi and nematodes. For example, determination of the presence of nematodes that are quarantine pests may be made using a combination of microscopy and molecular techniques on samples of wood taken from consignments.

Guidance on inspection and sampling is provided in ISPM 23 and ISPM 31.

2.5 Pest free areas, pest free places of production and areas of low pest prevalence

Pest free areas, pest free places of production and areas of low pest prevalence may be established to manage the pest risk associated with wood, where feasible. Relevant guidance is presented in ISPM 4 (*Requirements for the establishment of pest free areas*), ISPM 8 (*Determination of pest status in an area*), ISPM 10 (*Requirements for the establishment of pest free places of production and pest free production sites*), ISPM 22 (*Requirements for the establishment of areas of low pest prevalence*) and ISPM 29 (*Recognition of pest free areas and areas of low pest prevalence*). However, the use of pest free places of production or pest free production sites may be limited to specific situations such as forest plantations located within agricultural or suburban areas. Biological control may be used as an option for achieving the requirements for an area of low pest prevalence.

2.6 Systems approaches

The pest risk of the international movement of wood may be managed effectively by developing systems approaches that integrate measures for pest risk management as described in ISPM 14 (*The use of integrated measures in a systems approach for pest risk management*). Existing forest management systems, both pre- and post-harvest, including processing, storage and transportation, may include activities such as site selection in pest free areas, inspection to ensure the wood is free from pests, treatments, physical barriers (e.g. wrapping wood), and other measures which when integrated in a systems approach are effective in pest risk management.

Some of the pest risk associated with round wood (in particular that of deep wood borers and certain nematodes) is difficult to manage through the application of a single phytosanitary measure. In these situations, a combination of phytosanitary measures in a systems approach may be applied.

In accordance with ISPM 14, the NPPO of the importing country may implement additional measures within its territory for transporting, storing or processing wood after import. For example, round wood with bark that may harbour bark beetles that are quarantine pests may be permitted to enter the importing country only during a period when the bark beetles are not active. In this case, processing in the importing country to remove the pest risk may be required to occur before organisms develop to the active stage. Requirements that the wood be debarked and the bark or wood residue be used as a biofuel or otherwise destroyed before the active period of the beetles commences may be used to sufficiently prevent the risk of introduction and spread of the bark beetles that are quarantine pests.

The pest risk associated with fungi may be managed effectively through selection of wood from pest free areas or pest free places of production, application of appropriate harvesting (e.g. visual selection of wood free from signs of infestation) and processing measures and treatments (e.g. surface fungicide).

3. Intended Use

The intended use of wood may affect its pest risk, because some intended uses (e.g. round wood as firewood, wood chips as biofuel or for horticultural purposes) may affect the probability of introduction and spread of quarantine pests (ISPM 32 (*Categorization of commodities according to their pest risk*)). Therefore, intended use should be taken into account when assessing or managing the pest risk associated with the international movement of wood.

4. Non-compliance

Relevant information on non-compliance notification and emergency action is provided in ISPM 13 (*Guidelines for the notification of non-compliance and emergency action*) and ISPM 20 (*Guidelines for phytosanitary import regulatory system*).

This appendix is for reference purposes only and is not a prescriptive part of the standard.

APPENDIX 1: Illustrations of bark and wood

Illustrations are provided below to assist in better differentiating wood and cambium from bark.

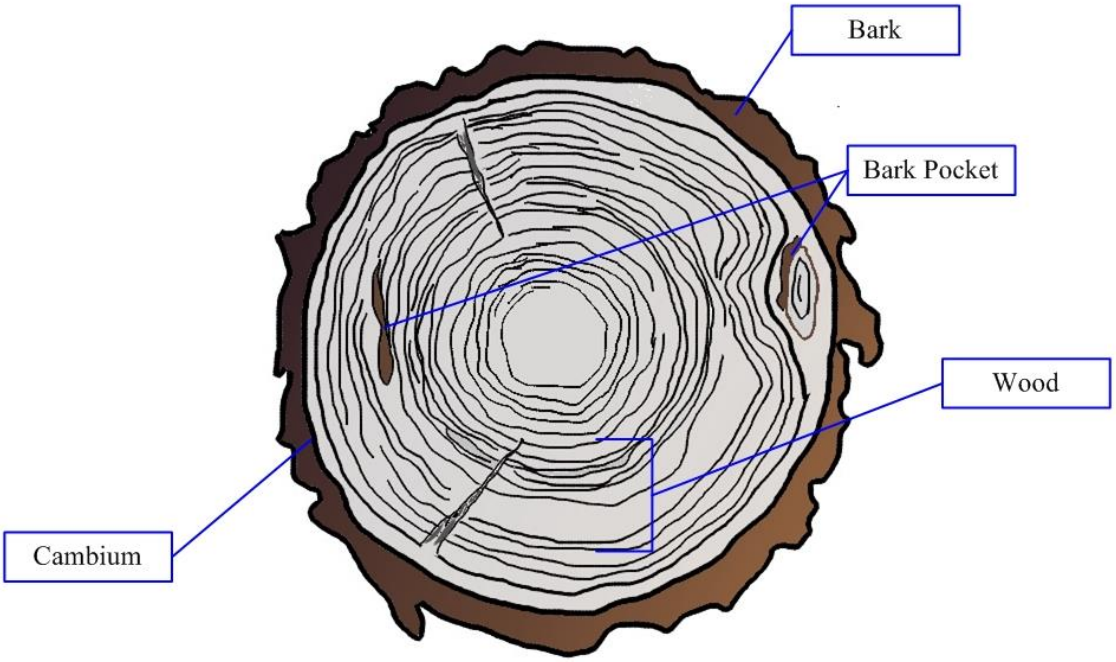

Figure 1. Cross-section of round wood.
Drawing courtesy S. Sela, Canadian Food Inspection Agency.

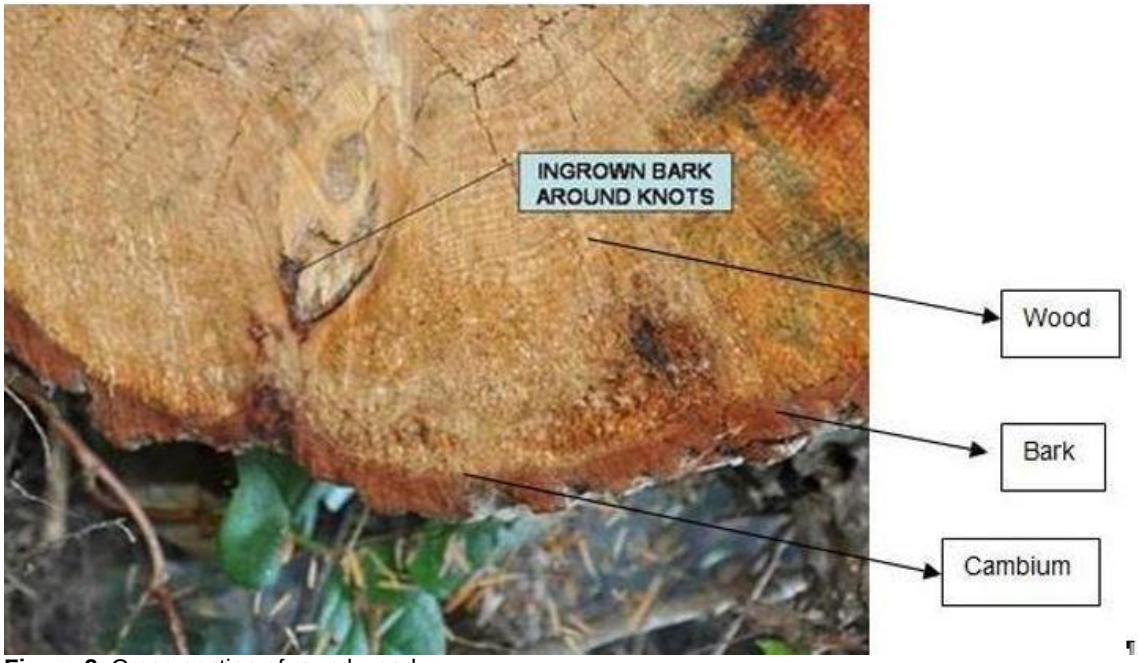

Figure 2. Cross-section of round wood.
Photo courtesy S. Sela, Canadian Food Inspection Agency.

Figure 3. Sawn wood.
Photo courtesy C. Dentelbeck, Canadian Lumber Standards Accreditation Board, Ottawa.

APPENDIX 2: Treatments that may be used to mitigate the pest risk of wood

1. Fumigation

Fumigation may be used to control pests associated with wood.

Despite the proven effectiveness of some fumigants against certain pests, there are limitations to their use to reduce pest risk. Fumigants vary in their ability to penetrate the wood and some are therefore effective only against pests in, on or just beneath the bark. The penetration depth for some fumigants may be limited to about 10 cm from the wood surface. Penetration is greater in dry than in fresh-cut wood.

For some fumigants, the removal of bark before fumigation may improve the efficacy of the treatment.

Before selecting fumigation as a phytosanitary measure, NPPOs should take into account the CPM Recommendation, *Replacement or reduction of the use of methyl bromide as a phytosanitary measure* (CPM, 2008).

2. Spraying or Dipping

Spraying with or dipping in chemicals may be used to control pests associated with wood, excluding wood chips, sawdust, wood wool, bark and wood residue.

In the process of spraying or dipping, liquid or dissolved chemicals are applied to wood at ambient pressure. This treatment results in limited penetration into the sapwood. Penetration depends on the species of the wood, the kind of wood (sapwood or heartwood), and the properties of the chemical product. Both removal of bark and application of heat increase the depth of penetration into the sapwood. The active ingredient of the chemical product may not prevent the emergence of pests already infesting the wood. Protection of the treated wood from subsequent pest infestation depends on the protective layer of chemical product remaining intact. Post-treatment infestation by some pests (e.g. dry wood borers) may take place if the wood is sawn after treatment and a portion of the cross-section has not been penetrated by the chemical product.

3. Chemical Pressure Impregnation

Chemical pressure impregnation may be used to control pests associated with wood, excluding wood chips, sawdust, wood wool, bark and wood residue.

The application of a preservative using vacuum, pressure or thermal processes results in a chemical product applied to the surface of the wood being forced deep into that wood.

Chemical pressure impregnation is commonly used to protect wood from infestation by pests after other treatments. It may also have some effect in preventing the emergence to the wood surface of pests that have survived treatment. The penetration of the chemical product into the wood is much greater than with spraying or dipping, but depends on the wood species and the properties of the chemical product. Penetration is generally throughout the sapwood and through a limited portion of the heartwood. Debarking or mechanical perforation of the wood may improve penetration of the chemical product. Penetration also depends on the moisture content of the wood, so drying wood before chemical pressure impregnation may improve penetration. Chemical pressure impregnation is effective against some wood-boring insects. In some impregnation processes, the chemical is applied at a temperature sufficiently high to be equivalent to a heat treatment. The protection of the treated wood from subsequent infestation depends on the protective layer of the chemical product remaining intact. Post-treatment infestation by some pests (e.g. dry wood borers) may take place if the wood is sawn after treatment and a portion of the cross-section has not been penetrated by the chemical product.

4. Heat Treatment

Heat treatment may be used to control pests associated with all wood commodities. The presence or absence of bark has no effect on the efficacy of heat treatment but should be taken into account if a heat treatment schedule specifies the maximum dimensions of the wood being treated.

The process of heat treatment involves heating wood to a temperature for a period of time (with or without moisture control) that is specific to the target pest. The minimum treatment time in the heat chamber necessary to reach the required temperature throughout the profile of the wood depends on the wood's dimensions, species, density and moisture content as well as on the capacity of the chamber and other factors. The heat may be produced in a conventional heat treatment chamber or by dielectric, solar or other means of heating.

The temperature required to kill pests associated with wood varies because heat tolerance varies across species. Heat-treated wood may still be susceptible to saprophytic moulds, particularly if moisture content remains high; however, mould should not be considered a phytosanitary concern.

5. Kiln-drying

Kiln-drying may be used for sawn wood and many other wood commodities.

Kiln-drying is an industrial process in which the moisture content in wood is reduced, by the application of heat, to achieve the prescribed moisture content for the intended use of the wood. Kiln-drying may be considered a heat treatment if carried out at sufficient temperatures and for sufficient durations. If lethal temperatures are not achieved throughout the relevant wood layers, kiln-drying on its own should not be considered a phytosanitary treatment.

Some species in the pest groups associated with wood commodities are dependent on moisture and therefore may be inactivated during kiln-drying. Kiln-drying also permanently alters the physical structure of the wood, which prevents subsequent resorption of sufficient moisture to sustain existing pests and reduces the incidence of post-harvest infestation. However, individuals of some species may be capable of completing their life cycle in the new environment of reduced moisture content. If favourable moisture conditions are re-established, many fungi and nematodes and some insect species may be capable of continuing their life cycle or infesting the wood after treatment.

6. Air-drying

Compared with kiln-drying, air-drying reduces wood moisture content only to ambient moisture levels and is therefore less effective against a broad range of pests. The pest risk remaining after treatment depends on the duration of drying and the moisture content and on the intended use of the wood. Moisture reduction through air-drying alone should not be considered a phytosanitary measure.

Although moisture reduction through air-drying or kiln-drying alone may not be a phytosanitary measure, wood dried to below the fibre saturation point may be unsuitable for infestation by many pests. Therefore, the likelihood of infestation of dried wood is very low for many pests.

7. Irradiation

The exposure of wood to ionizing radiation (e.g. accelerated electrons, x-rays, gamma rays) may be sufficient to kill, sterilize or inactivate pests (ISPM 18 (*Guidelines for the use of irradiation as a phytosanitary measure*)).

8. Modified Atmosphere Treatment

Modified atmosphere treatments may be applied to round wood, sawn wood, wood chips and bark.

In such treatments, wood is exposed to modified atmospheres (e.g. low oxygen, high carbon dioxide) for extended periods of time to kill or inactivate pests. Modified atmospheres can be artificially

generated in gas chambers or allowed to occur naturally, for instance during water storage or when the wood is wrapped in airtight plastic.

9. References

CPM. 2008. *Replacement or reduction of the use of methyl bromide as a phytosanitary measure*. CPM Recommendation. In: *Report of the Third Session of the Commission on Phytosanitary Measures*. Rome, 7–11 April 2008, Appendix 6. Rome, IPPC, FAO. Available at https://www.ippc.int/publications/500/ (last accessed 21 November 2016).

INTERNATIONAL STANDARDS FOR
PHYTOSANITARY MEASURES

ISPM 40

International movement of growing media in association with plants for planting

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This is not an official part of the standard

2004-11 SC recommended topic *Soil and growing media* (2005-004) be added to the work programme

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2014-11 SC revised and approved draft for CPM adoption

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ISPM 40. 2017. *International movement of growing media in association with plants for planting*. Rome, IPPC, FAO.

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CONTENTS

Adoption

This standard was adopted by the Twelfth Session of the Commission on Phytosanitary Measures in April 2017.

INTRODUCTION

Scope

This standard provides guidance for the assessment of the pest risk of growing media in association with plants for planting and describes phytosanitary measures to manage the pest risk of growing media associated with plants for planting in international movement.

Growing media moved as a separate commodity, contaminating a commodity or used as packaging material are not considered in this standard.

References

The present standard refers to International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

Definitions

Definitions of phytosanitary terms used in this standard can be found in ISPM 5 (*Glossary of phytosanitary terms*).

Outline of Requirements

Pest risk analysis (PRA) should provide the technical justification for phytosanitary import requirements for growing media in association with plants for planting.

The origin and the production method of components of growing media can affect the pest risk of the growing media associated with plants for planting. Growing media should be produced, stored and maintained under conditions that prevent contamination or infestation. These conditions will depend on the type of growing medium used. Growing media may need to be appropriately treated before use.

The production methods for plants for planting may affect the pest risk of growing media associated with these plants for planting.

Pest risk management options related to growing media in association with plants for planting – including phytosanitary measures such as treatment, inspection, sampling, testing, quarantine and prohibition – are described in this standard.

BACKGROUND

Soil as a growing medium is considered to be a high-risk pathway because it can harbour numerous quarantine pests and a number of other growing media are also recognized pathways for the introduction and spread of quarantine pests. The pest risk of growing media in association with plants for planting depends on factors related to both the production of the growing media and the production of the plants, as well as the interaction between the two.

Many countries have legislation in place to regulate the movement of growing media, particularly soil or soil as a component of growing media, but not necessarily for growing media associated with plants for planting. Growing media, particularly soil, are often prohibited. While it is possible to remove growing medium from some plants for planting, it may be difficult to completely avoid the movement of growing media in association with plants for planting. Some plants can survive transport only when moved in growing medium.

IMPACT ON BIODIVERSITY AND THE ENVIRONMENT

Pests associated with the international movement of growing media in association with plants for planting may have negative impacts on biodiversity. Implementation of this standard could significantly reduce the introduction and spread of quarantine pests associated with growing media and consequently reduce their negative impacts. In addition, the application of phytosanitary measures in accordance with this standard could also reduce the probability of introduction and spread of other organisms that may become invasive alien species in the importing country and thus affect biodiversity.

Certain phytosanitary measures (e.g. some treatments with fumigants) may have a negative impact on the environment. Countries are encouraged to promote the use of phytosanitary measures that have a minimal negative impact on the environment.

REQUIREMENTS

1. Pest Risk Analysis

This standard addresses the pest risk of quarantine pests in growing media, and only growing media that are associated with plants for planting. In some cases, however, regulated non-quarantine pests associated with those growing media may also need to be considered in the PRA.

Phytosanitary import requirements for growing media should be technically justified and based on a PRA in accordance with ISPM 2 (*Framework for pest risk analysis*), ISPM 11 (*Pest risk analysis for quarantine pests*) and ISPM 21 (*Pest risk analysis for regulated non-quarantine pests*). The PRA should include consideration of the factors that affect the pest risk of growing media, described in this standard, and factors related to the production of plants for planting, described in Annex 1 of ISPM 36 (*Integrated measures for plants for planting*). The pest risk posed by plants for planting, as well as that of the associated growing media in which the plants were grown, should be assessed together.

It should be noted that quarantine pests carried with growing medium in association with a plant may be pests of other plants, or may act as a vector for other pests.

2. Factors That Affect the Pest Risk of Growing Media

The production methods for plants for planting may affect the pest risk of the growing media used. While some growing media may pose a low pest risk by nature of their production, they may become contaminated or infested, depending on the type and composition of the growing medium during the production process of the commodity (i.e. growing media in association with plants for planting).

The national plant protection organization (NPPO) of the importing country may take into consideration the pest risk of growing media (as outlined in Annex 1, Annex 2 and Appendix 1) when conducting a PRA to identify appropriate phytosanitary measures. Based on the pests regulated by the importing country, the PRA should include consideration of the pest status in the importing and exporting countries. Furthermore, the pest risk may also depend on:

- whether the growing media are new or reused
- the origin of the growing media
- the components of the growing media
- the measures used in the production of the growing media, including the degree of processing and any treatments applied
- the measures to prevent contamination or infestation of the growing media before planting, such as during transportation and storage, as well as during plant propagation and production (e.g. use of clean starter plant stock, treatment of the irrigation water and avoiding exposure to high-risk growing media)
- the length of the plant's production cycle
- the quantity of growing media present in association with all plants for planting in a consignment.

In the assessment of pest risk, data on historical or existing importation of growing media and their geographical origin may be relevant.

The origin and production method of components of growing media affect the pest risk of growing media. Annex 1 lists common components of growing media and indicates their relative pest risk, assuming that they were not previously used as growing media and that they have been handled and stored in a way that prevents their contamination and recontamination.

Growing media containing organic components (including plant debris) may be more likely to harbour pests and so generally pose greater pest risk than purely mineral or synthetic growing media. If the growing medium consists of organic components, the pest risk may be particularly difficult to assess fully because of the likely presence of unknown organisms and it should be processed in a way that adequately addresses the pest risk.

3. Pest Risk Management Options

The following measures may be used singly or in combination to ensure the pest risk of growing media is adequately managed.

3.1 Growing media free from quarantine pests

Growing media free from quarantine pests may be achieved by:

- using growing media produced in a process that renders the growing media free from pests
- using growing media or their components collected from a pest free area or a pest free production site
- applying appropriate treatments to growing media that are not free from pests, before their use.

Growing media should be produced under a system that allows appropriate trace back and forward of both the media and their components, where appropriate.

Pest free growing media should be stored and maintained under conditions that keep them free from quarantine pests. The growing media should not be exposed to plants, pests, untreated soil, other untreated growing media or contaminated water. If this has not been achieved, the growing media should be treated appropriately before use.

Plants intended to be planted in the pest free growing media should be free from relevant quarantine pests.

The following measures may be used to prevent contamination or infestation of the growing media after planting the plants:

- using clean tools, clean equipment, clean containers, etc.
- keeping the growing media associated with the plants in a pest free area or a pest free place of production
- using water free from quarantine pests
- using physical isolation (e.g. protected conditions, prevention of pest transmission by wind, production on benches separated from contact with soil).

Examples of pest management measures to reduce pest risk that could be appropriate for growing media are available in ISPM 36.

3.2 Treatments

Treatments may be applied at various stages in the production cycle to mitigate the pest risk of growing media. Treatments that may be applied singly or in combination include:

- treatment of growing media before planting or after planting (e.g. steam treatment, heat treatment, chemical treatment, a combination of treatments)
- treatment of fields or planting beds intended for the production of plants for planting

- treatment (e.g. filtration, sterilization) of water or water-based nutrient solution used for irrigation or as a growing medium
- treatment of plants or propagative plant parts (e.g. seeds, bulbs, cuttings) before planting
- removal of growing media[1] (e.g. by root washing or plant shaking).

Factors such as temperature may affect the results of treatments. Also, some pesticides may only suppress, rather than eradicate, pest populations. Verification of the effectiveness of a treatment after application may be necessary.

After treatment, appropriate measures should be taken to avoid recontamination or reinfestation.

3.3 Inspection, sampling and testing

The places of production and the processing or treatment procedures for growing media may be inspected, monitored or approved by the NPPO of the exporting country, which should ensure that phytosanitary import requirements are met.

Plants for planting and associated growing media may need to be inspected to determine if pests are present or to determine compliance with phytosanitary import requirements (ISPM 23 (*Guidelines for inspection*)). However, most pests in growing media cannot be detected by inspection alone and testing may be required.

The NPPO of the importing country may require or undertake sampling and testing of the growing media associated with plants for planting (ISPM 20 (*Guidelines for a phytosanitary import regulatory system*); ISPM 31 (*Methodologies for sampling of consignments*)). However, sampling and testing may not detect some types of pests, in particular at low-level contamination or infestation of the growing media. To verify that required measures have been carried out, testing may include testing for indicator organisms (easily detectable organisms whose presence indicates that required measures failed to be effective or were not implemented).

3.4 Quarantine

The NPPO of the importing country may require quarantine for growing media attached to plants for planting, to reduce the pest risk. Quarantine allows for options such as testing, observation for signs or symptoms, and treatment for plants for planting and growing medium attached to the plants, during a quarantine period.

Quarantine may also be used for monitoring in cases where knowledge about the pest risk is incomplete or there is an indication of a failure of measures taken in the exporting country (e.g. a significant number of interceptions).

3.5 Prohibition

In cases where the measures outlined above are not deemed applicable, feasible or sufficient for growing media in association with certain plants for planting, the entry of growing medium in association with plants for planting may be prohibited.

[1] In some cases, removal of growing media may be followed by replanting in not previously used pest free growing media shortly before export, if accepted by the NPPO of the importing country.

This annex is a prescriptive part of the standard.

ANNEX 1: Common components of growing media ranked in order of increasing relative pest risk

The approximate ranking provided in this table is for components of growing media that have not previously been used for planting and have been handled and stored in a way that prevents contamination or infestation (e.g. they are free from soil).

The table outlines the relative pest risk posed by different components of growing media, but not in association with plants for planting.

Components of growing media	Facilitate pest survival	Comments
Baked clay pellets	No	Inert material
Synthetic media (e.g. glass wool, rock wool, polystyrene, floral foam, plastic particles, polyethylene, polymer stabilized starch, polyurethane, water-absorbing polymers)	No	Inert material
Vermiculite, perlite, volcanic rock, zeolite, scoria	No	Heat of production renders vermiculite and perlite virtually sterile
Clay	No	
Gravel, sand	No	
Paper, including corrugated cardboard	Yes	High level of processing
Tissue culture medium (agar-like)	Yes	Autoclaved or sterilized before use
Coconut fibres (coir/coco peat)	Yes	Pest risk depends on level of processing
Sawdust, wood shavings (excelsior)	Yes	Size of particles and heat treatment may affect the probability of pest survival
Water	Yes	Pest risk depends on source and treatment
Wood chips	Yes	Size of particles may affect the probability of pest survival
Cork	Yes	Pest risk depends on level of processing
Peat (excluding peat soil)	Yes	Pest risk is lower where the origin has had no agricultural exposure (e.g. certified bogs). Peat may contain seeds of plants as pests.
Non-viable moss (sphagnum)	Yes	Pest risk depends on level of processing. Living moss (sphagnum) may contain seeds of plants as pests.

Other plant material (e.g. rice hulls/chaff, grain hulls, coffee hulls, fallen leaves, sugar-cane refuse, grape marc, cocoa pods, oil palm shell charcoal)	Yes	Pest risk is reduced if treated or from a clean non-infested source
Bark	Yes	Pest risk depends on source (potential to harbour forest pests) and degree of processing or fermentation
Biowaste	Yes	Pest risk depends on source and degree of processing
Compost (e.g. municipal or agricultural composted waste, humus, leaf mould)	Yes	Pest risk depends on source and degree of processing or fermentation. Seeds of plants as pests are common.
Soil	Yes	Pest risk can be reduced if treated
Tree fern slabs	Yes	Pest risk depends on source and treatment
Vermicompost	Yes	May include remains of undigested organic material. Vermicompost should be prepared early as required, and treated to eliminate any organism before using as a growing medium.

This annex is a prescriptive part of the standard.

ANNEX 2: Examples of growing media and the measures that may effectively manage their pest risk when associated with plants for planting

Growing medium	Water and nutrients	Measures	Examples
Growing medium that has been sterilized (e.g. by heat to a specified temperature for a specified duration)	Sterilized, treated or filtered water supply (free from pests)	Maintained in conditions to prevent pest infestation	Plants grown from seed under protected conditions
Inert material such as perlite or vermiculite	Sterilized water-based nutrient solution	Maintained in conditions to prevent pest infestation	Plants for hydroponic cultivation where the absence of pests can be verified
Tissue culture medium	Incorporated in sterile medium	Maintained in aseptic conditions	Tissue cultured plants transported in closed containers
Water	Water or water-based nutrient solution	Sterilized, treated or filtered water may be required	Plants rooted in water

This appendix is for reference purposes only and is not a prescriptive part of the standard.

APPENDIX 1: Examples of common combinations of plants for planting and growing media moved internationally

Plant type	Growing media	Comments
Artificially dwarfed nursery stock	Soil	The plant roots are typically very difficult to wash free from soil. The plants may be transplanted to soil-free growing media and grown in greenhouses using integrated risk mitigation measures in an effort to minimize the pest risk associated with them.
Bare root nursery stock	Soil or none	Bare root is a technique of arboriculture whereby a field-grown tree or shrub is dug to be placed in a dormant state. The nursery stock may be shaken to remove some of the soil, or it may be washed free from all soil and growing media. The size and root structure of the plant and the type of soil has a large impact on whether soil can be removed from the root system.
Dormant bulbs and tubers, tuberous roots and herbaceous perennial roots	Soil, peat or none	Bulbs, tubers (including corms and rhizomes), tuberous roots and herbaceous perennial roots are generally propagated and grown in fields but shipped dormant and free from growing media. However, dormant bulbs may sometimes be packed as "growing kits", with growing media. These growing media may be considered as a separate commodity (packing material) provided the plants are not rooted in the media.
Epiphytic plants	Tree fern slabs, bark, non-viable moss (sphagnum), volcanic cinder, rock	Epiphytic plants, such as bromeliads and orchids, are often shipped in association with tree fern slabs, bark, wood, coconut husk, coconut fibre, non-viable moss (sphagnum), volcanic cinder, rock and so forth. These materials are generally intended for support and ornamentation rather than being true growing media.
Liners, whips	Various (including peat, vermiculite, soil as a contaminant)	These young plants are generally rooted in soil or in soil-free growing media in containers or trays.
Ornamental and flowering houseplants	Various (including synthetic media, vermiculite, perlite, coco peat)	The plants may be field-grown in soil, grown as containerized nursery stock, or grown as potted greenhouse plants in soil-free growing media
Plants grown from seed	Various (including peat, vermiculite, perlite)	Annuals and biennials are generally grown from seed in growing media and moved as rooted in growing media
Plants rooted in water or water-based nutrient solution	Water or water-based nutrient solution	Some plants may be grown from cuttings in water or in water-based nutrient solution, with or without synthetic growing media
Rooted herbaceous cuttings	Various (including peat, coco peat, synthetic media, non-viable moss (sphagnum))	Rooted herbaceous cuttings are generally rooted in soil-free growing media that may be contained in peat-pots or coco-pots. The roots are tender and the growing media cannot be removed without injuring the plants.
Tissue cultured plants	Sterile, agar-like	Tissue cultured plants are produced in association with sterile agar-like growing media. They may be shipped in sealed aseptic containers or ex-agar.

Plant type	Growing media	Comments
Trees and shrubs	Soil	Older trees and shrubs, including specimen trees, are often moved in the nursery trade as dug trees or “ball and burlap”
Turf or grass sod	Soil	Turf or grass sod contains a large amount of soil

INTERNATIONAL STANDARDS FOR
PHYTOSANITARY MEASURES

ISPM 41

International movement of used vehicles, machinery and equipment

Produced by the Secretariat of the
International Plant Protection Convention
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Publication history

This is not an official part of the standard

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2009-05 SC approved specification 48.

2013-05 EWG met and drafted ISPM.

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2016-11 SC revised draft and recommended to CPM-12 (2017) for adoption.

2017-04 Objection received.

2017-04 CPM-12 lifted the objection and adopted the standard.

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CONTENTS

Adoption

This standard was adopted by the Twelfth Session of the Commission on Phytosanitary Measures in April 2017.

INTRODUCTION

Scope

This standard identifies and categorizes the pest risk associated with used vehicles, machinery and equipment (VME) utilized in agriculture, forestry, horticulture, earth moving, surface mining, waste management and by the military being moved internationally and identifies appropriate phytosanitary measures.

This standard does not cover passenger and commercial transport vehicles moving under their own motive power.

References

The present standard refers to International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

Definitions

Definitions of phytosanitary terms used in this standard can be found in ISPM 5 (*Glossary of phytosanitary terms*).

Outline of requirements

This standard describes phytosanitary measures that may apply to used VME: cleaning and treatment, prevention from contamination, requirements for facilities and waste disposal, and verification procedures.

The standard also provides guidance to national plant protection organizations (NPPOs) working with the military on phytosanitary measures applicable to the international deployment of used military VME.

BACKGROUND

Used VME are frequently traded or otherwise moved between countries. They may have been used in agriculture and forestry, as well as for construction, industrial purposes, mining and waste management. They can also be used military VME that have been subject to international deployment. Depending on their use, storage or transportation before export, used VME may have become contaminated with quarantine pests or regulated articles. When moved internationally as either a traded commodity or an operational relocation (e.g. in the case of harvesters) used VME may carry soil, pests, plant debris or seeds, and they may therefore present a pest risk to the country of destination. Depending on their use in the country of destination, they may introduce quarantine pests to agricultural, forested, wilderness or other areas.

New VME may also be contaminated but they are not covered by this standard. However, this does not exclude the option that NPPOs of importing countries may require phytosanitary import requirements for new vehicles to prevent contamination, similar to those identified in section 2.2., if technically justified.

Examples of pests that may contaminate used VME are provided in Appendix 1.

Specific guidance is needed for NPPOs regarding the pest risk associated with the movement and storage of used VME and the phytosanitary measures that may be required in order to facilitate their safe movement. The phytosanitary measures may be applied with the aim of minimizing their negative effect on trade.

IMPACT ON BIODIVERSITY AND THE ENVIRONMENT

The decontamination of used VME may provide a means to prevent the entry of organisms into new areas that could be relevant to the biodiversity of those areas (invasive alien species).

REQUIREMENTS

1. Pest Risk

The main pest risk associated with used VME is contamination with soil, pests, plant debris, and seeds and other plant parts capable of propagation. Seeds and other plant parts capable of propagation may be of concern because the plant itself can be a pest or potentially harbour pests. Pests that have a resistant or dormant life stage allowing them to survive transport to endangered areas are a particular concern.

The pest risk from contamination of used VME is difficult to assess. Therefore, the normal process of undertaking pest risk analysis to determine if phytosanitary measures are necessary, and the strength of such measures, may not be possible. For this reason, in order to reduce the risk of introduction and spread of quarantine pests used VME moved internationally should be free from contamination in accordance with this standard.

1.1 Elements of pest risk categorization

The following elements of used VME may affect the level of pest risk:

- distance of movement: used VME moving on their own motive power over short distances across borders to be used immediately may pose a low pest risk
- type: used VME with more complex structure have more areas that may be contaminated
- origin and prior use: VME used on farms, in crop fields, in forests, in close proximity to vegetation or for transporting organic material are more likely to be contaminated
- storage: used VME stored outdoors and in close proximity to vegetation or lights that attract insects are more likely to be contaminated
- intended location or use: used VME that will be used in agricultural areas, in forests or in close proximity to vegetation are more likely to provide a pathway for the introduction of pests.

In the case of used military VME, exposure to kinetic forces and rigours of combat operations may result in external damage and internal penetration of contamination.

Examples of used VME, ranked in order of decreasing pest risk, together with examples of possible phytosanitary measures and verification procedures, are provided in Appendix 2.

2. Phytosanitary Measures

Used VME moved internationally should be free from contamination.

The main groups of phytosanitary measures that may be applied to used VME are described in the sections below.

NPPOs are encouraged to work with military authorities to develop procedures consistent with the guidance on the international movement of used military VME provided in Annex 1.

2.1 Cleaning and treatment

Some of the cleaning methods are:

- emptying water reservoirs
- removing debris or filters
- abrasive blasting
- pressure washing
- steam cleaning
- sweeping and vacuuming
- compressed air cleaning.

Treatments that may be used in addition to cleaning are:

- chemical treatment (e.g. fumigation, disinfestation)
- temperature treatment.

Partial or full dismantling of the used VME may be necessary for effective cleaning or treatment. It may be necessary to clean or treat the used VME while they are in operation to ensure that all moving parts can be accessed (e.g. agricultural equipment with moving parts such as conveyors or rollers).

2.2 Prevention of contamination

Where clean VME are moved to a storage area, packing area or port of loading or when they are transiting through another country, phytosanitary measures may be taken to prevent contamination. These include, as appropriate:

- storage in appropriate areas with reduced risk from contamination
- storage and handling on surfaces that prevent contact with soil
- keeping vegetation around storage areas, packing areas or ports of loading short by mowing or using weed control in order to reduce the risk of contamination by airborne seeds and other pests; consideration may be given to the erection of barriers to limit seed movement around storage and loading areas.

During seasonal pest emergence periods or occasional pest outbreaks, special consideration may be given to phytosanitary measures that prevent pests being attracted to storage and loading areas (e.g. restricting the use of artificial lights during night-time operations).

2.3 Facilities and waste disposal requirements

The type of equipment and nature of facilities necessary for cleaning and treatment of used VME depend on where these procedures take place. Inspection, cleaning and treatment will normally take place in the exporting country to fulfil the phytosanitary import requirements of the country of destination. Facilities in the exporting country may not need elaborate solid waste and wastewater management systems as the contamination may be of local origin.

Facilities required for the inspection, cleaning and treatment of used VME may include:

- surfaces that prevent contact with soil, including soil traps and wastewater management systems
- temperature treatment facilities
- fumigation or chemical treatment facilities.

Disposal of soil and contaminated washing water should be in accordance with national or local regulations.

Containment and disposal methods should be sufficient to prevent the spread of pests and may include: soil traps, bagging, deep burial, incineration, fumigation, chemical treatment, composting and wastewater management systems.

3. Verification Procedures

Requirements for documentation to attest that consignments have been cleaned, treated or inspected (e.g. cleaning declaration, treatment certificate, inspection declaration, phytosanitary certificate) should be determined by the NPPO of the country of destination, and should be proportionate to the identified pest risk and appropriate for the phytosanitary measures required.

An NPPO of a country of destination may conduct import inspections to verify that used VME are clean. Import inspections may include partial or full dismantling of used VME, and in some cases, collection of specimens for identification. Verification of cleanliness may also involve probing and flushing hidden areas (e.g. by using water under high pressure or compressed air).

The NPPO of the exporting country may authorize entities for the treatment of used VME. The cleaning of used VME may also be conducted by entities other than the NPPO.

The cleaning of used military VME may be performed and verified by military personnel when requested by the NPPO or in conformance with an agreement between the NPPO and military authorities.

4. Non-compliance and Phytosanitary Actions

Where non-compliance occurs, the NPPO of the country of destination may take phytosanitary action as outlined in ISPM 20 (*Guidelines for a phytosanitary import regulatory system*) and should notify the exporting country (ISPM 13 (*Guidelines for the notification of non-compliance and emergency action*)).

Examples of phytosanitary actions that may be taken are detention, cleaning, treatment or reshipment of the used VME found to be contaminated. Where contaminated used VME need to be transported to another location for cleaning and treatment, the NPPO should ensure that contamination is suitably contained (e.g. by containerization), in accordance with national or local regulations.

This annex is a prescriptive part of the standard.

ANNEX 1: Guidance on the international movement of used military vehicles, machinery and equipment

1. Background

The international movement of used military vehicles, machinery and equipment (VME) may present a risk for the introduction of pests with soil, pests, plant debris and seeds to the countries of both deployment and redeployment. Examples of pests that may contaminate used military VME are provided in Appendix 1 of this standard. Movements of used military VME occur continually around the world and encompass many different conveyances and cargo storage conditions.

The international movement of used military VME may present a practical problem to national plant protection organizations (NPPOs). In many countries, NPPOs have no or limited access to the military because of security issues. For this reason, the approach taken in managing the pest risk related to the commercial and private shipping of used VME may not be applicable to the military. Consequently, military authorities are encouraged to commit to using this guidance.

2. Objective

The objective of this guidance is that used military VME are clean of soil, pests, plant debris and seeds before they are moved internationally (e.g. for training, missions and deployment).

3. Guidance

Military authorities should ensure that used VME are cleaned according to the phytosanitary import requirements developed by the NPPO of the country of destination. Cleaning methods may consist of, for example:

- emptying water reservoirs
- removing debris or filters
- abrasive blasting
- pressure washing
- steam cleaning
- sweeping and vacuuming
- compressed air cleaning.

These cleaning methods may need to be carried out in combination with partial or full dismantling of the used VME to ensure they are cleaned to a high standard. For specialized military VME, military authorities are encouraged to develop specific procedures and manuals.

Additional treatments may be required, such as:

- chemical treatment (e.g. fumigation, disinfestation)
- temperature treatment.

Wood packaging material associated with used military VME should be compliant with ISPM 15 (*Regulation of wood packaging material in international trade*).

Military authorities are encouraged to liaise with the NPPOs in their home country. Military authorities are also encouraged to liaise with the NPPO in the country of deployment, where practical. Contact information for NPPOs is available on the IPP (https://www.ippc.int).

Military authorities are encouraged to implement verification procedures to ensure the appropriate cleaning and treatment for used military VME has been carried out before deployment.

This appendix is for reference purposes only and is not a prescriptive part of the standard.

APPENDIX 1: Examples of pests that may contaminate used vehicles, machinery and equipment

- *Achatina fulica*, as aestivating adults
- *Beet necrotic yellow vein virus*, transmitted through soil via spores of its vector *Polymyxa betae*
- *Chromolaena odorata*, as seeds or in soil
- *Clavibacter michiganensis* subsp. *sepedonicus*, in plant residues
- *Coptotermes formosanus*, in wood and soil
- *Fusarium guttiforme*, in soil and host plant residues
- *Fusarium oxysporum*, in soil and host plant residues
- *Globodera* spp., in soil and host plant residues
- *Halyomorpha halys*, as overwintering adults
- *Lymantria dispar*, as diapausing egg masses
- *Miconia calvescens*, as seeds in soil
- *Orgyia thyellina*, as diapausing pupae
- *Phytophthora ramorum*, in soil
- *Solenopsis invicta*, as eggs, larvae and adults, and nests
- *Sorghum halepense*, as rhizomes and seeds
- *Tilletia indica*, as spores in soil and on wheat seed residues

This appendix is for reference purposes only and is not a prescriptive part of the standard.

APPENDIX 2: Examples of used vehicles, machinery and equipment, ranked in order of decreasing pest risk, together with examples of possible phytosanitary measures and verification procedures

Category	Contamination notes	Phytosanitary measures	Verification procedures
Agricultural, forestry and horticultural used VME, such as: - harvesters - sawmill machinery - logging trucks - animal transport vehicles - compost and manure trailers - tractors - tools. Reconditioned or field-tested used VME are included. This category is usually considered to be high pest risk.	Contaminants: - soil - pests - plant debris - seeds	Abrasive blasting Emptying open water reservoirs, removing debris Pressure washing Steam cleaning Sweeping and vacuuming Compressed air cleaning Chemical treatment (e.g. fumigation, disinfestation) Temperature treatment	Cleaning declaration Treatment certificate Inspection (may include dismantling and testing) Phytosanitary certificate Authorization and audit
Earth moving used VME, such as: - bulldozers - graders - surface mining equipment. Reconditioned or field-tested used VME are included. Pest risk is variable, but high levels of contamination may occur in this category.	Soil is the main contaminant; pests, plant debris and seeds can also be contaminants	Abrasive blasting Emptying open water reservoirs, removing debris Pressure washing Steam cleaning Sweeping and vacuuming Compressed air cleaning Chemical treatment (e.g. fumigation, disinfestation)	Cleaning declaration Treatment certificate Inspection (may include dismantling and testing) Phytosanitary certificate Authorization and audit
Used military VME, such as: - trucks - tanks - personnel carriers - rolling stock. Pest risk is variable, but used military VME are often used off-road and stored outdoors, leading to a higher risk.	Contaminants: - soil - pests - plant debris - seeds	Emptying open water reservoirs, removing debris Pressure washing Steam cleaning Compressed air cleaning Chemical treatment (e.g. fumigation, disinfestation	(See Annex 1 of this standard)
Waste management used VME, such as: - rubbish/garbage/waste trucks - waste sorting equipment. Reconditioned used VME are included. Bulldozers used in landfills	Organic waste debris is the main contaminant, including: - soil - pests - plant debris	Abrasive blasting Emptying open water reservoirs, removing debris Pressure washing Steam cleaning Sweeping and vacuuming Chemical treatment (e.g. fumigation, disinfestation)	Cleaning declaration Treatment certificate Inspection (may include dismantling and testing) Phytosanitary certificate Authorization and audit

Category	Contamination notes	Phytosanitary measures	Verification procedures
are considered under earth moving VME.			
Deep mining used VME. The most likely contaminants are soil and to a lesser extent pests. Pest risk is generally low unless used VME are contaminated with surface soil. It can be difficult to determine the prior use and whether or not used VME were used for surface mining.		Abrasive blasting Emptying open water reservoirs, removing debris Pressure washing Steam cleaning	Cleaning declaration Inspection (may include dismantling and testing)
Used industrial VME used outdoors, such as: - cranes - forklifts. Pest risk is variable, but generally low unless used VME are used in close proximity to vegetation or are contaminated with soil.		Abrasive blasting Emptying open water reservoirs, removing debris Pressure washing Steam cleaning	Cleaning declaration Inspection
Used vehicles, such as: - cars, vans, trucks, buses - off-road vehicles (e.g. motorbikes, quad bikes, four-wheel drives) - locomotives and engines - used parts - trailers - attached tyres. Extremely variable pest risk, with some used vehicles at higher risk but many at low risk. This category has a large volume of used, traded vehicles.	Contaminants: - soil - pests - plant debris - seeds	Abrasive blasting Emptying open water reservoirs, removing debris Pressure washing Steam cleaning Sweeping and vacuuming Chemical treatment (e.g. fumigation, disinfestation) Temperature treatment	Cleaning declaration Treatment certificate Inspection (may include dismantling and testing)

VME, vehicles, machinery and equipment.

This phytosanitary treatment was adopted by the Twelfth Session of the Commission on Phytosanitary Measures in 2017.
The annex is a prescriptive part of ISPM 28.

ISPM 28
Phytosanitary treatments for regulated pests

PT 22: Sulphuryl fluoride fumigation treatment for insects in debarked wood

Adopted 2017; published 2017

Scope of the treatment

This treatment describes the fumigation of debarked wood using sulphuryl fluoride to reduce the risk of introduction and spread of insect pests[1].

Treatment description

Name of treatment	Sulphuryl fluoride fumigation treatment for insects in debarked wood
Active ingredient	Sulphuryl fluoride (also known as sulfuryl fluoride, sulphur dioxide difluoride, sulphuryl difluoride)
Treatment type	Fumigation
Target pests	Wood-borne life stages of insects, including *Anoplophora glabripennis* (Motschulsky, 1853) (Coleoptera: Cerambycidae), *Anobium punctatum* (De Geer, 1774) (Coleoptera: Anobiidae) and *Arhopalus tristis* (Fabricius, 1787) (Coleoptera: Cerambycidae)
Target regulated articles	Debarked wood not exceeding 20 cm in cross-section at its smallest dimension and 75% moisture content (dry basis)

Treatment schedule

Fumigation of debarked wood not exceeding 20 cm in cross-section at its smallest dimension and 75% moisture content (dry basis) in accordance with a schedule that achieves the minimum concentration–time product (CT) within a single 24 hour period at the temperature and final residual concentration specified in Table 1.

[1] The scope of phytosanitary treatments does not include issues related to pesticide registration or other domestic requirements for contracting parties' approval of treatments. Treatments adopted by the Commission on Phytosanitary Measures may not provide information on specific effects on human health or food safety, which should be addressed using domestic procedures before contracting parties approve a treatment. In addition, potential effects of treatments on product quality are considered for some host commodities before their international adoption. However, evaluation of any effects of a treatment on the quality of commodities may require additional consideration. There is no obligation for a contracting party to approve, register or adopt the treatments for use in its territory.

Table 1. Minimum concentration–time product (CT) within a single 24 hour period for debarked wood fumigated with sulphuryl fluoride

Temperature	Minimum required CT (g·h/m^3)	Minimum concentration (g/m^3)
15 °C or above	3 200	93
20 °C or above	2 300	67
25 °C or above	1 500	44
30 °C or above	1 400	41

This treatment schedule is effective against all wood-borne life stages of insect pests. There is 95% confidence that the treatment according to this schedule achieves the following levels of mortality for the wood-borne life stages of the following insect pests:

- *Anoplophora glabripennis* (larvae and pupae) to not less than 99.99683%[2]
- *Anobium punctatum* (all life stages) to not less than 99.7462%
- *Arhopalus tristis* (all life stages) to not less than 99%.

The measured temperature of the product (including at the wood core) or the ambient air (whichever is lower) is used to calculate the sulphuryl fluoride dose and must be at least 15 °C throughout the duration of the treatment.

Other relevant information

One example of a schedule that achieves the minimum required CT for debarked wood treated with sulphuryl fluoride is shown in Table 2.

Table 2. Example of a treatment schedule that achieves the minimum required concentration–time product (CT) for debarked wood treated with sulphuryl fluoride (SF)

Minimum temperature during treatment	Minimum required CT (g·h/m^3)	SF dose† (g/m^3)	Minimum concentration (g/m^3) at hour:				
			0.5	2	4	12	24
15 °C or above	3 200	183	188	176	163	131	93
20 °C or above	2 300	131	136	128	118	95	67
25 °C or above	1 500	88	94	83	78	62	44
30 °C or above	1 400	82	87	78	73	58	41

† Initial doses may need to be higher in conditions of high sorption or leakage.

[2] The minimum level of mortality achieved by the treatment for this species has been estimated by extrapolation from a model fitted to the experimental data.

The Technical Panel on Phytosanitary Treatments based its evaluation of this treatment for *A. glabripennis* on the research reported by Barak *et al*. (2006).

The general effectiveness of this treatment against other pests has been supported by Barak *et al*. (2010), Binker *et al*. (1999), Ducom *et al*. (2003), La Fage *et al*. (1982), Mizobuchi *et al*. (1996), Osbrink *et al*. (1987), Soma *et al*. (1996, 1997), Williams and Sprenkel (1990) and Zhang (2006).

If the CT is not achieved within a single 24 hour period (even if the minimum concentration is achieved), corrective action will need to be taken. The treatment may be extended for a maximum of two hours without adding more sulphuryl fluoride, or it may be restarted.

References

The present annex to the standard may refer to International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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Zhang, Z. 2006. Use of sulfuryl fluoride as an alternative fumigant to methyl bromide in export log fumigation. *New Zealand Plant Protection,* 59: 223–227.

Publication history

This is not an official part of the standard

2006-04 CPM-1 (2006) added topic *Revision of ISPM 15 (Regulation of wood packaging material in international trade)* (2006-011).

2006-09 Treatment submitted in response to 2006-08 call for treatments.

2006-12 TPPT reviewed treatment.

2007-07 Revised draft considered by TPFQ.

2007-12 Further revised draft submitted to TPPT.

2008-12 TPFQ discussion.

2009-01 TPPT reviewed draft.

2009-07 Amended draft considered by TPFQ.

2010-07 Draft updated and recommended to SC.

2010-09 TPFQ discussion.

2011-04 SC e-decision.

2011-05 SC via e-discussion returned to TPPT.

2011-07 TPPT revised draft based on SC comments.

2011-10 TPPT reviewed draft.

2012-02 TPFQ discussion.

2012-12 TPPT reviewed draft.

2013-07 TPPT reviewed draft based on additional information from Submitter.

2014-01 TPPT deferred draft review pending information from specialists.

2014-06 TPPT reviewed draft based on information from specialists; TPPT recommended topic *Sulphuryl fluoride fumigation of wood packaging material* (2007-101) be split into two topics (one for insects and one for nematodes and insects); TPPT recommended drafts to SC for consultation.

2014-09 SC approved draft for consultation via e-decision (2014_eSC_Nov_09).

2014-11 SC agreed to split *Sulphuryl fluoride fumigation of wood packaging material* (2007-101) into separate topics: *Sulphuryl fluoride fumigation of insects in debarked wood* (2007-101A) and *Sulphuryl fluoride fumigation of nematodes and insects in debarked wood* (2007-101B).

2015-07 First consultation.

2016-09 TPPT recommended to SC for adoption.

2016-11 SC recommended to CPM-12 for adoption vie e-decision (2016_eSC_Nov_15).

2017-04 CPM-12 adopted the phytosanitary treatment.

ISPM 28. **Annex 22**. *Sulphuryl fluoride fumigation treatment for insects in debarked wood* (2017). Rome, IPPC, FAO.

Publication history last updated: 2017-04

This phytosanitary treatment was adopted by the Twelfth Session of the Commission on Phytosanitary Measures in 2017.
The annex is a prescriptive part of ISPM 28.

ISPM 28
Phytosanitary treatments for regulated pests

PT 23: Sulphuryl fluoride fumigation treatment for nematodes and insects in debarked wood

Adopted 2017; published 2017

Scope of the treatment

This treatment describes the fumigation of debarked wood using sulphuryl fluoride to reduce the risk of introduction and spread of *Bursaphelenchus xylophilus* and insect pests[1].

Treatment description

Name of treatment	Sulphuryl fluoride fumigation treatment for nematodes and insects in debarked wood
Active ingredient	Sulphuryl fluoride (also known as sulfuryl fluoride, sulphur dioxide difluoride, sulphuryl difluoride)
Treatment type	Fumigation
Target pests	Wood-borne life stages of *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970 (Nematoda: Aphelenchoididae) and insects, including *Anoplophora glabripennis* (Motschulsky, 1853) (Coleoptera: Cerambycidae), *Anobium punctatum* (De Geer, 1774) (Coleoptera: Anobiidae) and *Arhopalus tristis* (Fabricius, 1787) (Coleoptera: Cerambycidae)
Target regulated articles	Debarked wood not exceeding 20 cm in cross-section at its smallest dimension and 75% moisture content (dry basis)

Treatment schedule

Fumigation of debarked wood not exceeding 20 cm in cross-section at its smallest dimension and 75% moisture content (dry basis) in accordance with a schedule that achieves the minimum concentration–time product (CT) within a single 24 or 48 hour period at the temperature and final residual concentration specified in Table 1.

[1] The scope of phytosanitary treatments does not include issues related to pesticide registration or other domestic requirements for contracting parties' approval of treatments. Treatments adopted by the Commission on Phytosanitary Measures may not provide information on specific effects on human health or food safety, which should be addressed using domestic procedures before contracting parties approve a treatment. In addition, potential effects of treatments on product quality are considered for some host commodities before their international adoption. However, evaluation of any effects of a treatment on the quality of commodities may require additional consideration. There is no obligation for a contracting party to approve, register or adopt the treatments for use in its territory.

Table 1. Minimum concentration–time product (CT) within a single 24 or 48 hour period for debarked wood fumigated with sulphuryl fluoride

Temperature	Duration (hours)	Minimum required CT ($g \cdot h/m^3$)	Minimum concentration (g/m^3)
20 °C or above	48	3 000	29
30 °C or above	24	1 400	41

This treatment schedule is effective against all wood-borne life stages of nematode and insect pests. There is 95% confidence that the treatment according to this schedule achieves the following levels of mortality for the wood-borne life stages of the following nematode and insect pests:

- *Bursaphelenchus xylophilus* to not less than 99.99683%
- *Anoplophora glabripennis* (larvae and pupae) to not less than 99.99683%[2]
- *Anobium punctatum* (all life stages) to not less than 99.7462%
- *Arhopalus tristis* (all life stages) to not less than 99%.

The measured temperature of the product (including at the wood core) or the ambient air (whichever is lower) is used to calculate the sulphuryl fluoride dose and must be at least 20 °C throughout the duration of the treatment.

Other relevant information

One example of a schedule that achieves the minimum required CT for debarked wood treated with sulphuryl fluoride is shown in Table 2.

Table 2. Example of a treatment schedule that achieves the minimum required concentration–time product (CT) for debarked wood treated with sulphuryl fluoride (SF)

Minimum temperature during treatment	Minimum required CT ($g \cdot h/m^3$)	SF dose† (g/m^3)	Minimum concentration (g/m^3) at hour:						
			0.5	2	4	12	24	36	48
20 °C or above	3 000	120	124	112	104	82	58	41	29
30 °C or above	1 400	82	87	78	73	58	41	n/a	n/a

† Initial doses may need to be higher in conditions of high sorption or leakage.

n/a, not applicable.

The Technical Panel on Phytosanitary Treatments based its evaluation of this treatment for *B. xylophilus* and insects on the research reported by Barak *et al*. (2006), Bonifacio *et al*. (2013) and Sousa *et al*. (2010, 2011).

The general effectiveness of this treatment has been supported by Barak *et al.* (2010), Binker *et al*. (1999), Bonifacio *et al*. (2013), Ducom *et al.* (2003), Dwinell *et al.* (2005), La Fage *et al.* (1982), Mizobuchi *et al*. (1996), Osbrink *et al*. (1987), Soma *et al*. (1996, 1997, 2001), Williams and Sprenkel (1990) and Zhang (2006).

[2] The minimum level of mortality achieved by the treatment for this species has been estimated by extrapolation from a model fitted to the experimental data.

If the CT is not achieved within a single 24–48 hour period (even if the minimum concentration is achieved), corrective action will need to be taken. The treatment may be extended for a maximum of two hours without adding more sulphuryl fluoride, or it may be restarted.

References

The present annex to the standard may refer to International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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Soma, Y., Mizobuchi, M., Oogita, T., Misumi, T., Kishono, H., Akagawa, T. & Kawakami, F. 1997. Susceptibility of forest insect pests to sulfuryl fluoride. 3. Susceptibility to sulfuryl fluoride at 25 °C. *Research Bulletin of the Plant Protection Service Japan*, 33: 25–30.

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Zhang, Z. 2006.Use of sulfuryl fluoride as an alternative fumigant to methyl bromide in export log fumigation. *New Zealand Plant Protection*, 59: 223–227.

Publication history

This is not an official part of the standard

2006-04 CPM-1 (2006) added topic *Revision of ISPM 15 (Regulation of wood packaging material in international trade)* (2006-011).

2006-09 Treatment submitted in response to 2006-08 call for treatments.

2006-12 TPPT reviewed treatment.

2007-07 Revised draft considered by TPFQ.

2007-12 Further revised draft submitted to TPPT.

2008-12 TPFQ discussion.

2009-01 TPPT reviewed draft.

2009-07 Amended draft considered by TPFQ.

2010-07 Draft updated and recommended to SC.

2010-09 TPFQ discussion.

2011-04 SC e-decision.

2011-05 SC via e-discussion returned to TPPT.

2011-07 TPPT revised draft based on SC comments.

2011-10 TPPT reviewed draft.

2012-02 TPFQ discussion.

2012-12 TPPT reviewed draft.

2013-07 TPPT reviewed draft based on additional information from Submitter.

2014-01 TPPT deferred draft review pending information from specialists.

2014-06 TPPT reviewed draft based on information from specialists; TPPT recommended topic *Sulphuryl fluoride fumigation of wood packaging material* (2007-101) be split into two topics (one for insects and one for nematodes and insects); TPPT recommended draft to SC for member consultation.

2014-09 SC approved draft for member consultation via e-decision (2014_eSC_Nov_09).

2014-11 SC agreed to split *Sulphuryl fluoride fumigation of wood packaging material* (2007-101) into two topics: *Sulphuryl fluoride fumigation of insects in debarked wood* (2007-101A) and *Sulphuryl fluoride fumigation of nematodes and insects in debarked wood* (2007-101B).

2015-07 First consultation.

2016-09 TPPT recommended to SC for adoption.

2016-11 SC recommended to CPM-12 for adoption via e-decision (2016_eSC_Nov_16).

2017-04 CPM-12 adopted the phytosanitary treatment.

ISPM 28. **Annex 23**. *Sulphuryl fluoride fumigation treatment for nematodes and insects in debarked wood* (2017). Rome, IPPC, FAO.

Publication history last updated: 2017-04

This phytosanitary treatment was adopted by the Twelfth Session of the Commission on Phytosanitary Measures in 2017.
The annex is a prescriptive part of ISPM 28.

ISPM 28
Phytosanitary treatments for regulated pests

PT 24: Cold treatment for *Ceratitis capitata* on *Citrus sinensis*

Adopted 2017; published 2017

Scope of the treatment

This treatment describes the cold treatment of fruit of *Citrus sinensis*[1] (orange) to result in the mortality of eggs and larvae of *Ceratitis capitata* at the stated efficacy[2].

Treatment description

Name of treatment	Cold treatment for *Ceratitis capitata* on *Citrus sinensis*
Active ingredient	n/a
Treatment type	Physical (cold)
Target pest	*Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae)
Target regulated articles	Fruit of *Citrus sinensis*

Treatment schedule

Schedule 1: 2 °C or below for 16 continuous days

There is 95% confidence that the treatment according to this schedule kills not less than 99.9937% of eggs and larvae of *Ceratitis capitata*.

Schedule 2: 2 °C or below for 18 continuous days

There is 95% confidence that the treatment according to this schedule kills not less than 99.999% of eggs and larvae of *Ceratitis capitata*.

Schedule 3: 3 °C or below for 20 continuous days

There is 95% confidence that the treatment according to this schedule kills not less than 99.9989% of eggs and larvae of *Ceratitis capitata*.

The fruit must reach the treatment temperature before treatment exposure time commences. The fruit temperature should be monitored and recorded, and the temperature should not exceed the stated level throughout the duration of the treatment.

[1] *Citrus* species and hybrids are named according to the nomenclature in Cottin, R. 2002. *Citrus of the world: A citrus directory*, version 2.0. France, SRA INRA-CIRAD.

[2] The scope of phytosanitary treatments does not include issues related to pesticide registration or other domestic requirements for contracting parties' approval of treatments. Treatments adopted by the Commission on Phytosanitary Measures may not provide information on specific effects on human health or food safety, which should be addressed using domestic procedures before contracting parties approve a treatment. In addition, potential effects of treatments on product quality are considered for some host commodities before their international adoption. However, evaluation of any effects of a treatment on the quality of commodities may require additional consideration. There is no obligation for a contracting party to approve, register or adopt the treatments for use in its territory.

Other relevant information

In evaluating this treatment the Technical Panel on Phytosanitary Treatments considered issues associated with temperature regimes and thermal conditioning, taking into account the work of Hallman and Mangan (1997).

Schedule 1 was based on the work of Laborda *et al.* (1997) and Santaballa *et al.* (1995), using larval mortality.

Schedules 2 and 3 were based on the work of De Lima *et al*. (2007), using failure to pupariate as the measure of mortality.

References

The present annex to the standard may refer to International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

De Lima, C.P.F., Jessup, A.J., Cruickshank, L., Walsh, C.J. & Mansfield, E.R. 2007. Cold disinfestation of citrus (*Citrus* spp.) for Mediterranean fruit fly (*Ceratitis capitata*) and Queensland fruit fly (*Bactrocera tryoni*) (Diptera: Tephritidae). *New Zealand Journal of Crop and Horticultural Science,* 35: 39–50.

Hallman, G.J. & Mangan, R.L. 1997. Concerns with temperature quarantine treatment research. In: G.L. Obenauf, ed. *1997 Annual International Research Conference on Methyl Bromide Alternatives and Emissions Reduction*. San Diego, CA, 3–5 November 1997, pp. 79-1–79-4.

Laborda, R., Cerdá, M., Santaballa, E. & Dalmau, A. 1997. *Report of quarantine cold treatment to control* Ceratitis capitata *(Wied) to export Salustiana oranges to Japan.* Valencia, Spain, Universidad Politécnica de Valencia. 16 pp.

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Publication history

This is not an official part of the standard

2007-09 Treatment submitted.

2007-12 TPPT combined *Cold treatment of* Citrus sinensis *for* Ceratitis capitata (2007-TPPT-106) and 2007-TPPT-109 to create 2007-206A.

2008-04 CPM-3 added subject under the topic *Fruit fly treatments.*

2008-09 SC approved for member consultation via e-decision.

2009-06 Member consultation.

2010-07 TPPT meeting revised draft and recommended to SC for adoption.

2011-11 SC commented by e-decision (2011_SC_Nov_03).

2012-12 TPPT revised draft and recommended to SC for adoption.

2013-11 SC recommended to CPM-9 for adoption via e-decision (2013_eSC_Nov_01).

2014-04 Treatment received formal objection before CPM-9.

2015-11 SC assigned the status “pending”.

2016-09 TPPT agreed that there are no fruit fly population differences in relation to cold treatment and no varietal or cultivar effects for *Citrus*, thus recommended merging draft annex to ISPM 28 2010-103 with 2007-206A; TPPT agreed that there are no fruit fly population differences in relation to cold treatment and no varietal or cultivar effects.

2016-09 TPPT recommended to SC for adoption.

2016-11 SC recommended to CPM-12 for adoption via e-decision (2016_eSC_Nov_05).

2017-04 CPM-12 adopted the phytosanitary treatment.

ISPM 28. **Annex 24**. *Cold treatment for* Ceratitis capitata *on* Citrus sinensis (2017). Rome, IPPC, FAO.

Publication history last updated: 2017-04

This phytosanitary treatment was adopted by the Twelfth Session of the Commission on Phytosanitary Measures in 2017.
The annex is a prescriptive part of ISPM 28.

ISPM 28
Phytosanitary treatments for regulated pests

PT 25: Cold treatment for *Ceratitis capitata* on *Citrus reticulata* × *C. sinensis*

Adopted 2017; published 2017

Scope of the treatment

This treatment describes the cold treatment of fruit of *Citrus reticulata* × *Citrus sinensis*[1] to result in the mortality of eggs and larvae of *Ceratitis capitata* at the stated efficacy[2].

Treatment description

Name of treatment	Cold treatment for *Ceratitis capitata* on *Citrus reticulata* × *Citrus sinensis*
Active ingredient	n/a
Treatment type	Physical (cold)
Target pest	*Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae)
Target regulated articles	Fruit of *Citrus reticulata* × *Citrus sinensis*

Treatment schedule

Schedule 1: 2 °C or below for 18 continuous days

There is 95% confidence that the treatment according to this schedule kills not less than 99.9987% of eggs and larvae of *Ceratitis capitata*.

Schedule 2: 3 °C or below for 20 continuous days

There is 95% confidence that the treatment according to this schedule kills not less than 99.9987% of eggs and larvae of *Ceratitis capitata*.

The fruit must reach the treatment temperature before treatment exposure time commences. The fruit temperature should be monitored and recorded, and the temperature should not exceed the stated level throughout the duration of the treatment.

[1] *Citrus* species and hybrids are named according to the nomenclature in Cottin, R. 2002. *Citrus of the world: A citrus directory*, version 2.0. France, SRA INRA-CIRAD.

[2] The scope of phytosanitary treatments does not include issues related to pesticide registration or other domestic requirements for contracting parties' approval of treatments. Treatments adopted by the Commission on Phytosanitary Measures may not provide information on specific effects on human health or food safety, which should be addressed using domestic procedures before contracting parties approve a treatment. In addition, potential effects of treatments on product quality are considered for some host commodities before their international adoption. However, evaluation of any effects of a treatment on the quality of commodities may require additional consideration. There is no obligation for a contracting party to approve, register or adopt the treatments for use in its territory.

Other relevant information

In evaluating this treatment the Technical Panel on Phytosanitary Treatments considered issues associated with temperature regimes and thermal conditioning, taking into account the work of Hallman and Mangan (1997).

Schedules 1 and 2 were based on the work of De Lima *et al*. (2007) and were developed using the cultivars "Ellendale" and "Murcott", and using failure to pupariate as the measure of mortality.

References

The present annex to the standard may refer to International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

De Lima, C.P.F., Jessup, A.J., Cruickshank, L., Walsh, C.J. & Mansfield, E.R. 2007. Cold disinfestation of citrus (*Citrus* spp.) for Mediterranean fruit fly (*Ceratitis capitata*) and Queensland fruit fly (*Bactrocera tryoni*) (Diptera: Tephritidae). *New Zealand Journal of Crop and Horticultural Science*, 35: 39–50.

Hallman, G.J. & Mangan, R.L. 1997. Concerns with temperature quarantine treatment research. In: G.L. Obenauf, ed. *1997 Annual International Research Conference on Methyl Bromide Alternatives and Emissions Reduction*. San Diego, CA, 3–5 November 1997, pp. 79-1–79-4.

Publication history

This is not an official part of the standard

2007-09 Treatment submitted.

2007-12 TPPT combined *Cold treatment of* Citrus reticulata × C. sinensis *for* Ceratitis capitata (2007-106) and 2007-206D to create 2007-206B.

2008-04 CPM-3 added subject under the topic *Fruit fly treatments.*

2008-09 SC approved for member consultation via e-decision.

2009-06 Member consultation.

2010-07 TPPT revised draft and recommended to SC for adoption.

2011-11 SC commented by e-decision.

2012-12 TPPT revised draft and recommended to SC for adoption.

2013-06 SC recommended to CPM-9 for adoption.

2014-04 Treatment received formal objection before CPM-9.

2015-11 SC assigned the status "pending".

2016-09 TPPT noted that the schedules presented for adoption were for "Murcott", and agreed that there are no varietal differences on *C. reticula* and therefore recalculated the efficacy levels to encompass both varieties (as submitted), TPPT agreed that there are no fruit fly population differences in relation to cold treatment.

2016-11 PPT recommended to SC for adoption.

2016-11 SC recommended to CPM-12 for adoption via e-decision (2016_eSC_Nov_06).

2017-04 CPM adopted the phytosanitary treatment.

ISPM 28. **Annex 25**. *Cold treatment for* Ceratitis *capitata on* Citrus reticulata × C. sinensis (2017). Rome, IPPC, FAO.

Publication history last updated: 2017-04

This phytosanitary treatment was adopted by the Twelfth Session of the Commission on Phytosanitary Measures in 2017.
The annex is a prescriptive part of ISPM 28.

ISPM 28
Phytosanitary treatments for regulated pests

PT 26: Cold treatment for *Ceratitis capitata* on *Citrus limon*

Adopted 2017; published 2017

Scope of the treatment

This treatment describes the cold treatment of fruit of *Citrus limon*[1] to result in the mortality of eggs and larvae of *Ceratitis capitata* at the stated efficacy[2].

Treatment description

Name of treatment	Cold treatment for *Ceratitis capitata* on *Citrus limon*
Active ingredient	n/a
Treatment type	Physical (cold)
Target pest	*Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae)
Target regulated articles	Fruit of *Citrus limon*

Treatment schedule

Schedule 1: 2 °C or below for 16 continuous days

There is 95% confidence that the treatment according to this schedule kills not less than 99.9975% of eggs and larvae of *Ceratitis capitata*.

Schedule 2: 3 °C or below for 18 continuous days

There is 95% confidence that the treatment according to this schedule kills not less than 99.9973% of eggs and larvae of *Ceratitis capitata*.

The fruit must reach the treatment temperature before treatment exposure time commences. The fruit temperature should be monitored and recorded, and the temperature should not exceed the stated level throughout the duration of the treatment.

Other relevant information

C. limon is considered to be a conditional host of *C. capitata.*

[1] *Citrus* species and hybrids are named according to the nomenclature in Cottin, R. 2002. *Citrus of the world: A citrus directory*, version 2.0. France, SRA INRA-CIRAD.

[2] The scope of phytosanitary treatments does not include issues related to pesticide registration or other domestic requirements for contracting parties' approval of treatments. Treatments adopted by the Commission on Phytosanitary Measures may not provide information on specific effects on human health or food safety, which should be addressed using domestic procedures before contracting parties approve a treatment. In addition, potential effects of treatments on product quality are considered for some host commodities before their international adoption. However, evaluation of any effects of a treatment on the quality of commodities may require additional consideration. There is no obligation for a contracting party to approve, register or adopt the treatments for use in its territory.

In evaluating this treatment the Technical Panel on Phytosanitary Treatments (TPPT) considered issues associated with temperature regimes and thermal conditioning, taking into account the work of Hallman and Mangan (1997).

Schedules 1 and 2 were based on the work of De Lima *et al.* (2007) and were developed using the cultivar "Lisbon", and using failure to pupariate as the measure of mortality.

The TPPT also considered issues associated with chilling injury in lemons (TPPT, 2012).

References

The present annex to the standard may refer to International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

De Lima, C.P.F., Jessup, A.J., Cruickshank, L., Walsh, C.J. & Mansfield, E.R. 2007. Cold disinfestation of citrus (*Citrus* spp.) for Mediterranean fruit fly (*Ceratitis capitata*) and Queensland fruit fly (*Bactrocera tryoni*) (Diptera: Tephritidae). *New Zealand Journal of Crop and Horticultural Science*, 35: 39–50.

Hallman, G.J. & Mangan, R.L. 1997. Concerns with temperature quarantine treatment research. In: G.L. Obenauf, ed. *1997 Annual International Research Conference on Methyl Bromide Alternatives and Emissions Reduction*. San Diego, CA, 3–5 November 1997, pp. 79-1–79-4.

TPPT (Technical Panel on Phytosanitary Treatments). 2012. TPPT response to SC's concerns about chilling injury in lemons during in-transit cold disinfestation. Appendix 9, TPPT meeting report, December 2012, pp. 55–57.

Publication history

This is not an official part of the standard

2007-09 Treatment submitted.

2007-12 TPPT split *Cold treatment of* Citrus limon *for* Ceratitis capitata from 2007-TPPT-106 to create 2007-206C.

2008-04 CPM-3 added subject under the topic *Fruit fly treatments.*

2008-09 SC approved for member consultation via e-decision.

2009-06 Member consultation.

2010-07 TPPT revised draft and recommended to SC for adoption.

2011-11 SC commented by e-decision.

2012-12 TPPT finalized response to concern about chilling injury, revised draft and recommended to SC for adoption.

2013-06 SC did not reach consensus during the forum discussion and agreed to discuss draft at SC 2013-11.

2013-11 SC recommended to CPM-9 for adoption.

2014-04 Treatment received formal objection before CPM-9.

2015-11 SC assigned the status "pending".

2016-09 TPPT agreed that there are no fruit fly population differences in relation to cold treatment and no varietal or cultivar effects.

2016-09 TPPT recommended to SC for adoption.

2016-11 SC recommended to CPM-12 for adoption via e-decision (2016_eSC_Nov_07).

2017-04 CPM adopted the phytosanitary treatment.

ISPM 28. **Annex 26**. *Cold treatment for* Ceratitis *capitata on* Citrus limon (2017). Rome, IPPC, FAO.

Publication history last updated: 2017-04

This phytosanitary treatment was adopted by the Twelfth Session of the Commission on Phytosanitary Measures in 2017.
The annex is a prescriptive part of ISPM 28.

ISPM 28
Phytosanitary treatments for regulated pests

PT 27: Cold treatment for *Ceratitis capitata* on *Citrus paradisi*

Adopted 2017; published 2017

Scope of the treatment

This treatment describes the cold treatment of fruit of *Citrus paradisi*[1] to result in the mortality of eggs and larvae of *Ceratitis capitata* at the stated efficacy[2].

Treatment description

Name of treatment	Cold treatment for *Ceratitis capitata* on *Citrus paradisi*
Active ingredient	n/a
Treatment type	Physical (cold)
Target pest	*Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae)
Target regulated articles	Fruit of *Citrus paradisi*

Treatment schedule

Schedule 1: 2 °C or below for 19 continuous days

There is 95% confidence that the treatment according to this schedule kills not less than 99.9917% of eggs and larvae of *Ceratitis capitata.*

Schedule 2: 3 °C or below for 23 continuous days

There is 95% confidence that the treatment according to this schedule kills not less than 99.9916% of eggs and larvae of *Ceratitis capitata.*

The fruit must reach the treatment temperature before treatment exposure time commences. The fruit temperature should be monitored and recorded, and the temperature should not exceed the stated level throughout the duration of the treatment.

[1] *Citrus* species and hybrids are named according to the nomenclature in Cottin, R. 2002. *Citrus of the world: A citrus directory* version 2.0. France, SRA INRA-CIRAD.

[2] The scope of phytosanitary treatments does not include issues related to pesticide registration or other domestic requirements for contracting parties' approval of treatments. Treatments adopted by the Commission on Phytosanitary Measures may not provide information on specific effects on human health or food safety, which should be addressed using domestic procedures before contracting parties approve a treatment. In addition, potential effects of treatments on product quality are considered for some host commodities before their international adoption. However, evaluation of any effects of a treatment on the quality of commodities may require additional consideration. There is no obligation for a contracting party to approve, register or adopt the treatments for use in its territory.

Other relevant information

In evaluating this treatment the Technical Panel on Phytosanitary Treatments considered issues associated with temperature regimes and thermal conditioning, taking into account the work of Hallman and Mangan (1997).

Schedules 1 and 2 were based on the work of Anonymous (2007a, b), Gastaminza *et al.* (2007) and Willink *et al.* (2007), using larval mortality.

Schedule 1 was developed using the cultivars "Marsh Seedless", "Star Ruby", "Henninger's Ruby" and "Rouge la Toma".

Schedule 2 was developed using the cultivar "Henninger's Ruby".

References

The present annex to the standard may refer to International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

Anonymous. 2007a. Technical Panel on Phytosanitary Treatments – 110a. Quarantine cold treatment of grapefruit for medfly (*Ceratitis capitata* Wied). Document provided by the National Plant Protection Organization of Argentina.

Anonymous. 2007b. Technical Panel on Phytosanitary Treatments – 111a. Quarantine cold treatment of grapefruit for medfly (*Ceratitis capitata* Wied). Document provided by the National Plant Protection Organization of Argentina.

Gastaminza, G., Willink, E., Gramajo, M.C., Salvatore, A., Villagrán, M.E., Carrizo, B., Macián, A., Avila, R., Favre, P., Toledo, S., García Degano, M.F., Socias, M.G. & Oviedo, A. 2007. Tratamientos con frío para el control de *Ceratitis capitata* y *Anastrepha fraterculus* para la exportación de cítricos. In: Moscas de los frutos y su relevancia cuarentenaria en la citricultura del Noroeste Argentino: once años de investigaciones 1996–2007. E. Willink, G. Gastaminza, L. Augier & B. Stein, eds. Centro de Investigaciones Cuarentenarias, Sección Zoología Agrícola, Estación Experimental Agroindustrial Obispo Colombres, Las Talitas, Tucumán, Argentina. Available at http://www.eeaoc.org.ar (last accessed 1 September 2016).

Hallman, G.J. & Mangan, R.L. 1997. Concerns with temperature quarantine treatment research. In: G.L. Obenauf, ed. *1997 Annual International Research Conference on Methyl Bromide Alternatives and Emissions Reduction*. San Diego, CA, 3–5 November 1997, pp. 79-1–79-4.

Willink, E., Gastaminza, G., Gramajo, M.C., Salvatore, A., Villagrán, M.E., Carrizo, B., Macián, A., Avila, R. & Favre, P. 2007. Estudios básicos para el desarrollo de tratamientos cuarentenarios con frío para *Ceratitis capitata* y *Anastrepha fraterculus* en cítricos de Argentina. In: Moscas de los frutos y su relevancia cuarentenaria en la citricultura del Noroeste Argentino: once años de investigaciones 1996–2007. E. Willink, G. Gastaminza, L. Augier & B. Stein, eds. Centro de Investigaciones Cuarentenarias, Sección Zoología Agrícola, Estación Experimental Agroindustrial Obispo Colombres, Las Talitas, Tucumán, Argentina. Available at http://www.eeaoc.org.ar (last accessed 1 September 2016).

Publication history

This is not an official part of the standard

2007-09 Treatment submitted.

2007-12 TPPT revised draft Cold treatment of *Citrus paradisi* for *Ceratitis capitata.*

2008-04 CPM-3 added subject under the topic *Fruit fly treatments*.

2008-09 SC approved for member consultation via e-decision.

2009-06 Member consultation.

2010-07 TPPT revised draft and recommended to SC for adoption.

2011-11 SC recommended to CPM-7 for adoption.

2012-03 Treatment received formal objections.

2012-09 TPPT drafted response to formal objections (no revision recommended with formal objections).

2012-12 TPPT reviewed draft (no changes made) and recommended to SC for adoption.

2013-06 SC recommended to CPM-9 for adoption.

2014-04 Treatment received formal objections before CPM-9.

2014-06 TPPT revised draft.

2014-09 TPPT responded to some formal objections.

2015-11 SC assigned the status “pending”.

2016-09 TPPT agreed that there are no fruit fly population differences in relation to cold treatment and no varietal or cultivar effects.

2016-09 TPPT recommended to SC for adoption.

2016-11 SC recommended to CPM-12 for adoption via e-decision (2016_eSC_Nov_08).

2017-04 CPM adopted the phytosanitary treatment.

ISPM 28. **Annex 27**. *Cold treatment for* Ceratitis capitata *on* Citrus paradisi (2017). Rome, IPPC, FAO.

Publication history last updated: 2017-04

This phytosanitary treatment was adopted by the Twelfth Session of the Commission on Phytosanitary Measures in 2017.
The annex is a prescriptive part of ISPM 28.

ISPM 28
Phytosanitary treatments for regulated pests

PT 28: Cold treatment for *Ceratitis capitata* on *Citrus reticulata*

Adopted 2017; published 2017

Scope of the treatment

This treatment describes the cold treatment of fruit of *Citrus reticulata*[1] to result in the mortality of eggs and larvae of *Ceratitis capitata* at the stated efficacy[2].

Treatment description

Name of treatment	Cold treatment for *Ceratitis capitata* on *Citrus reticulata*
Active ingredient	n/a
Treatment type	Physical (cold)
Target pest	*Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae)
Target regulated articles	Fruit of *Citrus reticulata*

Treatment schedule

2 °C or below for 23 continuous days.

There is 95% confidence that the treatment according to this schedule kills not less than 99.9918% of eggs and larvae of *Ceratitis capitata.*

The fruit must reach the treatment temperature before treatment exposure time commences. The fruit temperature should be monitored and recorded, and the temperature should not exceed the stated level throughout the duration of the treatment.

Other relevant information

In evaluating this treatment the Technical Panel on Phytosanitary Treatments considered issues associated with temperature regimes and thermal conditioning, taking into account the work of Hallman and Mangan (1997).

This schedule was based on the work of Gastaminza *et al.* (2007) and Willink *et al.* (2007) and was developed using the cultivar "Nova" (*C. reticulata*) and using larval mortality.

[1] Citrus species and hybrids are named according to the nomenclature in Cottin, R. 2002. *Citrus of the world: A citrus directory*, version 2.0. France, SRA INRA-CIRAD.

[2] The scope of phytosanitary treatments does not include issues related to pesticide registration or other domestic requirements for contracting parties' approval of treatments. Treatments adopted by the Commission on Phytosanitary Measures may not provide information on specific effects on human health or food safety, which should be addressed using domestic procedures before contracting parties approve a treatment. In addition, potential effects of treatments on product quality are considered for some host commodities before their international adoption. However, evaluation of any effects of a treatment on the quality of commodities may require additional consideration. There is no obligation for a contracting party to approve, register or adopt the treatments for use in its territory.

References

The present annex to the standard may refer to International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

Gastaminza, G., Willink, E., Gramajo, M.C., Salvatore, A., Villagrán, M.E., Carrizo, B., Macián, A., Avila, R., Favre, P., Toledo, S., García Degano, M.F., Socias, M.G. & Oviedo, A. 2007. Tratamientos con frío para el control de *Ceratitis capitata* y *Anastrepha fraterculus* para la exportación de cítricos. In: Moscas de los frutos y su relevancia cuarentenaria en la citricultura del Noroeste Argentino: once años de investigaciones 1996–2007. E. Willink, G. Gastaminza, L. Augier & B. Stein, eds. Centro de Investigaciones Cuarentenarias, Sección Zoología Agrícola, Estación Experimental Agroindustrial Obispo Colombres, Las Talitas, Tucumán, Argentina. Available at http://www.eeaoc.org.ar (last accessed 1 September 2016).

Hallman, G.J. & Mangan, R.L. 1997. Concerns with temperature quarantine treatment research. In: G.L. Obenauf, ed. *1997 Annual International Research Conference on Methyl Bromide Alternatives and Emissions Reduction*. San Diego, CA, 3–5 November 1997, pp. 79-1–79-4.

Willink, E., Gastaminza, G., Gramajo, M.C., Salvatore, A., Villagrán, M.E., Carrizo, B., Macián, A., Avila, R. & Favre, P. 2007. Estudios básicos para el desarrollo de tratamientos cuarentenarios con frío para *Ceratitis capitata* y *Anastepha fraterculus* en cítricos de Argentina. In: Moscas de los frutos y su relevancia cuarentenaria en la citricultura del Noroeste Argentino: once años de investigaciones 1996–2007. E. Willink, G. Gastaminza, L. Augier & B. Stein, eds. Centro de Investigaciones Cuarentenarias, Sección Zoología Agrícola, Estación Experimental Agroindustrial Obispo Colombres, Las Talitas, Tucumán, Argentina. Available at http://www.eeaoc.org.ar (last accessed 1 September 2016).

Publication history

This is not an official part of the standard

2007-09 Treatment submitted in response to call for treatments.

2007-12 TPPT revised draft *Cold treatment of* Citrus reticulata × C. sinensis *for* Ceratitis capitata (2007-212).

2008-04 CPM-3 added subject under the topic *Fruit fly treatments.*

2008-09 SC approved for member consultation via e-decision.

2009-06 Member consultation.

2010-07 TPPT revised draft and recommended to SC for adoption.

2011-11 SC recommended to CPM-7 for adoption.

2012-03 Treatment received formal objections.

2012-09 TPPT drafted response to formal objections (no revision recommended with formal objections).

2012-12 TPPT reviewed draft (no changes made) and recommended to SC for adoption.

2013-06 SC did not reach consensus during the forum discussion and agreed to discuss draft at SC 2013-11.

2013-11 SC agreed to request TPPT to address SC concerns.

2015-11 SC assigned the status “pending”.

2016-09 TPPT agreed that there are no fruit fly population differences in relation to cold treatment and no varietal/cultivar effects, thus TPPT recommended title change.

2016-09 TPPT recommended to SC for adoption.

2016-11 SC recommended to CPM-12 for adoption via e-decision (2016_eSC_Nov_09).

2017-04 CPM-12 adopted the phytosanitary treatment.

ISPM 28. **Annex 28**. *Cold treatment for* Ceratitis capitata *on* Citrus reticulata (2017). Rome, IPPC, FAO.

Publication history last updated: 2017-04

This phytosanitary treatment was adopted by the Twelfth Session of the Commission on Phytosanitary Measures in 2017.
The annex is a prescriptive part of ISPM 28.

ISPM 28
Phytosanitary treatments for regulated pests

PT 29: Cold treatment for *Ceratitis capitata* on *Citrus clementina*

Adopted 2017; published 2017

Scope of the treatment

This treatment describes the cold treatment of fruit of *Citrus clementina*[1] to result in the mortality of eggs and larvae of *Ceratitis capitata* at the stated efficacy[2].

Treatment description

Name of treatment	Cold treatment for *Ceratitis capitata* on *Citrus clementina*
Active ingredient	n/a
Treatment type	Physical (cold)
Target pest	*Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae)
Target regulated articles	Fruit of *Citrus clementina* Hort. ex Tanaka

Treatment schedule

2 °C (maximum fruit core temperature) or below for 16 continuous days.

There is 95% confidence that the treatment according to this schedule kills not less than 99.9900% of eggs and larvae of *Ceratitis capitata*.

The fruit must reach the treatment temperature before treatment exposure time commences. The fruit temperature should be monitored and recorded, and the temperature should not exceed the stated level throughout the duration of the treatment.

Other relevant information

This schedule is based on the work of Santaballa *et al.* (2009) and was developed using the variety "Clemenules", and using larval mortality.

[1] Citrus species and hybrids are named according to the nomenclature in Cottin, R. 2002. *Citrus of the world: A citrus directory*, version 2.0. France, SRA INRA-CIRAD.

[2] The scope of phytosanitary treatments does not include issues related to pesticide registration or other domestic requirements for contracting parties' approval of treatments. Treatments adopted by the Commission on Phytosanitary Measures may not provide information on specific effects on human health or food safety, which should be addressed using domestic procedures before contracting parties approve a treatment. In addition, potential effects of treatments on product quality are considered for some host commodities before their international adoption. However, evaluation of any effects of a treatment on the quality of commodities may require additional consideration. There is no obligation for a contracting party to approve, register or adopt the treatments for use in its territory.

References

The present annex to the standard may refer to International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

Santaballa, E., Laborda, R. & Cerdá, M. 2009. Quarantine cold treatment against *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) to export clementine mandarins to Japan. *Boletín de Sanidad Vegetal Plagas*, 35: 501–512 (in English).

Publication history

This is not an official part of the standard

2010-04 Cold treatment for *Ceratitis capitata* on *Citrus clementina* var. *Clemenules* treatment submitted (2010-102).

2010-07 TPPT reviewed treatment and requested additional information.

2012-05 TPPT received additional information.

2012-12 TPPT requested additional information from Submitter.

2013-02 TPPT sent letter to Submitter through Secretariat.

2013-05 Submitter responded.

2013-07 TPPT recommended to SC for member consultation only for var. *Clemenules.*

2013-09 TPPT approved treatment schedule (virtual meeting).

2014-02 SC e-decision for approval for member consultation.

2014-06 Member consultation.

2015-02 Member consultation comments reviewed by TPPT.

2015-11 SC assigned the status “pending”.

2016-07 Modified by Treatment lead (EW) in response to country comments.

2016-09 TPPT meeting (TPPT agreed to change title (removing “varieties”) and invited SC to note the change in title from *Cold treatment for* Ceratitis capitata *on* Citrus clementina *var.* Clemenules (2010-102) to *Cold treatment for* Ceratitis capitata *on* Citrus clementina (2010-102); TPPT agreed that there are no fruit fly population differences in relation to cold treatment).

2016-09 TPPT recommended to SC for adoption.

2016-11 SC recommended to CPM-12 for adoption via e-decision (2016_eSC_Nov_11).

2017-04 CPM-12 adopted the phytosanitary treatment.

ISPM 28. **Annex 29**. *Cold treatment for* Ceratitis capitata *on* Citrus clementina (2017). Rome, IPPC, FAO.

Publication history last updated: 2017-04

This phytosanitary treatment was adopted by the Twelfth Session of the Commission on Phytosanitary Measures in 2017.
The annex is a prescriptive part of ISPM 28.

ISPM 28
Phytosanitary treatments for regulated pests

PT 29: Cold treatment for *Ceratitis capitata* on *Citrus clementina*

Adopted 2017; published 2017

Scope of the treatment

This treatment describes the cold treatment of fruit of *Citrus clementina*[1] to result in the mortality of eggs and larvae of *Ceratitis capitata* at the stated efficacy[2].

Treatment description

Name of treatment	Cold treatment for *Ceratitis capitata* on *Citrus clementina*
Active ingredient	n/a
Treatment type	Physical (cold)
Target pest	*Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae)
Target regulated articles	Fruit of *Citrus clementina* Hort. ex Tanaka

Treatment schedule

2 °C (maximum fruit core temperature) or below for 16 continuous days.

There is 95% confidence that the treatment according to this schedule kills not less than 99.9900% of eggs and larvae of *Ceratitis capitata*.

The fruit must reach the treatment temperature before treatment exposure time commences. The fruit temperature should be monitored and recorded, and the temperature should not exceed the stated level throughout the duration of the treatment.

Other relevant information

This schedule is based on the work of Santaballa *et al.* (2009) and was developed using the variety "Clemenules", and using larval mortality.

[1] Citrus species and hybrids are named according to the nomenclature in Cottin, R. 2002. *Citrus of the world: A citrus directory*, version 2.0. France, SRA INRA-CIRAD.

[2] The scope of phytosanitary treatments does not include issues related to pesticide registration or other domestic requirements for contracting parties' approval of treatments. Treatments adopted by the Commission on Phytosanitary Measures may not provide information on specific effects on human health or food safety, which should be addressed using domestic procedures before contracting parties approve a treatment. In addition, potential effects of treatments on product quality are considered for some host commodities before their international adoption. However, evaluation of any effects of a treatment on the quality of commodities may require additional consideration. There is no obligation for a contracting party to approve, register or adopt the treatments for use in its territory.

References

The present annex to the standard may refer to International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

Santaballa, E., Laborda, R. & Cerdá, M. 2009. Quarantine cold treatment against *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) to export clementine mandarins to Japan. *Boletín de Sanidad Vegetal Plagas*, 35: 501–512 (in English).

Publication history

This is not an official part of the standard

2010-04 Cold treatment for *Ceratitis capitata* on *Citrus clementina* var. *Clemenules* treatment submitted (2010-102).

2010-07 TPPT reviewed treatment and requested additional information.

2012-05 TPPT received additional information.

2012-12 TPPT requested additional information from Submitter.

2013-02 TPPT sent letter to Submitter through Secretariat.

2013-05 Submitter responded.

2013-07 TPPT recommended to SC for member consultation only for var. *Clemenules.*

2013-09 TPPT approved treatment schedule (virtual meeting).

2014-02 SC e-decision for approval for member consultation.

2014-06 Member consultation.

2015-02 Member consultation comments reviewed by TPPT.

2015-11 SC assigned the status “pending”.

2016-07 Modified by Treatment lead (EW) in response to country comments.

2016-09 TPPT meeting (TPPT agreed to change title (removing “varieties”) and invited SC to note the change in title from *Cold treatment for* Ceratitis capitata *on* Citrus clementina *var.* Clemenules (2010-102) to *Cold treatment for* Ceratitis capitata *on* Citrus clementina (2010-102); TPPT agreed that there are no fruit fly population differences in relation to cold treatment).

2016-09 TPPT recommended to SC for adoption.

2016-11 SC recommended to CPM-12 for adoption via e-decision (2016_eSC_Nov_11).

2017-04 CPM-12 adopted the phytosanitary treatment.

ISPM 28. **Annex 29**. *Cold treatment for* Ceratitis capitata *on* Citrus clementina (2017). Rome, IPPC, FAO.

Publication history last updated: 2017-04

This phytosanitary treatment was adopted by the Twelfth Session of the Commission on Phytosanitary Measures in 2017.
The annex is a prescriptive part of ISPM 28.

ISPM 28
Phytosanitary treatments for regulated pests

PT 30: Vapour heat treatment for *Ceratitis capitata* on *Mangifera indica*

Adopted 2017; published 2017

Scope of the treatment

This treatment describes the vapour heat treatment of fruit of *Mangifera indica* to result in the mortality of eggs and larvae of *Ceratitis capitata* at the stated efficacy[1].

Treatment description

Name of treatment	Vapour heat treatment for *Ceratitis capitata* on *Mangifera indica*
Active ingredient	n/a
Treatment type	Physical (vapour heat)
Target pest	*Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae)
Target regulated articles	Fruit of *Mangifera indica* L.

Treatment schedule

Exposure in a vapour heat chamber:

- at a minimum of 95% relative humidity
- with air temperature increasing from room temperature to 47 °C or above
- for at least two hours or until fruit core temperature reaches 46.5 °C
- followed by ten minutes at a minimum of 95% relative humidity in a minimum air temperature of 47 °C and with fruit core temperature maintained at a minimum of 46.5 °C (of largest fruit).

Once the treatment is complete, fruits may be hydro-cooled to reach ambient temperature.

There is 95% confidence that the treatment according to this schedule kills not less than 99.9968% of eggs and larvae of *Ceratitis capitata*.

Other relevant information

In evaluating this treatment the Technical Panel on Phytosanitary Treatments considered issues associated with temperature regimes and thermal conditioning, taking into account the work of Hallman and Mangan (1997).

This schedule was based on the work of Heather *et al.* (1997) and was developed using the cultivar "Kensington Pride", and using failure to pupariate as the measure of mortality.

[1] The scope of phytosanitary treatments does not include issues related to pesticide registration or other domestic requirements for contracting parties' approval of treatments. Treatments adopted by the Commission on Phytosanitary Measures may not provide information on specific effects on human health or food safety, which should be addressed using domestic procedures before contracting parties approve a treatment. In addition, potential effects of treatments on product quality are considered for some host commodities before their international adoption. However, evaluation of any effects of a treatment on the quality of commodities may require additional consideration. There is no obligation for a contracting party to approve, register or adopt the treatments for use in its territory.

The egg stage was found to be the most thermotolerant among pre-puparial stages of *C. capitata* at temperatures from 41 ºC to 44 ºC; however, at 45 ºC, the third instar appeared to be slightly more thermotolerant.

References

The present annex to the standard may refer to International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

Hallman, G.J. & Mangan, R.L. 1997. Concerns with temperature quarantine treatment research. In: G.L. Obenauf, ed. *1997 Annual International Research Conference on Methyl Bromide Alternatives and Emissions Reduction*, San Diego, CA, 3–5 November, pp. 79-1–79-4.

Heather, N.W., Corcoran, R.J. & Kopittke, R.A. 1997. Hot air disinfestation of Australian 'Kensington' mangoes against two fruit flies (Diptera: Tephritidae). *Postharvest Biology and Technology*, 10: 99–105.

Publication history

This is not an official part of the standard

2007-03 CPM-2 added topic *Fruit fly treatments*.

2010-04 Vapour heat treatment for *Ceratitis capitata* on *Mangifera indica* (2010-106) submitted in response to 2009-12 call for treatments.
2010-07 TPPT reviewed treatment and requested additional information from the Submitter.
2012-02 TPPT requested additional information from Submitter.
2012-12 TPPT requested additional information from Submitter.
2013-02 TPPT sent final notice letter to Submitter through Secretariat.
2013-05 Submitter provided additional information.
2013-07 TPPT reviewed the draft and the additional information provided by the Submitter and recommended to SC for member consultation.
2014-02 SC approved for member consultation via e-decision (2014_eSC_May_04).
2014-07 Member consultation.
2015-11 SC assigned "pending" status.
2016-07 Modified by Treatment lead in response to consultation comments.
2016-09 TPPT decided that despite any possible differences in reaction to VHT existing among populations of *C. capitata*, the robustness of this treatment as exemplified by the very large number (> 165,000) of eggs (the most tolerant stage) treated in confirmatory testing compensated for any differences and thus recommended it to the SC.
2016-09 TPPT approval of responses to consultation comments via e-decision (2016_eTPPT_Sep_01).
2016-11 SC recommended to CPM-12 for adoption via e-decision (2016_eSC_Nov_12).
2017-04 CPM adopted the phytosanitary treatment.

ISPM 28. **Annex 30**. *Vapour heat treatment for* Ceratitis *capitata on* Mangifera indica (2017). Rome, IPPC, FAO.

Publication history last updated: 2017-04

This phytosanitary treatment was adopted by the Twelfth Session of the Commission on Phytosanitary Measures in 2017.
The annex is a prescriptive part of ISPM 28.

ISPM 28
Phytosanitary treatments for regulated pests

PT 31: Vapour heat treatment for *Bactrocera tryoni* on *Mangifera indica*

Adopted 2017; published 2017

Scope of the treatment

This treatment describes the vapour heat treatment of fruit of *Mangifera indica* to result in the mortality of eggs and larvae of *Bactrocera tryoni* at the stated efficacy[1].

Treatment description

Name of treatment	Vapour heat treatment for *Bactrocera tryoni* on *Mangifera indica*
Active ingredient	n/a
Treatment type	Physical (vapour heat)
Target pest	*Bactrocera tryoni* (Froggatt, 1897) (Diptera: Tephritidae)
Target regulated articles	Fruit of *Mangifera indica* L.

Treatment schedule

Exposure in a vapour heat chamber:

- with air temperature increasing from room temperature to 48 °C or above
- with air temperature held at 48 °C or above at a minimum of 95% relative humidity for a minimum of 90 minutes to achieve fruit core temperature of 47 °C or above
- followed by 15 minutes at a minimum of 95% relative humidity in a minimum air temperature of 48 °C and with fruit core temperature maintained at a minimum of 47 °C (of the largest fruit).

Once the treatment is complete, fruit may be air-cooled or cooled by an ambient temperature water drench.

There is 95% confidence that the treatment according to this schedule kills not less than 99.9968% of eggs and larvae of *Bactrocera tryoni*.

Other relevant information

This schedule was based on the work of Corcoran (2002), Corcoran *et al.* (2000), Heather *et al.* (1991, 1994, 1997) and Queensland Department of Primary Industries (1999) and was developed using the cultivars "Kensington Pride" and "Keitt", and using failure to pupariate as the measure of mortality.

[1] The scope of phytosanitary treatments does not include issues related to pesticide registration or other domestic requirements for contracting parties' approval of treatments. Treatments adopted by the Commission on Phytosanitary Measures may not provide information on specific effects on human health or food safety, which should be addressed using domestic procedures before contracting parties approve a treatment. In addition, potential effects of treatments on product quality are considered for some host commodities before their international adoption. However, evaluation of any effects of a treatment on the quality of commodities may require additional consideration. There is no obligation for a contracting party to approve, register or adopt the treatments for use in its territory.

References

The present annex to the standard may refer to International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

Corcoran, R.J. 2002. *Fruit fly (Diptera: Tephritidae) responses to quarantine heat treatment.* The University of Queensland, Brisbane, Australia. (PhD thesis)

Corcoran, R.J., Jordan, R.A., Peterson, P.M., Eelkema, M., Heslin, L.M. & Jen, E.V. 2000. *Disinfestation of additional mango varieties for export to Japan*. Gordon, Australia, Horticultural Research and Development Corporation.

Heather, N.W., Corcoran, R.L., Heard, T., Jacobi, K. & Coates, L. 1991. *Disinfestation of mangoes against Queensland fruit fly by vapour heat.* A Queensland Department of Primary Industries report to the Japanese Ministry of Agriculture, Forestry and Fisheries through the Commonwealth of Australia Department of Primary Industries and Energy.

Heather, N.W., Corcoran, R.J. & Kopittke, R.A. 1997. Hot air disinfestation of Australian 'Kensington' mangoes against two fruit flies (Diptera: Tephritidae). *Postharvest Biology and Technology*, 10: 99–105.

Heather, N.W., Jordan, R. & Corcoran, R.J. 1994. *Verification trials for vapour heat disinfestation of mangoes infested with fruit flies.* A Queensland Department of Primary Industries report to the Japanese Ministry of Agriculture, Forestry and Fisheries through the Commonwealth of Australia Department of Primary Industries and Energy.

Queensland Department of Primary Industries. 1999. *Verification trial against Queensland fruit fly,* Bactrocera tryoni *(Frogatt), in Keitt mangoes using vapour heat treatment.* A Queensland Department of Primary Industries report to the Japanese Ministry of Agriculture, Forestry and Fisheries through the Commonwealth of Australia Department of Primary Industries and Energy.

Publication history

This is not an official part of the standard

2007-03 CPM-2 added topic *Fruit fly treatments.*

2010-04 Vapour heat treatment for *Bactrocera tryoni* on *Mangifera indica* (2010-107) submitted in response to 2009-12 call for treatments.

2010-07 TPPT reviewed the draft and requested additional information from Submitter.

2012-02 TPPT reviewed Submitter response and requested further information.

2013-07 TPPT reviewed Submitter response and requested further information.

2014-06 TPPT reviewed Submitter response and recommended draft to SC for member consultation.

2014-08 SC approved for member consultation via e-decision (2014_eSC_Nov_08).

2015-07 Member consultation.

2016-09 TPPT agreed that there were no differences for mango varieties, but that the differences in treatment effectiveness were given by weight and shape of the fruit, thus TPPT modified the treatment to include a requirement on the ramp-up time and recommended it to the SC for adoption.

2016-11 SC recommended to CPM-12 for adoption via e-decision (2016_eSC_Nov_13) 2017-04 CPM adopted the phytosanitary treatment.

ISPM 28. **Annex 31**. *Vapour heat treatment for* Bacatrocera tryoni *on* Mangifera indica (2017). Rome, IPPC, FAO.

Publication history last updated: 2017-04

This diagnostic protocol was adopted by the Standards Committee on behalf of the Commission on Phytosanitary Measures in August 2016.
The annex is a prescriptive part of ISPM 27.

ISPM 27
Diagnostic protocols for regulated pests

DP 13: *Erwinia amylovora*

Adopted 2016; published 2016

CONTENTS

1. Pest Information

Erwinia amylovora is the causal agent of fire blight, a disease that affects most species of the subfamily Maloideae of the family Rosaceae (Spiraeoideae). It was the first bacterium described as the causal agent of a plant disease (Burrill, 1883). *E. amylovora* is considered to be native to North America and was first detected outside North America in New Zealand in 1920. Fire blight was reported in England in 1957 and since then the bacterium has been detected in most areas of Europe where susceptible hosts are cultivated. *E. amylovora* is now present in more than 40 countries. It has not been recorded in South America and most African and Asian countries (with the exception of countries surrounding the Mediterranean Sea), and it has been eradicated in Australia after one report there (van der Zwet, 2004). It represents a threat to the pome fruit industry of all these countries (Bonn and van der Zwet, 2000). Details on geographic distribution can be found in the European and Mediterranean Plant Protection Organization (EPPO) Plant Quarantine Data Retrieval System (EPPO, n.d.).

The most important host plants from both economic and epidemiological viewpoints are in the genera *Chaenomeles*, *Cotoneaster*, *Crataegus*, *Cydonia*, *Eriobotrya*, *Malus*, *Mespilus*, *Pyracantha*, *Pyrus*, *Sorbus* and *Stranvaesia* (Bradbury, 1986). The *E. amylovora* strains isolated from *Rubus* sp. in the United States are distinct from the strains on other hosts (Starr *et al.*, 1951; Powney *et al.*, 2011b).

Fire blight is probably the most serious bacterial disease affecting *Pyrus communis* (pear) and *Malus domestica* (apple) cultivars in many countries. Epidemics are sporadic and are dependent on a number of factors, including favourable environmental conditions, sufficient inoculum level present in the orchard and host susceptibility. The disease is easily dispersed by birds, insects, rain or wind (Thomson, 2000). The development of fire blight symptoms follows the seasonal growth development of the host plant. The disease begins in spring with the production of the primary inoculum from bacteria overwintering in cankers (Thomson, 2000) causing blossom infection, continues into summer with shoot and fruit infection, and ends in winter with the development of cankers throughout the dormant period of the host (van der Zwet and Beer, 1995; Thomson, 2000).

2. Taxonomic Information

Name:	*Erwinia amylovora* (Burrill, 1883) Winslow *et al.*, 1920
Synonyms:	*Micrococcus amylovorus* Burrill, 1883, *Bacillus amylovorus* (Burrill, 1883) Trevisan, 1889, "*Bacterium amylovorus*" [sic] (Burrill, 1883) Chester, 1897, *Erwinia amylovora* f.sp. *rubi* (Starr *et al.*, 1951)
Taxonomic position:	Proteobacteria, Y subdivision, Enterobacteriales, Enterobacteriaceae
Common name:	Fire blight (EPPO, 2013)

3. Detection

Diagnosis of fire blight can be achieved using isolation and serological and molecular tests. The assays indicated below are recommended after having been evaluated in one or more of the following ring tests: in 2003 in a Diagnostic Protocols for Organisms Harmful to Plants (DIAGPRO) project involving ten laboratories (López *et al.*, 2006); in 2009 in a European Phytosanitary Research Coordination (EUPHRESCO) project involving five laboratories (Dreo *et al*., 2009); and in 2010 by fourteen laboratories worldwide (López *et al*., 2010). The tests indicated in Figures 1 and 2 are the minimum requirements for the diagnosis, but further tests may be required by the national plant protection organization (NPPO), especially for the first report in a country. For example, serological tests may facilitate a presumptive diagnosis of symptomatic plant material based on the detection of a specific protein; however, an additional test based on a different biological principle should be used for detection. In all tests, positive and negative controls must be included.

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

3.1 Detection in plants with symptoms

The recommended screening tests are indicated in the flow diagram in Figure 1.

3.1.1 Symptoms

Symptoms of fire blight on the most common hosts such as *P. communis* (pear), *M. domestica,* (apple), *Cydonia* spp. (quince), *Eriobotrya japonica* (loquat), *Cotoneaster* spp. (cotoneaster), *Pyracantha* spp. (pyracantha) and *Crataegus* spp. (hawthorn) are similar and easily recognized. The name of the disease is descriptive of its major characteristic: the brownish, necrotic appearance of twigs, flowers and leaves, as though they had been burned by fire. The typical symptoms are the brown to black colour of leaves on affected branches, the production of exudate, and the characteristic "shepherd's crook" of terminal shoots. Depending on the affected plant part, the disease produces blossom blight, shoot or twig blight, leaf blight, fruit blight, limb or trunk blight, or collar or rootstock blight (van der Zwet and Keil, 1979; van der Zwet and Beer, 1995).

In apple and pear trees the first symptoms usually appear in early spring when the average temperature rises above 15 °C, during humid weather. Infected blossoms become soaked with water, then wilt, shrivel, and turn orange or brown to black. Peduncles may also appear water-soaked, and become dark green and finally brown or black, sometimes oozing droplets of sticky bacterial exudate. Infected leaves wilt and shrivel, and entire spurs turn brown in apples and dark brown to black in pears, but remain attached to the tree for some time. Upon infection young fruitlets turn brown but also remain attached to the tree. Immature fruit lesions appear oily or water-soaked, become brown to black, and often ooze droplets of bacterial exudate. Characteristic reddish-brown streaks are often found in the subcortical tissues when the bark is peeled from infected limbs or twigs (van der Zwet and Keil, 1979; Thomson, 2000). Brown to black slightly depressed cankers form in the bark of twigs, branches or the trunk of infected trees. These cankers later become defined by cracks near the margin of diseased and healthy tissue (Thomson, 2000).

Confusion may occur between fire blight and blight- or blast-like symptoms – especially in blossoms and buds – caused by other pathogenic bacteria and fungi, insect damage or physiological disorders. Other bacteria that cause fire blight-like symptoms include *Erwinia pyrifoliae*, the causal agent of bacterial shoot blight of *Pyrus pyrifolia* (Asian pear) (Kim *et al.*, 1999); *Erwinia piriflorinigrans*, isolated from necrotic pear blossoms in Spain (López *et al.*, 2011); *Erwinia uzenensis*, recently described in Japan (Matsuura *et al*., 2012); other *Erwinia* spp. reported in Japan that cause bacterial shoot blight (Tanii *et al.*, 1981; Kim *et al*., 2001a, 2001b; Palacio-Bielsa *et al*., 2012); and *Pseudomonas syringae* pv. *syringae*, the causal agent of blossom blast. A definitive diagnosis of fire blight should always be obtained through laboratory analysis.

3.1.2 Sampling and sample preparation

Plant material should be analysed as soon as possible after collection, but may be stored at 4–8 ºC for up to one week until processing. Precautions to avoid cross-contamination should be taken when collecting samples, during transport and processing, and especially while isolating the bacterium or extracting DNA.

The samples should be processed with a general procedure valid for isolation, serological tests and polymerase chain reaction (PCR) analysis. The use of freshly prepared antioxidant maceration buffer (polyvinylpyrrolidone (PVP)-10, 20 g; mannitol, 10 g; ascorbic acid, 1.76 g; reduced glutathione, 3 g; phosphate-buffered saline (PBS), 10 mM, 1 litre; pH 7.2; sterilized by filtration) is required for

successful enrichment, as indicated by Gorris *et al*. (1996). The samples can be processed also in sterile distilled water or in PBS, pH 7.2 (NaCl, 8 g; KCl, 0.2 g; $Na_2HPO_4 \cdot 12H_2O$, 2.9 g; KH_2PO_4, 0.2 g; distilled water, 1 litre) but for direct isolation, immunofluorescence or PCR.

Plant parts (flowers, shoots, twigs, leaves or fruit) showing the most typical symptoms, and with bacterial exudate if possible, are carefully selected. Material for processing is selected from the leading edge of disease lesions. The plant tissue is cut into pieces of approximately 0.1–1.0 g, crushed lightly in antioxidant maceration buffer, PBS or sterile distilled water (as described in the previous paragraph) at 1:50 (w/v), left to stand for at least 5 min, and placed on ice for a few minutes. Triplicate samples (1 ml each) of each macerate are transferred to sterile microcentrifuge tubes, with one tube stored at –20 ºC for subsequent analysis by PCR and another tube's contents adjusted to 30% glycerol and stored at –80 ºC for confirmation testing, if necessary. The third tube is kept on ice for performing enrichment before enzyme-linked immunosorbent assay (ELISA) or PCR, and isolation on selective media (Figure 1). If immunofluorescence is to be performed (i.e. immunofluorescence analysis is optional), the slides are prepared and fixed on the same day that the samples are macerated. The PCR analysis should be performed as soon as is convenient, using the macerated sample stored at –20 ºC.

3.1.3 Isolation

3.1.3.1 Isolation from symptomatic samples

In general, plating on three media is advised for maximum likelihood of recovery of *E. amylovora*, especially when samples are not in good condition. Depending on the amount and composition of the microbiota of the sample, each medium can be more or less efficient. Three media (CCT, King's B and levan) have been validated in two ring tests, with levan having the highest plating efficiency.

When symptoms are very advanced or the environmental conditions after infection are not favourable for bacterial multiplication, the number of culturable *E. amylovora* cells can be very low. Isolation under these conditions can result in plates with few cells of the pathogen and that can be overcrowded with saprophytic and antagonistic bacteria. If this is suspected, the sample should be re-tested and/or enriched before isolation. The induction of the reversible viable but non-culturable state (VBNC) has been described for *E. amylovora in vitro* using copper treatments and in fruits (Ordax *et al*., 2009), and it can be the cause of false negative isolation results.The recipes for the recommended media are described below:

- CCT medium is prepared in two parts. Part 1 consists of: sucrose, 100 g; sorbitol, 10 g; Niaproof,4 1.2 ml; crystal violet, 2 ml (solvent 0.1% ethanol); nutrient agar, 23 g; distilled water, 1 litre; pH 7.0–7.2; sterilized by autoclaving at 115 ºC for 10 min. The autoclaved medium is cooled to approximately 45 ºC. Part 2 consists of: thallium nitrate, 2 ml (1% w/v aqueous solution); cycloheximide, 0.05 g; sterilized by filtration. Part 2 is added to 1 litre sterile Part 1 (Ishimaru and Klos, 1984).
- King's B medium consists of: proteose peptone no. 3, 20 g; glycerol, 10 ml; K_2HPO_4, 1.5 g; $MgSO_4.7H_2O$, 1.5 g; agar, 15 g; distilled water, 1 litre; pH 7.0–7.2; sterilized by autoclaving at 120 ºC for 20 min (King *et al*., 1954).
- Levan medium consists of: yeast extract, 2 g; bactopeptone, 5 g; NaCl, 5 g; sucrose, 50 g; agar, 20 g; distilled water, 1 litre; pH 7.0–7.2; sterilized by autoclaving at 120 ºC for 20 min.

Cycloheximide is added at 0.05 g/litre to King's B and levan media when fungi are expected in the isolation. Dilutions of 1:10 and 1:100 of each macerate are prepared in PBS (NaCl, 8 g; KCl, 0.2 g; $Na_2HPO_4 \cdot 12H_2O$, 2.9 g; KH_2PO_4, 0.2 g; distilled water, 1 litre).

Preferably 100 µl of the macerates and their dilutions is spread, by triple streaking, in 130 mm plates, or 50 µl is spread in standard 90 mm Petri dishes. Plates are incubated at 25 ºC for up to four days. The final reading is usually taken at 72 h. Colonies of *E. amylovora* on CCT medium are pale violet, circular, high convex to domed, smooth and mucoid, and they grow more slowly than on King's B or levan media. Colonies on King's B medium are creamy white, circular and non-fluorescent under

ultraviolet (UV) light at 366 nm. Colonies on levan medium are white, circular, domed, smooth and mucoid. Levan-negative colonies of *E. amylovora* have been reported (Bereswill *et al*., 1997).

Pure cultures are obtained from individual suspect colonies of each sample by dilution and streaking onto King's B medium. Presumptive colonies of *E. amylovora* are identified preferably by double antibody sandwich indirect (DASI)-ELISA, PCR or by other appropriate tests (e.g. biochemical, immunofluorescence, fatty acid profile), or by inoculating susceptible organs of any available *E. amylovora* host to test pathogenicity, as indicated in section 4.

When analysing symptomatic samples, good correlation is expected between isolation, immunofluorescence, enrichment-DASI-ELISA (section 3.1.4.1) and PCR.

In the 2003 and 2010 ring tests, the accuracy of isolation was 0.88 and 0.81 for King's B, 0.92 and 0.89 for levan, and 0.92 and 0.95 for CCT media, respectively (López *et al.*, 2006; M.M. Lopez, personal communication, 2012). In the 2009 ring test, accuracy of isolation was 0.96 for CCT (Dreo *et al*., 2009).

3.1.3.2 Enrichment-isolation

Enrichment is used to multiply the initial population of culturable *E. amylovora* in a sample and to perform enrichment-DASI-ELISA or enrichment-PCR. It should be carried out before isolation (even for symptomatic samples) when a low number of culturable *E. amylovora* cells is expected to be present (e.g. for copper-treated samples, samples with old symptoms, samples collected during unfavourable weather conditions for fire blight such as in winter). The enrichment step greatly increases the sensitivity of DASI-ELISA. The use of two validated liquid media for enrichment – one non-selective (King's B) and one semi-selective (CCT) – is advised because the composition and population size of the microbiota are unknown.

The tissue sample is macerated as described in section 3.1.2 and 0.9 ml is immediately dispensed into each of two sterile 10–15 ml tubes (to ensure sufficient aeration) containing 0.9 ml of each liquid enrichment medium (King's B without agar, and CCT made with nutrient broth instead of nutrient agar). The tubes are incubated at 25 ºC for 48–72 h without shaking. A longer incubation is recommended when processing plant samples collected in winter. Both enrichment broths and dilutions (1:10 and 1:100) prepared in PBS are spread onto CCT plates, by triple streaking, to obtain isolated colonies. Plates are incubated at 25 ºC for 72–96 h. Final reading of the CCT plates is at 72 h and must be followed by purification of colonies and identification.

The use of semi-selective medium for plating and dilution is advised because the enrichment step will permit growth of the pathogen but will also allow abundant multiplication of other bacteria. The accuracy of the enrichment isolation on King's B and CCT was 0.97 in the 2010 ring test.

3.1.4 Serological detection

3.1.4.1 Enrichment-DASI-ELISA

A kit for enrichment-DASI-ELISA has been validated in two ring tests and is available commercially from Plant Print Diagnòstics SL[1]. It is based on the mixture of two specific monoclonal antibodies described in Gorris *et al*. (1996) and requires prior enrichment of the samples, as previously described. The following protocol must be followed strictly for maximum accuracy. Before ELISA, the required amount of the enriched extracts and controls is treated by incubation in a water bath at 100 ºC for 10 min. This treatment is necessary for optimum specificity. The boiled samples are processed (at

[1] In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

room temperature) by ELISA on the same day (or stored at –20 ºC for subsequent analysis) following the instructions provided by the manufacturer of the commercial kit.

The ELISA is negative if the average optical density (OD) reading from duplicate sample wells is <2× the OD in the negative sample extract control wells (providing the OD for the positive control wells are above 1.0 after 90 min incubation and are greater than twice the OD obtained for the negative sample extracts). The ELISA is positive if the average OD reading from duplicate sample wells is >2× the OD in the negative sample extract control wells (providing all negative control wells are lower than 2× the average OD reading of the positive control wells).

Negative ELISA readings in positive control wells indicate that the test has not been performed correctly and/or the reagents were not well prepared. Positive ELISA readings in negative control wells indicate cross-contamination or non-specific antibody binding. In both cases, the test should be repeated or a second test based on a different biological principle, such as PCR, should be performed.

In the 2003 and 2010 ring tests the accuracy of the DASI-ELISA was 0.79 and 0.82, respectively, for enrichment in King's B medium (King's B-DASI-ELISA), and 0.83 and 0.77, respectively, for enrichment in CCT medium (CCT-DASI-ELISA) (López *et al.*, 2006, 2010).

3.1.4.2 Direct tissue print-ELISA

To make tissue prints, freshly cut plant sections are pressed carefully against a nitrocellulose membrane. Prints are prepared for positive and negative controls. Printed membranes can be kept for several months in a dry place at room temperature. A validated source of antibodies to *E. amylovora* such as the Plant Print Diagnòstics SL kit[1] should be used. To develop prints, the manufacturer's instructions should be followed. The prints are observed under low power magnification (×10 or ×20). The test is positive when purple–violet precipitates appear in the sections of plant tissue that are printed on the membrane and not in the plant tissue print of the negative control. If exudates or colonies are printed they should appear violet when positive. The test is negative when no purple–violet precipitates appear, as in the negative control.

3.1.4.3 Immunofluorescence

Immunofluorescence is a recommended alternative serological method, and it is easy to follow the standard protocol (Anonymous, 1998). A validated source of antibodies to *E. amylovora* should be used. Two commercial antibodies have been validated in one ring test: one monoclonal antibody is available through Plant Print Diagnòstics SL[1] and one polyclonal antibody is available from Loewe Biochemicals[1].

Immunofluorescence should be performed on fresh sample extracts fixed onto slides. Undiluted macerates and dilutions of 1:10 and 1:100 in PBS are used to spot windows of the immunofluorescence slides. The monoclonal or polyclonal antibody is used at the appropriate dilution in PBS. The appropriate fluorescein isothiocyanate (FITC) conjugate is diluted in PBS: goat anti-mouse for monoclonal antibody (GAM-FITC), and goat anti-rabbit (GAR-FITC) or anti-goat for polyclonal antibody.

The test on a sample is negative if green fluorescing cells with morphology typical of *E. amylovora* are observed in the positive controls, but not in the sample windows. The test on a sample is positive if green fluorescing cells with typical morphology are observed in the positive controls and in the sample windows, but not in the negative controls. As a population of 10^3 cells/ml is considered the limit for reliable detection by immunofluorescence, for samples with $>10^3$ cells/ml, the immunofluorescence test is considered positive. For samples with $<10^3$ cells/ml, or weakly fluorescing cells, the result of the immunofluorescence may be considered uncertain.

The accuracy of immunofluorescence in the 2003 ring test was 0.70 for the Plant Print Diagnòstics SL[1] monoclonal antibody, and 0.72 for the Loewe Biochemicals[1] polyclonal antibodies confirming that the sensitivity of the technique is approximately 10^3 colony-forming units (c.f.u.)/ml.

3.1.4.4 Lateral flow immunoassay

Two lateral flow devices are available commercially for rapid analysis of plant material: Ea AgriStrip (Bioreba[1]) and Pocket Diagnostics (Forsite Diagnostics[1]). Following the manufacturers' instructions their accuracy in the 2009 and 2010 ring tests was 0.66 and 0.55, respectively, for Ea AgriStrip[1] and 0.64 and 0.56, respectively, for Pocket Diagnostics[1]. These results were obtained for the detection of *E. amylovora* in samples from 1 to10^6 c.f.u./g, but the accuracy was approximately 1.0 when analysing samples with 10^5 to 10^6 c.f.u./g, the minimum number expected in symptomatic samples (López *et al.*, 2010). The kits are recommended for use only with symptomatic samples.

3.1.5 Molecular detection

Several PCR methods and one loop-mediated isothermal amplification (LAMP) protocol[2], available for the detection of *E. amylovora*, were evaluated extensively in ring testing by several laboratories (Lopez *et al.*, 2010; M.M. Lopez, personal communication, 2012). The specificity of some of these methods has been evaluated by Powney *et al*. (2011a). Conventional PCR methods may be more expensive and time consuming and usually require more training than serological methods, and for these reasons, as well as the risk of contamination, they are not always appropriate for large-scale testing. However, real-time PCR and some conventional PCR and nested PCR in one tube protocols have provided highly accurate results and they are therefore recommended molecular methods. All PCR assays should be performed using DNA extracted from the samples because of the high amount of inhibitors of *E. amylovora* hosts, or from enriched samples, which have increased reliability of detection.

3.1.5.1 Controls for molecular tests

For the test result obtained to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid isolation and amplification of the target nucleic acid. For PCR a positive nucleic acid control, an internal control and a negative amplification control (no template control) are the minimum controls that should be used.

Positive nucleic acid control

This control is used to monitor the efficiency of the test method (apart from the extraction), and specifically the amplification. Pre-prepared (stored) nucleic acid, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product) may be used.

Internal control

For conventional and real-time PCR, plant internal controls (e.g. a housekeeping gene (HKG) such as COX (Weller *et al.*, 2000) or 16S ribosomal (r)DNA (Weisberg *et al.*, 1991)) should be incorporated into the protocol to eliminate the possibility of PCR false negatives due to nucleic acid extraction failure or degradation or the presence of PCR inhibitors.

Negative amplification control (no template control)

This control is necessary for conventional and real-time PCR to rule out false positives due to contamination during preparation of the reaction mixture. PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage.

[2] When using LAMP on a regular basis in an area that has a patent system such as Japan (patent no.s 3 313 358, 3 974 441 and 4 139 424), the United States (US6 410 278, US6 974 670 and US7 494 790), the European Union (no.s 1 020 534, 1 873 260, 2 045 337 and 2 287 338), China (ZL008818262), the Republic of Korea (patent no. 10-0612551), Australia (no. 779160) and the Russian Federation (no. 2 252 964), it is necessary for users to obtain a licence from Eiken Chemical Co., Ltd before use to protect the intellectual property right.

Positive extraction control

This control is used to ensure that target nucleic acid extracted is of sufficient quantity and quality and that the target is detected. Nucleic acid is extracted from infected host tissue or healthy plant tissue that has been spiked with the target.

The positive control should be approximately one-tenth of the amount of leaf tissue used per plant for the DNA extraction.

For PCR, care needs to be taken to avoid cross-contamination due to aerosols from the positive control or from positive samples. If required, the positive control used in the laboratory should be sequenced so that this sequence can be readily compared with sequence obtained from PCR amplicons of the correct size. Alternatively, synthetic positive controls can be made with a known sequence that, again, can be compared with PCR amplicons of the correct size.

Negative extraction control

This control is used to monitor contamination during nucleic acid extraction and/or cross-reaction with the host tissue. The control compromises nucleic acid that is extracted from uninfected host tissue and subsequently amplified. Multiple controls are recommended to be included when large numbers of positive samples are expected.

3.1.5.2 DNA extraction

Three DNA extraction methods – Llop *et al.* (1999), Taylor *et al.* (2001) and the REDExtract-N-Amp Plant PCR Kit (Sigma-Aldrich[1]) – were evaluated in the 2009 ring test (Dreo *et al*., 2009) with four PCR protocols with accuracies ranging from 0.67 to 0.76. The methods showed comparable results in the 2010 ring test (Lopez *et al.*, 2010), as indicated below in the accuracies given for the different PCR methods. Their efficiencies did not improve after diluting the extracts 1:10, suggesting that few or no inhibitors were present. Based on these findings, the Llop *et al.* (1999) extraction method is recommended as it has been extensively tested in a number of countries and is cheap and easy to set up in the laboratory.

DNA extraction according to Llop* et al. *(1999)

One millilitre of a sample macerate prepared according to section 3.1.2 and/or 1 ml enriched macerate is centrifuged at 10 000 *g* for 5 min at room temperature. The supernatant is discarded, and the pellet is resuspended in 500 µl extraction buffer (Tris-HCl pH 7.5, 24.2 g; NaCl, 14.6 g; ethylenediaminetetraacetic acid (EDTA), 9.3 g; sodium dodecyl sulphate (SDS), 5 g; PVP-10, 20 g; distilled water, 1 litre; sterilized by filtration) and incubated for 1 h at room temperature before centrifugation at 4 000 *g* for 5 min. Approximately 450 µl supernatant is mixed with an equal volume of isopropanol, inverted, and left at room temperature for 30 min to 1 h. The precipitated nucleic acid is centrifuged at 10 000 *g* for 5 min, the supernatant is discarded and the pellet is air-dried. If there is still a coloured precipitate (brown or green) at the bottom of the tube, this is carefully removed while discarding the supernatant, thus obtaining a cleaner DNA pellet. The pellet is resuspended in 200 µl water. It should be used for PCR immediately or stored at –20 ºC.

3.1.5.3 DNA amplification by PCR

There are many PCR primers and protocols described for *E. amylovora* detection and some have shown specificity problems (Roselló *et al.*, 2006; Powney *et al.*, 2011a). The primers and protocols validated in ring tests were those of Bereswill *et al.* (1992) and Llop *et al.* (2000), with or without previous enrichment, in 2003; and those of Taylor *et al.* (2001), Stöger *et al.* (2006) and Obradovic *et al.* (2007) in 2009 and 2010. The discovery of fully virulent *E. amylovora* strains without the pEA29 plasmid (Llop *et al.*, 2006) and experiences from different countries (Powney *et al.*, 2011a) indicate that two PCR protocols should be used: one with primers based on pEA29 sequences, and another with primers targeting unique chromosomal sequences. If the PCR is negative with the protocol based on the pEA29 primers and positive with the protocol based on the chromosomal

primers, the PCR test can be considered as positive for *E. amylovora*. PCR can be carried out using the primers and conditions validated in the ring tests, although amplification conditions should be optimized for different thermocyclers.

PCR according to Bereswill* et al. *(1992)

The primers are:

A (forward): 5′-CGG TTT TTA ACG CTG GG-3′

B (reverse): 5′-GGG CAA ATA CTC GGA TT-3′

The targeted sequences are in the plasmid pEA29. The PCR mixture is composed of: ultrapure water, 17.4 µl; buffer 10×, 2.5 µl; $MgCl_2$ 50 mM, 1.5 µl; dNTPs 10 mM, 0.5 µl; primer A 10 pmol/µl, 0.25 µl; primer B 10 pmol/µl, 0.25 µl; and Taq DNA polymerase 5 U/µl, 0.1 µl. The extracted DNA sample volume is 2.5 µl, and should be added to 22.5 µl of the PCR mix. The cycling parameters are a denaturation step of 93 ºC for 5 min followed by 40 cycles of 93 ºC for 30 s, 52 ºC for 30 s and 72 ºC for 1 min 15 s, with a final elongation step at 72 ºC for 10 min. The amplicon size is 900 base pairs (bp) according to Bereswill *et al*. (1992), although variations in size can occur between 900 and 1 100 bp depending on the number of 8 bp repeats within the amplified fragment (Jones and Geider, 2001).

The accuracy was 0.51 in the 2003 ring test but increased to 0.74 and 0.78 after enrichment of the samples in King's B and CCT media, respectively (López *et al.*, 2006).

PCR according to Taylor* et al. *(2001)

The primers are:

G1-F: 5′-CCT GCA TAA ATC ACC GCT GAC AGC TCA ATG-3′

G2-R: 5′-GCT ACC ACT GAT CGC TCG AAT CAA ATC GGC-3′

The targeted sequences are chromosomal. The PCR mixture is composed of: ultrapure water, 14.3 µl; buffer 10×, 2.5 µl; $MgCl_2$ 50 mM, 0.75 µl; dNTPs 10 mM, 0.25 µl; G1-F 10pmol/µl, 1 µl; G2-R 10pmol/µl, 1 µl; and Taq DNA polymerase 5 U/µl, 0.2 µl. An extracted DNA sample of 5 µl is added to 45 µl PCR mix. The cycling parameters are 95 ºC for 3 min followed by 40 cycles of 94 ºC for 30 s, 60 ºC for 30 s and 72 ºC for 1 min, with a final extension step at 72 ºC for 5 min, and cooling at 15 ºC. The expected amplicon size is 187 bp.

The accuracy was 0.77 in the 2010 ring test using the Llop *et al.* (1999) DNA extraction procedure.

PCR according to Stöger* et al. *(2006)

The primers (from Llop *et al*., 2000) are:

PEANT1-F: 5′-TAT CCC TAA AAA CCT CAG TGC-3′

PEANT2-R: 5′-GCA ACC TTG TGC CCT TTA-3′

The targeted sequences are in the plasmid pEA29. Stöger *et al.* (2006) recommended this method be used with DNA extracted using the REDExtract-N-Amp Plant PCR Kit (Sigma-Aldrich[1]). The PCR mixture is composed of: ultrapure water, 5 µl; REDExtract-N-Amp PCR ReadyMix (Sigma-Aldrich[1]), 10 µl; PEANT1-F 10 pmol/µl, 0.5 µl; PEANT2-R 10 pmol/µl, 0.5 µl; and extracted DNA, 4 µl. The cycling parameters are 95 °C for 5 min followed by 35 cycles of 95 °C for 15 s, 58 °C for 30 s and 72 °C for 45 s, with a final extension step at 72 °C for 5 min, and cooling at 15 °C. The expected amplicon size is 391 bp.

The accuracy was 0.76 in the 2009 ring test and 0.72 in the 2010 ring test with the recommended DNA extraction kit.

PCR according to Gottsberger (2010) (adapted from Obradovic* et al. *(2007))

The primers are:

FER1-F: 5′-AGC AGC AAT TAA TGG CAA GTA TAG TCA-3′

rgER2-R: 5′-AAA AGA GAC ATC TGG ATT CAG ACA AT-3′

The targeted sequences are chromosomal. The PCR mixture is composed of: ultrapure water, 14.3 µl; buffer 10×, 2.5 µl; $MgCl_2$ 50 mM, 0.75 µl; dNTPs 10 mM, 0.25 µl; FER1-F 10 pmol/µl, 1 µl; rgER2-R 10 pmol/µl, 1 µl; Taq DNA polymerase 5 U/µl, 0.2 µl; and extracted DNA, 5 µl. The cycling parameters are 94 ºC for 3 min followed by 41 cycles of 94 ºC for 10 s, 60 ºC for 10 s and 72 ºC for 30 s, with a final extension step at 72 ºC for 5 min, and cooling at 15 ºC. The expected amplicon size is 458 bp.

The accuracy was 0.76 in the 2009 ring test and 0.68 in the 2010 ring test using the DNA extraction method described by Llop *et al.* (1999).

Nested PCR according to Llop* et al. *(2000)

The nested PCR of Llop *et al*. (2000) uses two sets of primers, which are combined in a single reaction tube. Because of the different annealing temperatures of the primers the two PCRs are run consecutively. The external primers are those designed by McManus and Jones (1995) and are based on sequences of the pEA29 plasmid. The internal primers are those described by Llop *et al*. (2000).

The external primers are:

AJ75-F: 5′-CGT ATT CAC GGC TTC GCA GAT-3′

AJ76-R: 5′-ACC CGC CAG GAT AGT CGC ATA-3′

The internal primers are:

PEANT1-F: 5′-TAT CCC TAA AAA CCT CAG TGC-3′

PEANT2-R: 5′-GCA ACC TTG TGC CCT TTA-3′

The PCR mixture is composed of: ultrapure water, 36.25 µl; buffer 10×, 5 µl; $MgCl_2$ 50 mM, 3 µl; dNTPs 10 mM, 0.5 µl; AJ75-F 0.1 pmol/µl, 0.32 µl; AJ76-R 0.1 pmol/µl, 0.32 µl; PEANT1-F 10 pmol/µl, 1 µl; PEANT2-R 10 pmol/µl, 1 µl; and Taq DNA polymerase 5 U/µl, 0.6 µl. A DNA sample volume of 2 µl should be added to 48 µl PCR mix. The cycling parameters are a denaturation step of 94 ºC for 4 min followed by 25 cycles of 94 ºC for 60 s and 72 ºC for 90 s. This first round PCR is followed in the same thermocycler by a second denaturation step of 94 ºC for 4 min and 40 cycles of 94 ºC for 60 s, 56 ºC for 60 s and 72 ºC for 60 s, with a final elongation step at 72 ºC for 10 min. The expected amplicon size is 391 bp, although variations in size can occur.

The accuracy was 0.69 and 0.72 in the 2003 and 2010 ring tests, respectively, but increased after enrichment to 0.84 (King's B medium) and 0.86 (CCT medium) in the 2003 ring test, and to 0.79 (King's B) and 0.88 (CCT) in 2010.

3.1.5.4 General considerations for PCR

The PCR protocols may need to be modified (optimized) when using different reagents or thermocyclers.

After PCR amplification the presence of *E. amylovora* can be confirmed by sequencing the PCR products or by restriction fragment length polymorphism (RFLP) analysis. The restriction pattern observed in the amplicons obtained with the primers of Bereswill *et al.* (1992) or with the nested PCR of Llop *et al.* (2000) can be used to confirm the specificity of the PCR analysis when compared with the restriction pattern of a known control strain. Restriction digestion should be performed with the endonucleases DraI and SmaI.

The test on a sample is negative if the E. amylovora-specific amplicon of the expected size (and the restriction enzyme pattern or amplicon sequence, when applicable) is not detected in the sample but is detected in all positive controls. The test on a sample is positive if the E. amylovora-specific amplicon of the expected size is detected, providing there is no amplification from any of the negative controls and the restriction enzyme pattern or amplicon sequence (when applicable) is indicative of E. amylovora.

3.1.5.5 Real-time PCR

Based on an evaluation of real-time PCR protocols in the ring tests in 2009 and 2010 (Dreo *et al.*, 2009; Lopez *et al.*, 2010) the protocol described by Pirc *et al.* (2009), which targets chromosomal sequences, was recommended. A duplex real-time PCR based on chromosomal sequences is also available but has not been ring tested (Lehman *et al.*, 2008).

Real-time PCR according to Pirc et al. (2009)

The following oligonucleotides are used:

Ams116F primer: 5′-TCC CAC ATA CTG TGA ATC ATC CA-3′

Ams189R primer: 5′-GGG TAT TTG CGC TAA TTT TAT TCG-3′

Ams141T probe: FAM-CCA GAA TCT GGC CCG CGT ATA CCG-TAMRA

The reaction is carried out in a final volume of 25 µl. The PCR mixture is composed of: ultrapure water, 2.5 µl; 2× TaqMan Fast Universal PCR Master Mix (Applied Biosystems[1]), 12.5 µl; Ams116F 10 pmol/µl, 2.25 µl; Ams189R 10 pmol/µl, 2.25 µl; FAM-labelled Ams141T 10 pmol/µl, 0.5 µl; and 5 µl DNA extract (added to the 20 µl PCR mix). The cycling parameters are: 2 min at 50 ºC; 10 min at 95 ºC; and 40 cycles of 15 s at 95 ºC and 1 min at 60 ºC. The standard mode for temperature ramping rates on analysers 7900HT and 7900HT Fast (Applied Biosystems[1]) are: 1.6 ºC/s up and 1.6 ºC/s down. It is possible to run reactions at slower ramp rates, but with faster ramp rates (up and down at approximately 3.5 ºC/s) the results were not acceptable. The expected amplicon size is 74 bp.

For analysis of the real-time PCR results, there are usually different options available, automatic or manual, for setting the signal and noise limits. The instructions for the appropriate software should be followed. The baseline should be set automatically, and the threshold should be set manually crossing the exponential phase of the control amplification curves.

The accuracy in the 2010 ring test was 0.80, 0.85 and 0.76 with the DNA extraction method of Llop *et al.* (1999), the REDExtract-N-Amp Plant PCR Kit (Sigma-Aldrich[1]) and Taylor *et al.* (2001), respectively.

Real-time PCR according to Gottsberger (2010)

The following oligonucleotides that target the *E. amylovora* chromosome are used:

hpEaF primer: 5′-CCG TGG AGA CCG ATC TTT TA-3′

hpEaR primer: 5′-AAG TTT CTC CGC CCT ACG AT-3′

hpEaP probe: FAM-TCG TCG AAT GCT GCC TCT CT-MGB

The reaction is carried out in a final volume of 20 µl. The PCR mixture is composed of: ultrapure water, 6 µl; 2× TaqMan Universal PCR Master Mix (Applied Biosystems[1]), 10 µl; hpEaF 10 pmol/µl, 1 µl; hpEaR 10 pmol/µl, 1 µl; hpEaP 1 pmol/µl, 1 µl; and 1 µl DNA extract (added to the 19 µl PCR mix). The cycling parameters are: 2 min at 50 °C; 10 min at 95 °C; and 50 cycles of 15 s at 95 °C and 1 min at 60°C. The expected amplicon size is 138 bp.

For analysis of the real-time PCR results, there are usually different options available, automatic or manual, for setting the signal and noise limits. The instructions for the appropriate software should be followed. The baseline should be set automatically, and the threshold should be set manually crossing the exponential phase of the control amplification curves.

The accuracy of this real-time PCR could not be tested in the 2010 ring test; however, it was tested in parallel with the real-time PCR of Pirc *et al.* (2009) by one laboratory and gave the same qualitative results with the DNA extraction from Llop *et al.* (1999).

3.1.5.6 Interpretation of results from PCR

Conventional PCR

The pathogen-specific PCR will be considered valid only if:

(1) the positive control produces the correct size amplicon for the bacterium

(2) no amplicons of the correct size for the bacterium are produced in the negative extraction control and the negative amplification control.

If the 16S rDNA internal control primers are also used, the negative (healthy plant tissue) control (if used), positive control and each of the test samples must produce a 1.6 kilobase (kb) amplicon (16S rDNA). Note that synthetic or plasmid-positive controls will not produce a 1.6 kb amplicon. Failure of the samples to amplify with the internal control primers suggests for example that the DNA extraction has failed, the nucleic acid has not been included in the reaction mixture, compounds inhibitory to PCR are present in the DNA extract or the DNA has degraded.

The test on a sample will be considered positive if it produces an amplicon of the correct size.

Real-time PCR

The real-time-PCR will be considered valid only if:

(1) the positive control produces an amplification curve with the pathogen-specific primers

(2) no amplification curve is produced (i.e. cycle threshold (Ct) value is 40) in the negative extraction control and the negative amplification control.

If the COX internal control primers are also used, the negative control (if used), positive control and each of the test samples must produce an amplification curve. Failure of the samples to produce an amplification curve with the internal control primers suggests for example that the DNA extraction has failed, the nucleic acid has not been included in the reaction mixture, compounds inhibitory to PCR are present in the DNA extract or the DNA has degraded.

The test on a sample will be considered positive if it produces a typical amplification curve in an exponential manner. The Ct value needs to be verified in each laboratory when implementing the test for the first time.

3.1.5.7 Loop-mediated isothermal amplification

The LAMP protocol was developed and described by Temple *et al.* (2008) and Temple and Johnson (2011). It was evaluated in the 2010 ring test because it was considered appropriate for laboratories not equipped for PCR and it is easy to perform. In the ring test, the LAMP protocol using primers to detect the chromosomal gene *amsL* of *E. amylovora* was found to lack appropriate sensitivity for analysis of samples with low bacterial populations. Consequently, the LAMP protocol described below to detect chromosomal *amsL* is recommended only for the analysis of symptomatic samples with more than 10^5–10^6 c.f.u./ml. The protocol from Temple and Johnson (2011) using primers to detect pEA29 was not evaluated in the ring test.

The LAMP primers to detect *amsL* Bare:

ALB Fip: 5′-CTG CCT GAG TAC GCA GCT GAT TGC ACG TTT TAC AGC TCG CT-3′

ALB Bip: 5′-TCG TCG GTA AAG TGA TGG GTG CCC AGC TTA AGG GGC TGA AG-3′

ALB F: 5′-GCC CAC ATT CGA ATT TGA CC-3′

ALB B: 5′-CGG TTA ATC ACC GGT GTC A-3′

Primers Fip and Bip were used at 2.4 µM and primers F and B at 0.2 µM final concentrations. Melting temperatures for primers were between 58 and 60 °C. The LAMP reaction mixture is composed of: 10× ThermoPol buffer (New England Biolabs[1]), 5 µl; dNTPs 10 mM, 5 µl; $MgSO_4$ 100 mM, 2 µl; bovine serum albumin (BSA) 10 mg/ml, 2 µl; ALB Fip 100 µM, 1.2 µl; ALB Bip 100 µM, 1.2 µl; ALB F 10 µM, 1 µl; ALB B 10 µM, 1 µl; *Bst* DNA polymerase 8 U/µl, 2 µl; template DNA, 5 µl; and ultrapure water, 24.6 µl. Note that the *Bst* DNA polymerase, template DNA and ultrapure water are not added to the master mix, but are added separately after aliquoting the master mix. Before starting the LAMP reaction, a water bath or a thermocycler is set at 65 °C. The mix is prepared and 18.4 µl is pipetted into each individual 0.2 ml PCR tube. The *Bst* DNA polymerase, template DNA and ultrapure water are then pipetted separately into each tube with master mix. The tubes are spun down in a plate spinner (1 000 r.p.m. for 30 s) and are placed in the water bath (65 °C) in a holder so the reaction end is submerged, or in the thermocycler (65 °C) for 55 min. The tubes are removed and allowed to cool for 10 s.

The test on a sample is positive if the presence of precipitate as cloudiness in the tube or the presence of a solid white magnesium pyrophosphate precipitate at the bottom of the tube is observed, as for the positive control. A clear solution indicates a negative test result, as should be observed for the negative control.

The accuracy in the 2010 ring test was 0.64, but for samples with 10^5–10^6 c.f.u./ml the accuracy was 0.80. For this reason LAMP is recommended only for the analysis of symptomatic samples.

3.2 Detection in asymptomatic plants

The recommended screening tests are indicated in the flow diagram in Figure 2.

3.2.1 Sampling and sample preparation

Asymptomatic samples can be processed individually (preferred) or in groups of up to 100 (EPPO, 2013). Precautions to avoid cross-contamination should be taken when collecting the samples and during the extraction process. Sampling and sample preparation can be performed following one of the following protocols:

- Blossoms, shoots, fruitlets or stem segments are collected in sterile bags or containers in summer or early autumn, after favourable conditions for the multiplication of *E. amylovora* have occurred and when average temperatures rise above about 15 ºC (van der Zwet and Beer, 1995). Young shoots approximately 20 cm in length, or blossoms when available, are cut from the suspect plant. If analyses need to be performed in winter, five to ten buds are collected per plant. In the laboratory, blossoms when available, the peduncle and base of the limb of several leaves from the base of the shoots, or the stem segments are cut from the selected plants. About 0.1–1.0 g plant material is weighed and macerated in antioxidant buffer following the protocol described in section 3.1.2.
- A sampling procedure reported but not validated for the analysis of twigs of asymptomatic woody material from nurseries is as follows. A sample comprises 100 twigs, each about 10 cm in length, from 100 plants. If there are several plant genera in the lot, these should be represented equally in the sample (with a maximum of three genera per sample). From each sample 30 twigs are randomly taken and each twig is cut into four pieces (producing 120 stem pieces). The samples are covered with sterile PBS containing 0.1% Tween 20 in Erlenmeyer flasks, and the flasks are stirred vigorously on a rotary shaker for 1.5 h at room temperature. The extract is filtered through filter paper held in a sintered glass filter using a vacuum pump, and the filtrate is collected. The filtrate is used directly for analysis or centrifuged at 10 000 *g* for 20 min. The pellet is suspended in 4.5 ml sterile PBS. The detection techniques indicated below are performed. A similar protocol can be applied for leaves, shoots, flowers and buds.

Depending on the timing of the sampling, the expected recovery of *E. amylovora* will vary, with maximum recovery in summer (providing weather conditions are favourable to *E. amylovora*) and reduced recovery in winter. Samples should be processed immediately by performing enrichment

followed by DASI-ELISA, PCR and isolation using the protocols described for each technique for symptomatic samples in López *et al.* (2006). Immunofluorescence is optional; if done, it must be done directly on the extracts, before enrichment.

3.2.2 Screening tests

Direct analysis of asymptomatic samples is normally negative for *E. amylovora* because of the low bacterial population. Consequently, when analysing asymptomatic material, it is an absolute requirement to perform enrichment from samples prepared in the antioxidant buffer (section 3.2.1) (Gorris *et al.*, 1996) for 72 h at approximately 25 ºC. It is advisable to perform at least two of these screening tests based on different biological principles:

- Enrichment-isolation. Follow the procedure for symptomatic samples (section 3.1.3.2).
- Enrichment-DASI-ELISA. Follow the procedure for symptomatic samples (section 3.1.4.1).
- Enrichment-PCR or enrichment-real-time PCR. Use 500–1000 µl of the samples enriched in King's B and/or CCT media for DNA extraction, then follow the procedure for amplification according to Taylor *et al.* (2001) or Llop *et al.* (2000) (section 3.1.5.3) or the real-time PCR protocols (section 3.1.5.5).

If any of the screening tests are positive but isolation is negative, isolation of the pathogen from the extract stored at –80 ºC with glycerol or from the enriched samples should be attempted. When three tests or more are positive and the isolation is negative, it is reasonable to strongly suspect the presence of *E. amylovora* in the sample, but identification and confirmation require isolation of the pathogen from new samples and subsequent identification of the bacterium.

4. Identification

Identification should be based on results obtained from several techniques because other species of *Erwinia* such as *E. piriflorinigrans* (López *et al.*, 2011), *E. pyrifoliae* (Kim *et al.*, 1999; Rhim *et al.*, 1999), *E. uzenensis* (Matsuura et al., 2012) and other *Erwinia* spp. (Kim *et al.*, 2001a, 2001b; Palacio-Bielsa *et al.*, 2012) share similar morphological, serological and molecular characteristics to that of *E. amylovora*. Differentiation of *E. amylovora* from these closely related *Erwinia* species (that can be found in similarly symptomatic tissues in some hosts) can be achieved with a combination of three techniques based on different biological principles:

- PCR based on chromosomal DNA (sections 3.1.5.2 and 4.3.1)
- DASI-ELISA using specific monoclonal antibodies as described for detection (section 3.1.4.1, excluding the enrichment step)
- Inoculation into fire blight hosts to fulfil the requirements of Koch's postulates, including re-isolation of the inoculated pathogen (section 4.4).

For identification of colonies, at least two of these three techniques are recommended to be used. Other tests can also be used depending on the experience of the laboratory; these are described below. When required, the final confirmation of a culture's identification should include a pathogenicity test.

The *E. amylovora* isolates recommended for use as positive controls are NCPPB 683 and CFBP 1430. The following collections, among others, can provide different *E. amylovora* reference strains: National Collection of Plant Pathogenic Bacteria (NCPPB), Fera, York, United Kingdom; Collection Française de Bactéries Phytopathogènes (CFBP), French National Institute for Agricultural Research (INRA), Station Phytobactériologie, Angers, France; Belgian Co-ordinated Collection of Micro-organisms BCCM/LMG Bacteria Collection, Ghent, Belgium; International Collection of Microorganisms from Plants (ICMP), Manaaki Whenua Landcare Research, Auckland, New Zealand; and American Type Culture Collection (ATTC), Manassas, VA, United States. The authenticity of the strains can be guaranteed only if directly obtained from the culture collections.

4.1 Nutritional and enzymatic identification

Key phenotypic tests are useful and are still used for identification, but it is advised to combine them with pathogenicity assays and a serological or molecular test. Members of the genus *Erwinia* are defined as Gram-negative, facultative anaerobes, motile by peritrichous flagella, rod-shaped, and able to produce acid from glucose, fructose, galactose and sucrose. Key phenotypic properties (Paulin, 2000) that are common to most strains in *E. amylovora*, according to the methods of Jones and Geider (2001), are: oxidase test (–), oxidative/fermentative (O/F) test (+/+), fluorescent pigment in King's B medium under UV light (–), levan production (+), nitrate reduction (–), citrate utilization (+), gelatine liquefaction (+), urease and indol (–) and colony morphology on CCT medium.

The following tests differentiate *E. amylovora* from *E. pyrifoliae* and *E. piriflorinigrans*, although some physiological and biochemical characteristics may vary for some strains (Table 1).

Table 1. Differences among *Erwinia amylovora*, *Erwinia pyrifoliae* and *Erwinia piriflorinigrans*

Microbiological test	*Erwinia amylovora*	*Erwinia pyrifoliae*	*Erwinia piriflorinigrans*
Gelatin hydrolysis	+	–	–
Inositol†	–	ND	+
Sorbitol†	+	+	–
Aesculin†	V	–	+
Melibiose†	–	–	+
D-Raffinose†	–	–	+
β-Gentiobiose†	+	–	+
Amplification with‡ EP16A/EPI62C CPS1/CPS2C	–	+	ND

† From Roselló *et al.* (2006) and López *et al.* (2011). Oxidation of substrates in API 50 CH strips (bioMérieux) using the method described by López *et al.* (2011). More than 90% of strains give the results indicated.

‡ According to Kim *et al.* (2001b).

ND, not determined; V, variable.

4.1.1 Biochemical characterization

4.1.1.1 Nutritional and enzymatic profiling

Identification of *E. amylovora* can be obtained biochemically by profiling on the API system 20 E and 50 CH strips (bioMérieux[1]).

API 20 E[1]. The manufacturer's instructions should be followed for preparing the suspension and inoculating the strip. The strip is incubated at 25–26 ºC. The reading after 48 h for a typical *E. amylovora* culture should be as follows: the tests lysine decarboxylase (LDC), ornithine decarboxylase (ODC), citrate utilization (CIT), H_2S production (SH_2), urease (URE), tryptophan deaminase (TDA), indole production (IND) and rhamnose oxidation (RHA) should be negative, while sucrose oxidation (SAC) should be positive. Other tests may vary by strain, according to Donat *et al.* (2007).

API 50 CH[1]. A suspension of OD 1.0 (at 600 nm wavelength) is prepared in PBS. One millilitre of the suspension is added to 20 ml Ayers medium ($NH_4H_2PO_4$, 1 g; KCl, 0.2 g; $MgSO_4$, 0.2 g; bromothymol blue 0.2%, 75 ml; distilled water, 1 litre; pH 7; sterilized at 120 ºC for 20 min) (Ayers *et al.*, 1919). The manufacturer's instructions should be followed for inoculating the strip. The strip is incubated at 25–26 ºC under aerobic conditions. Utilization of the different carbohydrates is observed by the

development of a yellow colour in the well. The reading after 72 h for a typical *E. amylovora* culture should be positive for L-arabinose, ribose, D-glucose, D-fructose, mannitol, sorbitol, N-acetylglucosamine, sucrose, trehalose and β-gentiobiose. The remaining sugars are not utilized by *E. amylovora* in these conditions, but some strains can utilize glycerol and D-fucose, according to Donat *et al.* (2007).

4.1.1.2 Automated identification

An automated identification system based on differential results of 94 phenotypic tests in a microtiter plate and accompanying analysis software are commercially available (OmniLog[1], Biolog[1]). The manufacturers' instructions should be followed for presumptive identification of suspected *E. amylovora* isolates.

4.1.1.3 Fatty acid profiling

In fatty acid profiling (FAP), levan-positive, non-fluorescent colonies are grown on commercially available trypticase soy agar at 28 ºC for 48 h (Sasser, 1990). An appropriate fatty acid extraction procedure is applied and the extract is analysed using the commercially available Sherlock Microbial Identification System (MIS) (MIDI[1]) or other appropriate software for presumptive identification of *E. amylovora*, according to Wells *et al.* (1994).

4.2 Serological identification

4.2.1 Agglutination

Suspected *E. amylovora* colonies can be presumptively identified by slide agglutination. A dense suspension of cells is mixed with a drop of PBS and a drop of *E. amylovora* specific antiserum (undiluted, or at 1:5 to 1:10 dilution only) on a slide. Monoclonal antibodies can be used providing they agglutinate the reference strains. The specificity of the antibodies must be established in advance.

4.2.2 Immunofluorescence

A suspension of approximately 10^6 cells/ml is prepared in PBS from levan-positive, non-fluorescent colonies and the immunofluorescence procedure described in section 3.1.4.3 is followed. The specificity of the antibodies must be established in advance.

4.2.3 ELISA

Direct tissue print-ELISA (section 3.1.4.2), DASI-ELISA (section 3.1.4.1) and indirect ELISA (see below) for isolate identification can be performed using specific monoclonal antibodies as described for detection. A mixture of monoclonal antibodies has been validated in two ring tests for DASI-ELISA. A suspension of approximately 10^8 cells/ml is prepared in PBS from suspected colonies. The DASI-ELISA procedure in section 3.1.4.1 can be used, but without the enrichment step.

Indirect ELISA

Pure cultures of the suspected isolates are treated at 100 ºC for 10 min in a water bath or on a heating block to reduce non-specific reactions with commercial monoclonal antibodies. Aliquots of 200 µl culture are mixed with an equal volume of carbonate buffer (Na_2CO_3, 1.59 g; $NaHCO_3$, 2.93 g; distilled water, 1 litre; pH 9.6) and this solution is applied to at least two wells of a microtiter plate. The plate is incubated at 37 ºC for 1 h or at 4 ºC overnight. Extracts are flicked out from the wells and the plate is washed three times with washing buffer (see the DASI-ELISA protocol). The specific commercial anti-*E. amylovora* antibodies from Plant Print Diagnòstics SL[1] are prepared at the recommended dilutions. To each well is added 200 µl of the diluted anti-*E. amylovora* antibody solution and the plate is incubated at 37 ºC for 1 h. The antibody solution is flicked out from the wells and the wells are washed as before. The appropriate dilution of secondary antibody-alkaline phosphatase conjugate (GAM-AP) is prepared in PBS containing 0.5% BSA. To each well is added 200 µl of the diluted conjugate antibody and the plate is incubated at 37 ºC for 1 h. The conjugated

antibody is flicked out from the wells and the wells are washed as before. A 1 mg/ml alkaline phosphatase substrate (p-nitrophenylphosphate) is prepared in substrate buffer (diethanol amine, 97 ml; 800 ml distilled water; adjusted to pH 9.8 with concentrated HCl; then the volume is adjusted to 1 000 ml with distilled water). To each well is added 200 µl alkaline phosphatase substrate solution. The plate is incubated in the dark at room temperature and read at 405 nm at regular intervals within 90 min. A positive test is indicated by substrate conversion to a yellow colour.

4.2.4 Lateral flow immunoassay

A suspension of 10^7 c.f.u./ml of the pure culture is prepared for presumptive identification. Buffers and procedures provided by the manufacturers of the kits are used, as described in section 3.1.4.4.

4.3 Molecular identification

4.3.1 PCR

A suspension of approximately 10^6 cells/ml is prepared in molecular grade sterile water from purified levan-positive, non-fluorescent colonies and is treated at 100 ºC for 10 min. The appropriate PCR procedures or the LAMP protocol are applied as described in sections 3.1.5.2 to 3.1.5.4 (directly, without DNA extraction). When using PCR to identify isolated colonies, 1 U of Taq DNA polymerase should be used (instead of 2 U as for plant material).

4.3.2 Macro-restriction and pulsed field gel electrophoresis

Pulsed field gel electrophoresis (PFGE) analysis of genomic DNA after *Xba*I digestion according to Jock *et al.* (2002) shows six patterns for *E. amylovora* European strains. The method can provide useful information for strain differentiation and has been applied to understanding the spread of fire blight in Europe (Jock *et al.*, 2002; Donat *et al.*, 2007).

4.4 Pathogenicity techniques

Suspected *E. amylovora* colonies should be inoculated back into host plants to fulfil Koch's postulates and verify their pathogenicity. For plant inoculation, susceptible cultivars of pear (e.g. Conference, Doyenne du Comice, Williams, Passa Crassane), apple (e.g. Fuji, Gala, Idared, Jonathan), loquat (e.g. Algerie, Tanaka), *Crataegus* spp., *Cotoneaster* spp. or *Pyracantha* spp. are used. Young shoots are inoculated by cutting across a young leaf through the central vein with scissors dipped in a 10^9 c.f.u./ml suspension of each isolate prepared in PBS. The plants are maintained at 20–25 ºC at approximately 80% relative humidity for one to two weeks. Detached young shoots that have been surface-sterilized (treated with 70% ethanol for 30 s then washed three times with sterile distilled water) from greenhouse-grown plants can also be inoculated in the same way and kept in tubes with sterile 1% agar. The tubes should be kept at 20–25 ºC with 16 h light per day.

Inoculation can also be performed on detached immature fruits of susceptible cultivars of pear, apple and loquat by placing 10 µl of 10^9 c.f.u./ml suspensions of the isolates in PBS into a fresh wound on the surface of disinfected fruits (treated with 70% commercial chlorine for 30 min then washed three times with sterile distilled water). The fruits should be incubated in a humid chamber at 25 ºC for three to five days.

E. amylovora-like colonies are re-isolated and characterized from inoculated organs showing typical fire blight symptoms. A positive test is evident by the oozing of bacteria and browning around the inoculation site after two to seven days, as seen in the positive *E. amylovora* control, providing no lesions are or only a small necrotic lesion is observed at the wound site in the negative control.

Other inoculation techniques are possible. Hypersensitive reactions in tobacco leaves may indicate expression of the *hrp* genes of *E. amylovora*, but this test may be positive for many other plant pathogenic bacteria. Tobacco plants of cultivars Xanthi or Samsun with more than five to six leaves should be used. Bacterial suspensions of 10^9 c.f.u./ml (OD at 600 nm, 1.0) are prepared and a needle and syringe used to inject the suspensions into the intracellular space of mature leaves. Complete

collapse of the infiltrated tissue after 24–48 h at room temperature is recorded as positive, as observed in the positive *E. amylovora* control.

5. Records

Records and evidence should be retained as described in section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*).

In cases where other contracting parties may be affected by the results of the diagnosis, in particular in cases of non-compliance (ISPM 13 (*Guidelines for the notification of non-compliance and emergency action*) and where the pest is found in an area for the first time, the following records and evidence and additional material should be kept for at least one year in a manner that ensures traceability: the original sample, culture(s) of the pest, preserved or slide-mounted specimens or test materials (e.g. photographs of gels, ELISA plate results printouts and PCR amplicons).

6. Contact Points for Further Information

Further information on this protocol can be obtained from:

Centro de Protección Vegetal, Instituto Valenciano de Investigaciones Agrarias (IVIA), Carretera Moncada-Náquera km 4.5, 46113 Moncada (Valencia), Spain (María M. López; e-mail: mlopez@ivia.es; tel.: +34 963424000; fax +34 963424001).

Plant Health and Environment Laboratory, Investigation and Diagnostic Centres, Ministry for Primary Industries, 231 Morrin Road, St Johns, Auckland 1140, New Zealand (Robert Taylor; e-mail: Robert.Taylor@mpi.govt.nz; tel.: +64 99093548; fax: +64 99095739).

A request for a revision to a diagnostic protocol may be submitted by NPPOs, regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will in turn forward it to the Technical Panel on Diagnostic Protocols (TPDP).

7. Acknowledgements

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Most techniques described were ring tested in a DIAGPRO project financed by the European Union in 2003, in an EUPHRESCO project in 2009 and in a Spanish project in 2010.

8. References

The present annex refers to ISPMs. ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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9. Figures

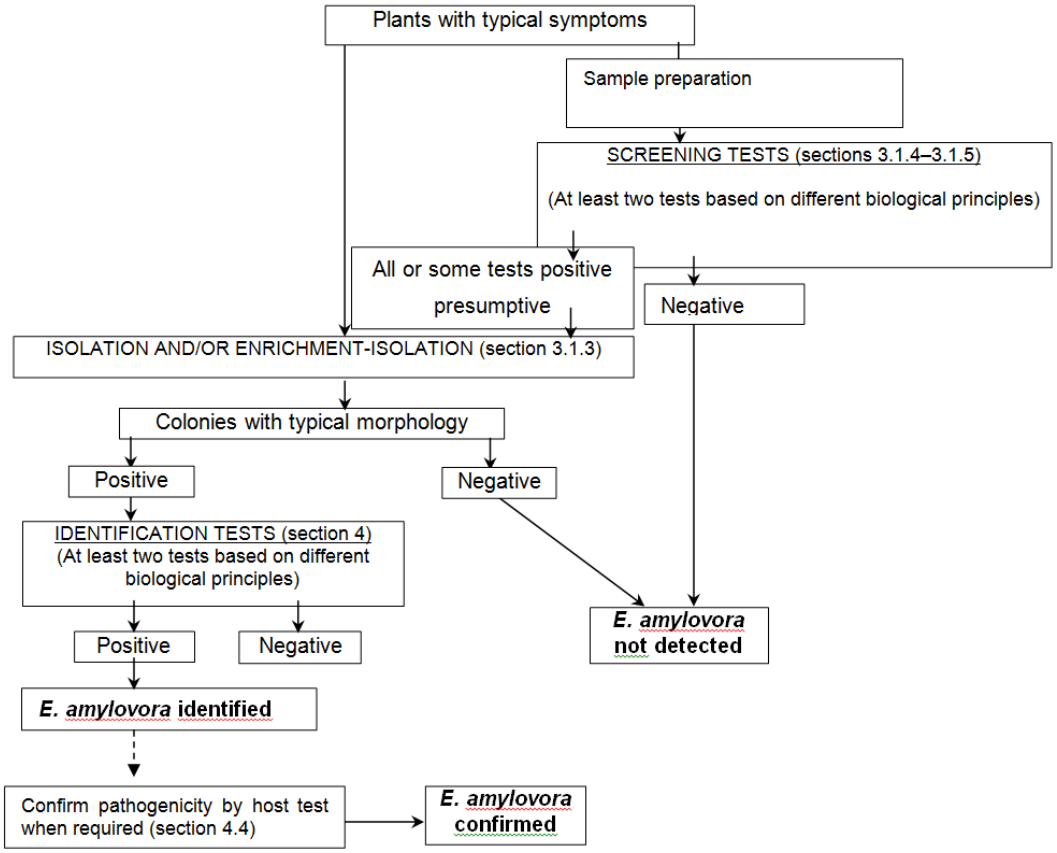

Figure 1. Flow chart for the identification of *Erwinia amylovora* in samples showing symptoms of fire blight.

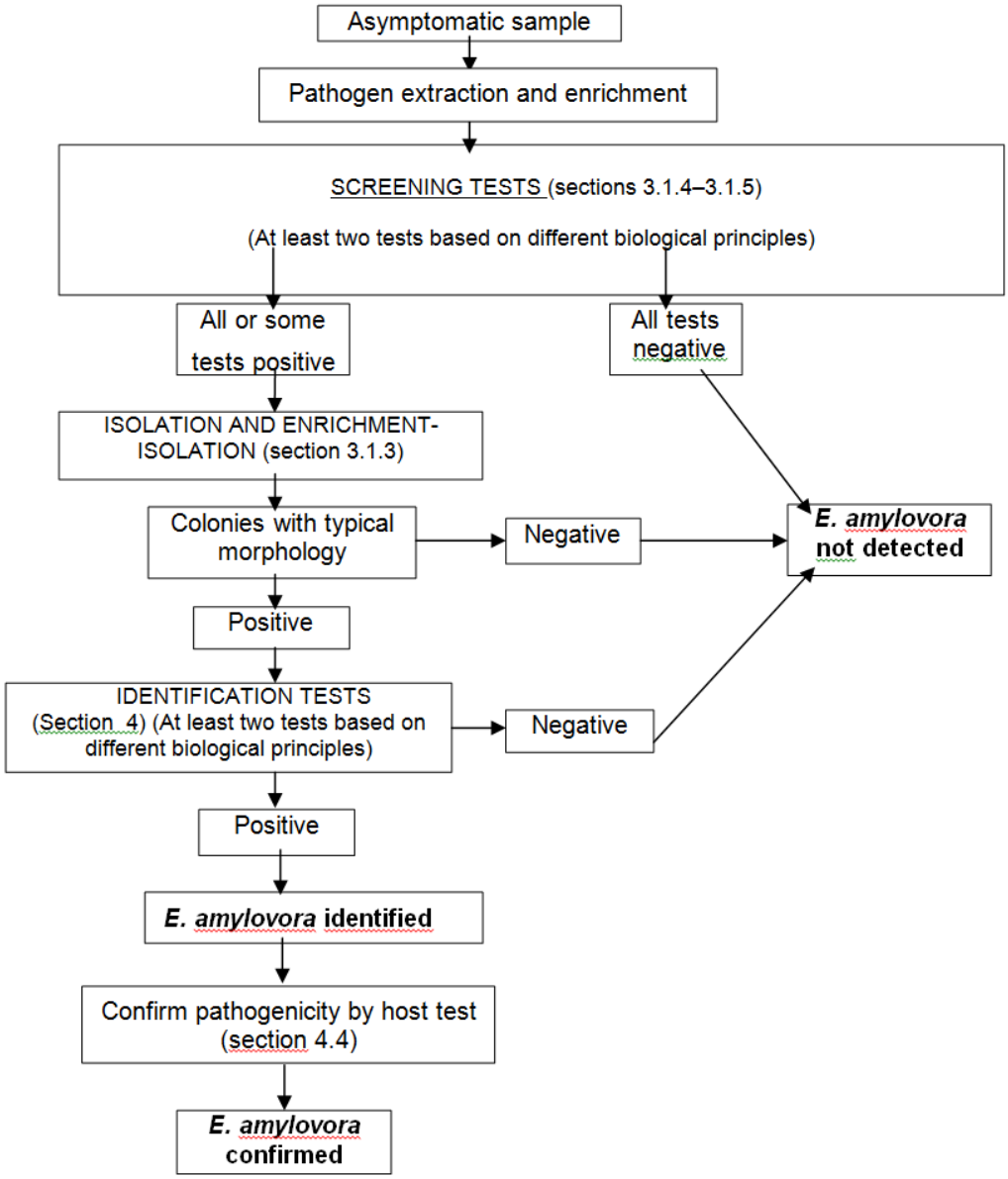

Figure 2. Flow chart for the identification of *Erwinia amylovora* in asymptomatic samples.
* It is reasonable to strongly suspect the presence of *E. amylovora* in the sample, but identification requires isolation of the pathogen from new samples and subsequent identification of the bacterium.

Publication history

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2015-12 DP drafting group reviewing draft DP and responses to member comments.

2016-03 TPDP e-decision for approval for adoption (2016_eTPDP_Mar_01).

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2016-07 DP notification period.

2016-08 SC adopted DP on behalf of CPM (with no objections received).

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Publication history last updated: 2016-10.

This diagnostic protocol was adopted by the Standards Committee on behalf of the Commission on Phytosanitary Measures in August 2016.
The annex is a prescriptive part of ISPM 27.

ISPM 27
Diagnostic protocols for regulated pests

DP 14: *Xanthomonas fragariae*

Adopted 2016; published 2016

CONTENTS

1. Pest Information

Xanthomonas fragariae Kennedy and King, 1962 is the causal agent of bacterial angular leaf spot disease of strawberry. The disease is prevalent mainly in North America and was first reported in the United States in 1962 (Kennedy and King, 1962; Hildebrand *et al*., 1967; Maas *et al*., 1995), but it has been subsequently reported in many strawberry growing areas around the world, including South America and Europe (CABI). *Fragaria* × *ananassa*, the predominant cultivated strawberry, is the primary host of *X. fragariae*. Commercial cultivars vary in susceptibility, and other *Fragaria* species, including *F. chiloensis*, *F. virginiana* and *F. vesca*, as well as *Potentilla fruticosa* and *P. glandulosa*, are also susceptible. Among *Fragaria* species only *F. moschata* is immune (Kennedy and King, 1962; Kennedy, 1965; Maas, 1998).

X. fragariae is readily transmitted via asymptomatic planting stock with latent infection. Inoculum sources for primary infection are infected but visually asymptomatic daughter plants that develop on runners from infected nursery plants and that are used for planting in fruit production fields. Although *X. fragariae* is not free-living in the soil, it can overwinter in the soil in association with previously infected plant material and persist there for long periods of time (Maas, 1998). Residues of infected leaves and crown infections on runners used for planting are also sources of inoculum for primary infection.

Analyses of *X. fragariae* strains isolated at different times in diverse locations around the world indicate some genetic and phenotypic diversity among these strains (Opgenorth *et al.*, 1996; Pooler *et al*., 1996; Roberts *et al*., 1996). In addition, some differential pathogenicity has been noted among *X. fragariae* strains (Maas *et al*., 2000). Nevertheless, there is a high degree of similarity among pathogenic strains of this phytopathogen, and there has been no correlation between genotypes or phenotypes and geographic origin of the strains. Currently known *X. fragariae* strains around the world are thus likely to represent a clonal population. Early detection of *X. fragariae* in infected but asymptomatic strawberry planting stock is critical for avoiding dissemination of the pathogen and disease development.

2. Taxonomic Information

Name: *Xanthomonas fragariae* Kennedy and King, 1962

Synonyms: None

Taxonomic position: Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae

Common names: Bacterial angular leaf spot

Note: *Xanthomonas fragariae* Kennedy and King, 1962 is a member of the gamma subdivision of the Proteobacteria (Stackebrandt *et al.*, 1988), Phenon 3 of Van den Mooter and Swings (1990), DNA-DNA homology Group 1 of Rademaker *et al.* (2000) and DNA Group 1 of Rademaker *et al.* (2005).

3. Detection

Diagnosis of bacterial angular leaf spot disease of strawberry caused by *X. fragariae* is based on inspection for diagnostic symptoms, direct or indirect isolation of the pathogen, serological tests (e.g. indirect immunofluorescence, enzyme-linked immunosorbent assay (ELISA)) and molecular methods. Several polymerase chain reaction (PCR) detection tests, each targeting different loci in the *X. fragariae* genome, have been developed (Roberts *et al.*, 1996; Zimmerman *et al.*, 2004; Weller *et al.*, 2007; Vandroemme *et al.*, 2008; Turechek *et al.*, 2008; Vermunt and van Beuningen, 2008). These tests can be used to confirm the presence of *X. fragariae* in symptomatic plant material, and several of them have also been used for the detection of latent *X. fragariae* infection (Mahuku and Goodwin, 1997; Zimmerman *et al.*, 2004; Moltman and Zimmerman, 2005). A detached leaf assay (Civerolo *et al.*, 1997a) is useful for presumptive diagnosis of *X. fragariae* in cases where direct isolation is very slow or inhibited. The methods described in this diagnostic protocol, with the exception of the nested PCR,

have been validated in a test performance study funded by the European Union (SMT-4-CT98-2252) (López *et al.*, 2005).

Direct isolation of *X. fragariae* is difficult, even in the presence of characteristic symptoms and bacterial exudates, because the bacterium grows very slowly on artificial nutrient media and is readily overgrown by saprophytic bacteria (Hazel and Civerolo 1980; López, *et al.*, 1985; Schaad *et al.*, 2001; Saddler and Bradbury, 2005). Specific procedures for direct isolation of *X. fragariae* are given in López *et al.* (2005). Selective enrichment of the pathogen *in planta* by inoculating detached strawberry leaves with aqueous extracts of diseased or suspected infected tissue can facilitate isolation of *X. fragariae in vitro* (Civerolo *et al*., 1997a).

Procedures for the detection of *X. fragariae* in symptomatic and asymptomatic plants are presented below.

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

3.1 Symptoms

Small (1–4 mm diameter) angular water-soaked spots (lesions) bounded by the smallest leaf veins appear initially on the lower leaf surface. In the early stages of infection, these spots are barely visible in the field and appear translucent yellow when viewed under transmitted light. The lesions enlarge and coalesce, eventually appearing on the upper leaf surface as angular water-soaked spots that become reddish brown (Figure 1). Viscous bacterial exudates that are white, milky, cream or yellow in colour develop from lesions under wet conditions or when the relative humidity is high (Figure 2). The exudates become dry scale-like masses that are opaque and whitish or silvery at first, then turn brown (Janse, 2005). As the disease progresses, coalesced reddish-brown lesions become necrotic. Necrotic lesion tissue may tear or break off the leaf, and diseased leaves may appear blighted or ragged. Leaf infections often develop and form long lesions along major veins. In advanced stages of disease development, the foliar tissue around old coalesced reddish-brown lesions is generally chlorotic (Kennedy and King, 1962; EPPO, 1997; Rat, 1993; Maas, 1998).

In contrast to angular leaf spot disease of strawberry, bacterial leaf blight of strawberry caused by *X. arboricola* pv. *fragariae* is characterized by small reddish-brown lesions on the lower leaf surface that are neither water-soaked nor translucent; reddish spots on the upper leaf surface; lesions coalescing into large, dry brown spots surrounded by a chlorotic halo; and large brown V-shaped lesions along the leaf margin, midrib and major veins (Janse *et al*., 2001). Also, no bacterial exudation is associated with bacterial leaf blight lesions (Janse *et al*., 2001). In advanced stages, bacterial angular leaf spot is difficult to distinguish from fungal leaf-spotting diseases such as common leaf spot (*Mycosphaerella fragariae*) and leaf scorch (*Diplocarpon earliana*) (Janse *et al.*, 2001).

Severe infections of *X. fragariae* may spread from the leaves to the crown, where discrete water-soaked areas develop (Hildebrand *et al*., 1967). Severe crown infection can result in plants with decreased vigour that may collapse and eventually die. Leaves that develop from infected crowns are often systemically infected, with lesions that appear along the veins at the base of the leaves. Bacterial exudate may ooze from vascular bundles when the crown is cut transversely.

In severe cases of disease, *X. fragariae* may attack flowers and cause blossom blight, but it does not directly infect fruits (Gubler *et al.*, 1999). Water-soaked lesions on infected calyx tissue are similar in appearance to foliar lesions (Figure 3). Fruit tissue near severely infected calyx tissue may also become water-soaked.

X. fragariae can move systemically into the roots, crowns and runners without exhibiting obvious symptoms (Stefani *et al.*, 1989; Milholland *et al.*, 1996; Mahuku and Goodwin, 1997). This infection

may result in the appearance of water-soaked areas at the base of newly emerged leaves followed shortly by sudden plant collapse and death. This type of infection is not usually seen.

3.2 Sampling

For plants with symptoms, leaves with initial water-soaked spots are preferred as samples for the diagnosis of bacterial angular leaf spot as they facilitate successful isolation of *X. fragariae*. Alternatively, leaves with dry spots and with or without exudates can be used. Crown tissue should also be examined.

X. fragariae is a very slow growing bacterium and plating and serological tests are not suitable for detecting small numbers of bacteria in symptomless plants. For symptomless plants, it is recommended that several entire plants be selected and small amounts of tissue be excised from their leaves, petioles and crowns (EPPO, 2006). These tissues can be used directly for PCR-based analyses, as described in section 3.9.

Samples should not be left in a wet condition after collection. Preferably, samples should be partially dried, wrapped in paper, placed in polythene bags and kept cool. Samples should be transported in a well-insulated container, stored at 4 °C upon arrival at their destination and processed as soon as possible.

3.3 Sample preparation

For symptomatic plants, the surfaces of leaf and stem plant tissue can be disinfested by wiping with 70% ethanol. If the plants show vascular symptoms, it is recommended that the roots and the leaves are removed, keeping the crown and petioles. Rinse the sample in tap water to remove excess soil and then disinfest by immersing for 1 min in 70% ethanol followed by rinsing three times in sterile distilled water. Add approximately 0.1 g of leaf or crown and petiole tissue per sample to 9 ml phosphate-buffered saline (PBS) (8 g NaCl, 0.2 g KCl, 2.9 g $Na_2HPO_4 \cdot 12H_2O$, 0.2 g KH_2PO_4, distilled water to 1 litre; pH 7.2). Homogenize the plant tissue and incubate it at room temperature for 15 min.

For asymptomatic plants, collect a 30 g sample at random, place it in 150 ml PBS and shake it for 30 min. Either use the washing liquid directly for detection, or centrifuge it at 10 000 g for 10 min then resuspend the pellet in sterile distilled water to obtain a final volume of 5 ml. Leave it to settle for 15 min then collect the upper clarified part and prepare dilutions (1:10 and 1:100) in sterile distilled water (EPPO, 2006). These sample tissue macerates are then used in ELISA, immunofluorescence and PCR.

3.4 Rapid screening tests

Rapid screening tests facilitate the detection of *X. fragariae*. As the bacterium is very difficult to isolate, three tests (ELISA, immunofluorescence and PCR) should be positive to confirm *X. fragariae* detection. The detached leaf assay is a supplemental test for confirming the presence of viable *X. fragariae*. The correlation among ELISA, PCR and detached leaf assay is usually high (Civerolo *et al.*, 1997b).

3.5 Isolation

Direct isolation of *X. fragariae* is difficult, even in the presence of symptoms and exudates, because *X. fragariae* grows very slowly on artificial nutrient media and is rapidly overgrown by saprophytic organisms. Two media are recommended for isolation. Isolation is more successful on Wilbrink's medium with nitrate (Wilbrink-N) (10 g sucrose, 5 g proteose peptone (L85; Oxoid[1]), 0.5 g K_2HPO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.25 g $NaNO_3$, 15 g purified agar, distilled water to 1 litre; pH 7.0–7.2) (Koike, 1965). Isolation on YPGA medium (5 g yeast extract, 5 g Bacto[1] peptone, 10 g glucose, 15 g purified

[1] In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

agar, distilled water to 1 litre; adjust pH to 7.0–7.2; add 5 ml filter-sterilized cycloheximide (stock solution: 5 g cycloheximide per 100 ml absolute ethanol) after autoclaving) is less successful but still recommended. A third medium, SPA (20 g sucrose, 5 g Bacto[1] peptone, 0.5 g K_2HPO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 15 g purified agar, distilled water to 1 litre; pH 7.2–7.4), may be useful for fastidious bacteria (Hayward, 1960). The use of purified agar (Oxoid[1] or Difco[1]) is recommended for all media as impurities in other commercial agars can inhibit the growth of *X. fragariae*.

3.5.1 Isolation method 1

For plants with symptoms, select leaves with initial lesions and disinfest the surface by wiping it with 70% ethanol. Isolations should be made from initial water-soaked lesions or from the margins of older lesions by excising a small piece of tissue (0.5–1.0 cm^2) with a sharp sterile scalpel.

Homogenize the tissue in a few millilitres of sterile distilled water or PBS and incubate it at room temperature (20–25 °C) for 10–15 min. Plate out aliquots (50–100 µl) of lesion tissue macerates as well as dilutions (1:10, 1:100, 1:1 000 and 1:10 000) onto the surface of Wilbrink-N, YPGA and/or SPA media. Similar aliquots of *X. fragariae* cell suspensions (10^4, 10^5 and 10^6 colony-forming units (cfu)/ml should also be plated out in order to verify the quality of the media and to compare the cultural characteristics of any bacterial colonies that develop. Incubate the plates at 25–27 °C for seven days, but mark the colonies appearing after two to three days as these will not be *X. fragariae*. Perform final readings after seven to ten days of incubation at 25–27 °C.

*X. fragari*ae colonies on Wilbrink-N medium are initially off-white, becoming pale yellow, circular, slightly convex, smooth and mucoid after four to six days. On YPGA and SPA media, the colonies are similar in morphology to those on Wilbrink-N, but they have a more intense yellow colour.

3.5.2 Isolation method 2

Excise pieces of leaf tissue with distinct water-soaked angular lesions and wash them in 50 ml tap water and a few drops of Tween 20. Incubate the leaf pieces at room temperature for 10 min, then rinse them in distilled water and blot dry. The surfaces of the leaf pieces can be disinfested in 70% ethanol for 5 s and blot-dried. Cut the leaf pieces into smaller pieces (1–4 mm^2) and place them in 5 ml of 0.1 M PBS. Mix and incubate at room temperature for 30 min to release any *X. fragariae* into the supernatant. Prepare a 1:100 dilution of the supernatant in 0.1 M PBS and add 20 µl aliquots of the undiluted sample and 1:100 dilution to separate wells of a multiwell microscope slide. Fix the bacterial cells to the slide by flaming for later immunofluorescence analysis (section 3.8). Place 200 µl undiluted supernatant in a microtube for later PCR analysis (section 3.9) and another 1 ml undiluted supernatant in a second microtube, adding glycerol to obtain a final concentration of at least 20 %, and store it at –20 °C or –80 °C for reference purposes. The remaining supernatant can be used for isolation by dilution plating as described above and for inoculation of detached strawberry leaves (section 3.6).

In addition to isolation methods 1 and 2 described above, isolation of *X. fragariae* from tissue may be performed from aliquots of fresh exudates from lesions directly onto Wilbrink-N, YPGA, SPA or other commonly used media.

3.5.3 Interpretation of isolation results

The isolation is negative if no bacterial colonies with morphology characteristic of *X. fragariae* colonies are observed after seven days on any of the three media (provided no growth inhibition due to competition or antagonism has occurred) and typical *X. fragariae* colonies are found in the positive controls.

The isolation is positive if presumptive *X. fragariae* colonies are isolated on at least one of the media used.

Considering that isolation of this bacterium frequently fails, if the ELISA, immunofluorescence and PCR tests are positive, the sample should be considered as presumptively positive for *X. fragariae*, pending final identification (section 4). The best isolation results are expected when using freshly

prepared sample extracts from young lesions. Isolation onto media can also be achieved by *in planta* enrichment, as described in section 3.6.

3.6 Detached leaf assay and biological enrichment

3.6.1 Detached leaf assay

Tissue sample preparations (section 3.3) can be used for inoculating detached strawberry leaves as soon as they are prepared in extraction buffer or distilled water (Civerolo *et al*., 1997a). Use young (7–14 day old) leaves of a cultivar susceptible to *X. fragariae* (e.g. Camarosa, Pajaro, Seascape, Selva, Korona) from greenhouse-grown, *X. fragariae*-free plants. The quality of the leaves and their age are essential considerations for a successful test.

Aseptically remove three leaves (each one with three leaflets) from the greenhouse-grown plants, cut off the basal portion of the petioles and immediately place the petioles in glass tubes containing sterile water.

Prepare a cell suspension of a reference *X. fragariae* strain (table 3) containing 10^5–10^6 cfu/ml in PBS or distilled water as a positive control. PBS or distilled water is used as a negative control. Infiltrate four sites on the abaxial surface of each leaflet (two on each side of the main vein) using a needleless syringe (3 cc plastic disposal BD[1], 2 mm orifice).

Rinse off the excess inoculum with sterile water 1 h after inoculation. Place the leaves with their petioles in the tubes in a humid chamber (relative humidity 95–100%) and incubate at 18–20 °C with a 12 h photoperiod for up to 21 days. The specified temperature and illumination during incubation is essential for avoiding false negative results. The inoculated leaves should not have visible injuries, and water-soaking caused by the inoculum infiltration should disappear within 24 h.

Specific symptoms (i.e. angular dark water-soaked lesions) similar to those observed on naturally infected leaves begin to appear a few days after inoculation. Record symptoms every two days for 14–21 days.

3.6.2 Interpretation of detached leaf assay results

The detached leaf assay is negative if no typical *X. fragariae* angular leaf spots (i.e. dark and water-soaked when viewed with reflected light; translucent yellow when viewed with transmitted light) and/or chlorotic halos appear at any of the inoculated sites after 21 days. No water-soaked spots that appear translucent yellow when viewed with transmitted light should appear within inoculation sites infiltrated with negative controls (Civerolo *et al*., 1997a).

The detached leaf assay is positive if typical *X. fragariae* angular leaf spots (i.e. dark and water-soaked when viewed with reflected light; translucent yellow when viewed with transmitted light) develop at the infiltration inoculation sites within 10 to 21 days. These should be similar in appearance to those that develop at inoculation sites infiltrated with the positive control suspensions. No water-soaked spots that appear translucent yellow when viewed with transmitted light should appear within inoculation sites infiltrated with negative controls (Civerolo *et al*., 1997a).

3.6.3 Enrichment in planta isolation

Select one leaf per sample from those inoculated in the detached leaf assay 48 h after inoculation for isolation onto nutrient media. Excise 10–12 small discs, 0.5 cm in diameter, from each inoculated site per inoculated detached leaf and crush it in 4.5 ml PBS. Prepare dilutions as for direct isolation (section 3.5) in PBS and streak 50 µl of each dilution onto the surface of Wilbrink-N medium in triplicate. Incubate the plates at 25–27 °C and check for *X. fragariae*-like colonies after five to seven days.

3.6.4 Enrichment in vitro-PCR from detached leaf assay

Use the Wilbrink-N medium plates streaked with extracts prepared for isolation following enrichment *in planta* as described in section 3.6.3 after incubation at 25–27 °C for four days. Wash bacterial colonies off the surface of the medium in 3–5 ml PBS and use them for PCR analysis (section 3.9). This is a modification of the bio-enrichment PCR described by Schaad *et al.* (1995).

3.7 ELISA

The specificity of ELISA with two commercially available polyclonal anti-*X. fragariae* sera has been validated (López *et al*., 2005). Rowhani *et al.* (1994) showed that ELISA using polyclonal antibodies could specifically detect 34 strains of *X. fragariae* and the antibodies did not cross-react with other closely related pathovars or other bacteria isolated from strawberry plants. A test sensitivity of 10^5 cfu/ml has been reported for ELISA detection of *X. fragariae* (Rowhani *et al.*, 1994; Civerolo *et al.*, 1997b).

Use cell suspensions prepared from pure cultures of *X. fragariae* and a non-*X. fragariae* strain as positive and negative controls in each microtiter plate. It is recommended that the appropriate working dilution of each polyclonal antiserum be determined.

3.7.1 Indirect ELISA

Mix 210 µl of each test sample, the positive *X. fragariae* cell suspension (approximately 10^9 cfu/ml), the negative non-*X. fragariae* cell suspension (approximately 10^9 cfu/ml) and the negative control (suspension of healthy strawberry material, see below) with 210 µl coating buffer (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, distilled water to 1 litre) and add 200 µl of the sample and buffer mixture to each of two wells of a microtiter plate (PolySorp (Nunc[1]) or equivalent). For the negative plant material control, crush approximately 0.1 g healthy strawberry leaf, petiole or crown tissue in 0.9 ml PBS and add 0.9 ml coating buffer.

Incubate the plate at 4 °C overnight. Wash the plate three times with PBS containing 0.05% Tween 20 (PBS-T) (8 g NaCl, 0.2 g KCl, 0.2 g $Na_2HPO_4 \cdot 12H_2O$, 2.9 g KH_2PO_4, 500 µl Tween 20, distilled water to 1 litre). After washing add 200 µl blocking buffer (PBS containing 1% bovine serum albumin (BSA) or non-fat milk powder) to each of the test wells and incubate at 37 °C for 1 h. Wash the plate three times with PBS-T.

Prepare the appropriate working dilution, according to the manufacturer's instructions, of the anti-*X. fragariae* serum in PBS and add 200 µl to each test well. Incubate at 37 °C for 2 h and then wash the plate three times with PBS-T. Add 200 µl of the antibody–enzyme conjugate at the appropriate dilution in PBS containing 0.2% BSA to each well. Incubate at 37 °C for 1 h and then wash the plate four times with PBS-T. Add 200 µl freshly prepared substrate (1 mg p-nitrophenylphosphate/ml substrate buffer, pH 9.8) to each test well. Incubate in the dark at room temperature for 15, 30 and 60 min, and read the absorbance at 405 nm.

3.7.2 DAS-ELISA

For double antibody sandwich (DAS)-ELISA, add 200 µl of an appropriate dilution of anti-*X. fragariae* serum in the coating buffer to each well of two microtiter plates (PolySorp (Nunc[1]) or equivalent). Incubate at 37 °C for 4 h and wash the wells three times with PBS-T. Add 200 µl of each tissue macerate sample, and a positive and a negative control, as described for indirect ELISA (section 3.7.1), to each of two wells of each plate and incubate at 4 °C overnight. After washing the plates three times with PBS-T, add 200 µl of an appropriate dilution of the enzyme–antibody conjugate in PBS containing 0.2% BSA to each well. Incubate at 37 °C for 3 h. After washing the plates four times with PBS-T add 200 µl of freshly prepared substrate (1 mg ρ-nitrophenylphosphate/ml substrate buffer, pH 9.8) to each test well. Incubate in the dark at room temperature for 15, 30 and 60 min, and read the absorbance at 405 nm.

3.7.3 Interpretation of ELISA results

The ELISA is negative if the average absorbance reading of duplicate wells containing tissue macerate is <2× the average absorbance reading of the negative control wells containing healthy strawberry tissue macerate.

The ELISA is positive if (1) the average absorbance reading of duplicate sample wells is >2× the average absorbance reading of the negative control wells containing healthy strawberry tissue macerate, and (2) the average absorbance reading of the positive control wells is >2× that of the average absorbance reading of the negative control wells.

Negative ELISA results for positive control wells indicate that the test was not performed correctly and/or the reagents have degraded or expired.

Positive ELISA results for negative control wells indicate that cross-contamination or non-specific antibody binding has occurred. The test should be repeated with fresh tissue or another test based on a different principle should be performed.

3.8 Immunofluorescence

Immunofluorescence procedures for identifying phytopathogenic bacteria are given in De Boer (1990) and EPPO (2009). Three commercially available polyclonal anti-*X. fragariae* sera (Table 1) have been validated using fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulins (López *et al.*, 2005). Immunofluorescence with these antibodies allows the detection of 10^3–10^4 cfu/ml *X. fragariae* in strawberry tissue (Calzolari and Mazzucchi, 1989).

Test samples consist of dilutions of tissue macerates (1:10, 1:100 and 1:1 000) and cell suspensions (10^6 cfu/ml) of a positive *X. fragariae* and a negative non-*X. fragariae* bacterial strain in PBS or distilled water. Negative controls should consist of healthy plant tissue extracts.

Add aliquots (20 µl) of test samples and positive and negative control suspensions to separate wells of a multiwell microscope slide. Air-dry the preparations and fix them by flaming or by soaking the slides in acetone for 10 min followed by air-drying. Slides can be stored at –20 °C until required. Dilute the primary *X. fragariae* antibody in PBS containing 10% skim milk powder. Select the lowest antibody concentration that gives good staining when there are up to 100 positive cells per microscope field. It is advisable that two dilutions of the antibody are used to detect cross-reactions with other bacteria. Apply 20 µl of the primary antibody to each well and incubate the slides in a moist chamber at room temperature or at 37 °C for 30–60 min. Rinse the slides in PBS and wash them by submerging them in the same buffer for 10 min. Dilute the FITC-conjugated secondary antibody in PBS (optimum dilutions usually vary between 1:20 and 1:200). Cover the wells of the slides with the secondary antibody and incubate in a moist chamber at room temperature or at 37 °C for 30–60 min. Repeat the washing step then air-dry the slides. Mount coverslips on the slides with mounting fluid (90 ml glycerol, 10 ml PBS) containing 1 mg ρ-phenylenediamine/ml and view the slides under oil immersion at 500–1 000× magnification. Count the cells that fluoresce and have a similar size to the cells of the reference *X. fragariae* strain (López *et al*., 2005).

3.8.1 Interpretation of immunofluorescence results

The immunofluorescence test is negative if green-fluorescing cells with characteristic morphology of *X. fragariae* are observed in positive control wells but not in test sample or negative control wells.

The immunofluorescence test is positive if green-fluorescing cells with characteristic morphology of *X. fragariae* are observed in positive control and test sample wells but not in negative control wells.

As a population of 10^3 cells/ml is considered the limit of reliable detection by immunofluorescence, samples with $>10^3$ cells/ml are considered positive (De Boer, 1990). The immunofluorescence test may be considered to be inconclusive for samples with $<10^3$ cells/ml. In this case, further testing or re-sampling should be performed. Samples with large numbers of incompletely or weakly fluorescing cells

compared with the positive control need further testing with different dilutions of antibody or another source of antibody.

Table 1. Polyclonal antibodies to *Xanthomonas fragariae* currently recommended for use in serological tests

Source	Recommended uses†
Neogen Europe[1]	Detection using immunofluorescence or double antibody sandwich-enzyme-linked immunosorbent assay
Plant Research International, Wageningen UR	Detection using immunofluorescence
Bioreba AG[1]	Detection using double antibody sandwich-enzyme-linked immunosorbent assay

† Validated in a test performance study in a European Union-funded project (SMT-4-CT98-2252) (López *et al.*, 2005).

3.9 PCR

The PCR methods described in this diagnostic protocol, with the exception of the nested PCR developed by Zimmerman *et al.* (2004), have been validated in a a test performance study funded by the European Union (SMT-4-CT98-2252) (López *et al.*, 2005). Nested PCR protocols have been reported to increase sensitivity up to 100 times compared with conventional PCR protocols (Roberts *et al.*, 1996; Zimmerman *et al.*, 2004).

Protocols for DNA extraction from plant samples and PCR described in Pooler *et al.* (1996) and Hartung and Pooler (1997) have been validated (López *et al.*, 2005). A modified protocol using the REDExtract-N-Amp Plant PCR Kit (Sigma[1]) has also been reported to be appropriate for DNA extraction before amplification for testing large numbers of samples of asymptomatic leaves (Stöger and Ruppitsch, 2004). Other commercial kits for extracting DNA and for nested PCR and PCR using other primers (Roberts *et al.*, 1996) are available; however, these have not yet been validated (López *et al.*, 2005).

Two sensitive real-time PCR tests have been described for the detection of *X. fragariae* (Weller *et al.*, 2007; Vandroemme *et al.*, 2008) in strawberry tissue. The real-time PCR test developed by Weller *et al.* (2007) will also differentiate between *X. fragariae* and *X. arboricola* pv. *fragariae*. The real-time PCR described by Weller *et al.* (2007) is based on primers designed within regions of the *gyrB* gene unique to *X. fragariae* and the *pep* gene unique to *X. arboricola* pv. *fragariae*. The real-time PCR developed by Vandroemme *et al.* (2008), yielding a 41 base pair (bp) amplicon, is based on primers designed from the 550 bp amplicon from the PCR described by Pooler *et al.* (1996). These methods are potentially useful for detecting low levels of *X. fragariae* in asymptomatic or latent infections.

3.9.1 DNA extraction

The DNeasy Plant Mini Kit (Qiagen[1]), as modified for mycoplasmalike organism (MLO) DNA extraction (Lopez *et al.*, 2005), provided the best results during the European Union ring test (SMT-4-CT98-2252).

For DNA extraction use 250 µl test sample tissue macerate(s); similarly prepared healthy strawberry plant material and sterile PBS or ultrapure water as negative controls; and a cell suspension of a pure culture of *X. fragariae* as a positive control. Add 250 µl cetyl trimethylammonium bromide (CTAB) extraction buffer (50 ml of 1 M Tris-HCI, 50 ml of 5 M ethylenediaminetetraacetic acid (EDTA), 40.9 g NaCl, 5 g polyvinylpyrrolidone (PVP)-40, 12.5 g CTAB, distilled water to 500 ml) and 4 µl RNase A (100 mg/ml), mix by inverting gently five times, and incubate at 65 °C for 10 min with occasional mixing by inversion. Then follow the manufacturer's instructions until the DNA elution step.

To elute the DNA, add 100 µl of 10 mM Tris-HCI, pH 9 (preheated to 65 °C) to the column and centrifuge at ≥6 000 *g* for 1 min. Add an additional 100 µl Tris-HCI and repeat the centrifugation step. Adjust the DNA solution to a total volume of 300 µl with Tris-EDTA (TE) buffer and add 200 µl of 5 M ammonium acetate and 1 ml absolute ethanol. Mix well and incubate at –20 °C for 1 h to overnight. After incubation, centrifuge at 17 000 *g* for 10 min. Discard the supernatant and wash the DNA pellet in 1 ml absolute ethanol and centrifuge at 16 000 *g* for 5 min. Discard the supernatant and wash the

DNA pellet in 500 µl of 80% ethanol and centrifuge at 16 000 *g* for 5 min. Discard the supernatant. After the pellet has dried, resuspend it in 50 µl sterile distilled water.

3.9.2 Multiplex PCR

3.9.2.1 Protocol of Hartung and Pooler (1997)

Specificity for this protocol was confirmed in a study with 30 isolates of *X. fragariae*, 36 isolates of *X. campestris* (representing 19 pathovars) and 62 isolates of epiphytic bacteria commonly isolated from strawberry. Only *X. fragariae* was detected (in all isolates). This multiplex PCR enabled detection to 10^3 cfucfu/ml in plant tissue (Pooler *et al.*, 1996; Hartung and Pooler 1997).

The three sets of primers described by Pooler *et al*. (1996) are:

241A: 5′-GCCCGACGCGAGTTGAATC-3′

241B: 5′-GCCCGACGCGCTACAGAC TC-3′

245A: 5′-CGCGTGCCAGTGGAGATCC-3′

245B: 5′-CGCGTGCCAGAACTAGCAG-3′

295A: 5′-CGT TCC TGGCCGATT AATAG-3′

295B: 5′-CGCGTTCCT GCG TTTTTT CG-3′

PCR is carried out in 25 µl reaction mixtures containing 2.5 µl buffer (PerkinElmer[1]) (containing 15 mM $MgCl_2$), 5.0 µl deoxyribonucleotide triphosphate (dNTP) (1 mM), 2.0 µl (0.4 µM) of each of the six primers, 0.5 µl Taq DNA polymerase and 5.0 µl sample DNA. The cycling parameters are an initial activation step of 95 °C for 15 min; 35 cycles of 95 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min; and a final extension step of 72 °C for 7 min. PCR products are analysed by 1.5% agarose gel electrophoresis in 0.5× Tris-acetate-EDTA (TAE) buffer (EPPO, 2006).

Specific PCR amplicons for *X. fragariae* are 300, 550 and 615 bp, as previously described (Pooler *et al.*, 1996; Hartung and Pooler, 1997). The 300 bp band is usually present when the extracts are from plants infected with *X fragariae* but the other bands (550 and 615 bp) may appear occasionally.

The primers 245A and 245B can be used in a conventional PCR, using the procedure described above, and will produce an amplicon of 300 bp.

3.9.3 Nested PCR

The nested PCR described by Moltmann and Zimmerman (2005) using primers developed by Pooler *et al*. (1996) and Zimmerman *et al.* (2004) is recommended for diagnosing *X. fragariae* in symptomatic strawberry plants as well as for testing asymptomatic strawberry plants (frigo and green plants). The nested PCR described by Roberts *et al.* (1996) offers an alternative method for confirmation.

3.9.3.1 Protocol of Moltmann and Zimmerman (2005)

Specificity for this protocol was confirmed in a study with 14 isolates of *X. fragariae*, 30 isolates of *X. campestris* (representing 14 pathovars) and 17 isolates of unidentified bacteria associated with strawberry leaves. In addition, the specificity of the external primer set was verified by Hartung and Pooler (1997) (section 3.9.2.1). No cross-reactions were observed with the isolates tested. This PCR has been successfully applied to testing of samples collected during a survey of strawberry plants and imported plants (Moltmann and Zimmerman, 2005). It enabled detection to 200 fg DNA per reaction and was 100 times more sensitive than conventional PCR (Zimmerman *et al.*, 2004).

Incubate leaf, petiole and crown tissue (30–70 g) in 10–20 ml of 0.01 M sodium phosphate buffer (pH 7.2) per gram of tissue at room temperature overnight. Extract DNA and analyse by single and nested PCR as described by Zimmerman *et al.* (2004).

The primers are:

245A: 5′-CGCGTGCCAGTGGAGATCC-3′

245B: 5′-CGCGTGCCAGAACTAGCAG-3′
245.5: 5′-GGTCCAGTGGAGATCCTGTG-3′
245.267: 5′-GTTTTCGTTACGCTGAGTACTG-3′

PCR is carried out in 25 µl reaction mixtures containing PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.08% Nonidet P-40, 2.5 mM $MgCl_2$), 0.2 mM each dNTP, 0.2 µM each primer and 0.5 µl Taq DNA polymerase. The cycling parameters are an initial denaturation step of 94 °C for 4 min; 35 cycles of 94 °C for 1 min, 68 °C for 1 min and 72 °C for 1 min; and a final extension step of 72 °C for 7 min. For nested PCR, after amplification of DNA with the first round primers (245A and 245B), 1 µl of the first PCR product is used as template in a second PCR with the internal primers 245.5 and 245.267. The same cycling parameters are used except the annealing temperature is 62 °C for the internal primers 245.5 and 245.267. PCR products are analysed by 1.2% agarose gel electrophoresis in 0.5× TAE buffer.

Specific PCR amplicons for *X. fragariae* are 300 bp in the first round PCR using the 245A and 245B primers, and 286 bp in the nested PCR using the internal primers 245.5 and 245.267. With high template concentrations, a second fragment of approximately 650 bp is sometimes amplified.

3.9.3.2 Protocol of Roberts *et al.* (1996)

Specificity for this protocol was confirmed in a study with 30 isolates of *X. fragariae*, 17 isolates of *X. campestris* (representing 16 pathovars) and 9 isolates of non-pathogenic xanthomonads isolated from strawberry. No cross-reactions were observed with the isolates tested. This nested PCR enabled detection to approximately 18 *X. fragariae* cells in plant tissue (Roberts *et al.*, 1996).

The semi-nested primers, as described by Roberts *et al.* (1996), are:

XF9: 5′-TGGGCCATGCCGGTGGAACTGTGTGG-3′
XF11: 5′-TACCCAGCCGTCGCAGACGACCGG-3′
XF12: 5′-TCCCAGCAACCCAGATCCG-3′

PCR is carried out in 25 µl reaction mixtures containing PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM each dNTP, 0.2 µM each primer and 0.5 µl Taq DNA polymerase. The cycling parameters are an initial denaturation step of 95 °C for 2 min; 30 cycles of 95 °C for 30 s, 65 °C for 30 s and 72 °C for 45 s; and a final extension step of 72 °C for 5 min. For the semi-nested PCR, after amplification of DNA with the first round primers (XF9 and XF11), 3 µl of the first PCR product is used as template in a second PCR with the primers XF9 and XF12. The same cycling parameters as described for the first round are performed except that the annealing temperature is 58 °C. PCR products are analysed by 1.5% agarose gel electrophoresis in 0.5× TAE buffer.

Specific PCR amplicons for *X. fragariae* are 537 bp in the first round PCR using the XF9 and XF11 primers, and 458 bp in the semi-nested PCR using the XF9 and XF12 primers.

3.9.4 Real-time PCR

3.9.4.1 Protocol of Weller *et al.* (2007)

Specificity for this protocol was confirmed in a study with 10 isolates of *X. fragariae* and 24 *Xanthomonas* isolates (representing 12 species and 17 pathovars). Only *X. fragariae* was detected (in all isolates). This real-time PCR enabled detection to 10^3 cfu per leaf disc (Weller *et al.*, 2007). This protocol has been further validated by a laboratory in the Netherlands; the validation data are available on the EPPO database on diagnostic expertise ().

The primers, based on sequences of the *gyrB* gene, and TaqMan probe, covalently labelled at the 5′ end with the reporter dye JOE and at the 3′ end with the quencher dye TAMRA, are:

Xf gyrB-F: 5'-CCG CAG CGA CGC TGA TC -3'
Xf gyrB-R: 5'-ACG CCC ATT GGC AAC ACT TGA-3'
Xf gyrB-P: 5'-TCC GCA GGC ACA TGG GCG AAG AAT TC-3'

PCR is carried out by adding 4 µl template DNA to a reaction mixture containing 1× TaqMan Buffer A (Applied Biosystems[1]), 5.5 mM $MgCl_2$, 200 µM dNTPs (Promega[1]), 300 nM each primer, 100 nM probe and 0.63 U AmpliTaq Gold DNA polymerase (Applied Biosystems[1]). The cycling parameters are an initial activation step of 2 min at 50 °C then 15 min at 95 °C followed by 40 cycles of 10 s at 95 °C and 1 min at 60 °C.

3.9.5 Interpretation of PCR results

3.9.5.1 Conventional PCR

The PCR test is negative if none of the *X. fragariae*-specific amplicons of expected size is detected for samples and negative controls but the amplicons are detected for all positive controls.

The PCR test is positive if at least one of the *X. fragariae*-specific amplicons of expected size is detected, providing that it is not amplified from any of the negative controls.

Inhibition of the PCR may be suspected if the expected amplicon is obtained from the positive control containing *X. fragariae* in water but negative results are obtained from positive controls with *X. fragariae* in plant extract. Repeating the PCR with 1:10, 1:100 and 1:1 000 dilutions of the extract or repeating the DNA extraction is recommended.

3.9.5.2 Real-time PCR

The real-time PCR test will be considered valid only if:

- the positive control produces an amplification curve with the pathogen-specific primers
- no amplification curve is seen (i.e. cycle threshold (Ct) value is 40) with the negative extraction control and the negative amplification control.

If the *COX* internal control primers are used, then the negative control (if used), positive control and each of the test samples must produce an amplification curve. Failure of the samples to produce an amplification curve with the internal control primers suggests, for example, that the DNA extraction failed, the DNA was not included in the reaction mixture, compounds inhibitory to PCR were present in the DNA extract, or the nucleic acid was degraded.

A sample will be considered positive if it produces a typical amplification curve. The Ct value needs to be verified in each laboratory when implementing the test for the first time.

3.9.6 Controls for molecular tests

For the test result obtained to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid isolation and amplification of the target pest or target nucleic acid. For PCR, a positive nucleic acid control, an internal control and a negative amplification control (no template control) are the minimum controls that should be used.

Positive controls should be prepared in a separate area than that in which the samples will be tested.

Positive nucleic acid control. This control is used to monitor the efficiency of PCR amplification. Pre-prepared (stored) nucleic acid, whole genome DNA or a synthetic control (e.g. cloned PCR product) may be used. For this protocol, a suspension of pure culture *X. fragariae* cells (10^4–10^6 cfu/ml) is recommended as a positive nucleic acid control.

Internal control. For conventional and real-time PCR, a plant housekeeping gene (HKG) such as *COX* (Weller *et al.*, 2000), 16S ribosomal (r)DNA (Weisberg *et al.*, 1991) or *GADPH* (Mafra *et al.*, 2012) should be incorporated into the protocol to eliminate the possibility of PCR false negatives due to nucleic acid extraction failure or degradation or the presence of PCR inhibitors.

Negative amplification control (no template control). This control is necessary for conventional and real-time PCR to rule out false positives due to contamination during preparation of the reaction mixture.

PCR-grade water that was used to prepare the reaction mixture or sterile PBS is added at the amplification stage.

Positive extraction control. This control is used to ensure that nucleic acid from the target is of sufficient quality for PCR amplification. Nucleic acid is extracted from infected host tissue or healthy plant tissue that has been spiked with the target near the concentration considered the detection limit of the protocol.

The positive control should be approximately one-tenth of the amount of leaf tissue used per plant for the DNA extraction. For this protocol, *X. fragariae* tissue macerates spiked with 10^6 cfucfu/ml of a reference *X. fragariae* strain are recommended as positive extraction controls.

For PCR, care needs to be taken to avoid cross-contamination due to aerosols from the positive control or from positive samples (in particular for nested PCR). If required, the positive control used in the laboratory should be sequenced so that this sequence can be readily compared with sequences obtained from PCR amplicons of the correct size. Alternatively, synthetic positive controls can be made with a known sequence that, again, can be compared with PCR amplicons of the correct size.

Negative extraction control. This control is used to monitor contamination during nucleic acid extraction and/or cross-reaction with the host tissue. The control comprises nucleic acid that is extracted from uninfected host tissue and subsequently amplified, or a tissue macerate sample extract previously tested negative for *X. fragariae*. Multiple controls are recommended to be included when large numbers of positive samples are expected.

4. Identification

The minimum requirements for identification are isolation of the bacterium and a positive result from each of the three detection techniques: (1) indirect ELISA, DAS-ELISA (section 3.7) or immunofluorescence (section 3.8) using polyclonal antibodies; (2) PCR (section 3.9); and (3) pathogenicity testing by inoculation of strawberry hosts to fulfil the requirements of Koch's postulates (sections 4.4 and 3.6). Additional tests (sections 4) may be done to further characterize the strain present. In all tests, positive and negative controls must be included.

In the case of latent infections or asymptomatic plants, after an initial screening test the pathogen should be isolated and its identity confirmed, including by pathogenicity testing with the pure culture and the fulfilment of Koch's postulates.

4.1 Biochemical and physiological tests

X. fragariae has the common cultural characteristics of all xanthomonads. Cells are Gram-negative, aerobic rods with a single polar flagellum. Nitrates not reduced, catalase test positive, and asparagine not used as a sole source of carbon and nitrogen (Bradbury, 1977; Bradbury, 1984; Schaad *et al*., 2001). Weak production of acids from carbohydrates. Colonies are mucoid, convex and shiny on YPGA and Wilbrink-N media (Dye, 1962; van den Mooter and Swings 1990; Swings *et al.*, 1993; Schaad *et al.*, 2001). *Xanthomonas* species are easily differentiated from the other genera of aerobic, Gram-negative rod-shaped and other yellow-pigmented bacteria by the characteristics shown in Table 2 as described by Schaad *et al*. (2001).

Table 2. Phenotypic characteristics for differentiating *Xanthomonas* from *Pseudomonas* and the yellow-pigmented bacteria *Flavobacterium* and *Pantoea* (Schaad et al. 2001)

Characteristic	*Xanthomonas*	*Pseudomonas*	*Flavobacterium*	*Pantoea*
Flagellation	1, polar	>1, polar	None	Peritrichous
Xanthomonadin	Yes	No	No	No
Fluorescence	No	Variable	No	No
Levan from sucrose	Yes	Variable	No	No
H_2S from cysteine	Yes	No	No	No
Oxidase	Negative or weak	Variable	Positive	Negative
Fermentation	No	No	No	Yes
Growth on 0.1% triphenyltetrazolium chloride (TTC)	No	Yes	Yes	Yes

The reference *X. fragariae* strains available from different collections that are presented in Table 3 are recommended for use as positive controls in biochemical and physiological tests.

Table 3. Reference strains for *Xanthomonas fragariae*

Strain	Source
ATCC 33239	American Type Culture Collection, Manassas, VA, United States
CFBP 2510	Collection Française de Bactéries Phytopathogènes, INRA Station Phytobactériologie, Angers, France
ICMP 5715	International Collection of Microorganisms from Plants, Auckland, New Zealand
BCCM/LMG 708	Belgian Co-ordinated Collections of Micro-organisms / Collection of the Laboratorium voor Microbiologie en Microbiele Genetica, Ghent, Belgium
NCPPB 1469	National Collection of Plant Pathogenic Bacteria, Central Science Laboratory, York, United Kingdom; Culture Collection of the Plant Protection Service (PD), Wageningen, Netherlands
NCPPB 1822	National Collection of Plant Pathogenic Bacteria, Central Science Laboratory, York, United Kingdom; Culture Collection of the Plant Protection Service (PD), Wageningen, Netherlands

The most relevant or useful characteristics for distinguishing *X. fragariae* from other *Xanthomonas* species (Schaad *et al.*, 2001; Janse *et al.*, 2001; EPPO, 2006) are shown in Table 4.

Table 4. Diagnostic tests to distinguish *Xanthomonas fragariae* from the *"Xanthomonas campestris* group" and *Xanthomonas arboricola* pv. *fragariae*

Test	*X. fragariae*	*X. campestris*	*X. arboricola* pv. *fragariae*
Growth at 35 °C	–	+	ND
Growth on 2% NaCl	–	+	+
Esculin hydrolysis	–	+	+
Gelatin liquefaction	+	V	+
Protein digestion	–	+	ND
Starch hydrolysis	+	V	+
Urease production	–	–	–
Acid from:			
Arabinose	–	+	ND
Galactose	–	+	+
Trehalose	–	+	ND
Cellobiose	–	+	+

ND, not determined; V, variable reaction.
Source: Janse et al. (2001) and EPPO (2006).

Biochemical characterization of isolated strains can be done using commercial systems and identification of *X. fragariae* can be obtained by specific profiling using API 20 NE and API 50 CH strips (BioMérieux[1]) (EPPO, 2006).

For the API 20 NE strips[1], follow the manufacturer's instructions for preparing suspensions from 48 h old test and reference strain cultures on Wilbrink-N medium and inoculate the strips. Incubate at 25–26 °C and read after 48 and 96 h. The readings after 48 h for enzymatic activity and 96 h for substrate utilization are compared with those characteristic of *X. fragariae* (Table 5).

Table 5. Reactions of *Xanthomonas fragariae* in API 20 NE strips

Test	Reaction (after 48 or 96 h)†
Glucose fermentation	–
Arginine	–
Urease	–
Esculin	+
Gelatin	+ (weakly)
Para-nitrophenyl-β-D-galactopyranosidase (PNPG)	+
Assimilation of:	
Glucose	+
Arabinose	–
Mannose	+
Mannitol	–
N-acetyl glucosamine	+
Maltose	–
Gluconate	–
Caprate	–
Adipate	–
Malate	+
Citrate	–
Phenyl acetate	–

† Common reactions from 90% of *X. fragariae* strains tested (López *et al.*, 2005).

For the API 50 CH strips[1], prepare bacterial cell suspensions of OD_{600nm} = 1.0 in PBS. Add 1 ml suspension to 20 ml modified medium C (0.5 g $NH_4H_2PO_4$, 0.5 g K_2HPO_4, 0.2 g $MgSO_4$, 5 g NaCl, 1 g yeast extract, 70 ml bromothymol blue (0.2%), distilled water to 1 litre; pH 6.8) (Dye, 1962). Follow the manufacturer's instructions for inoculation of the strips. Incubate at 25 °C under aerobic conditions and read after two, three and six days. Utilization of the different carbohydrates is indicated by a yellow colour in the wells after the incubation period (Table 6).

Table 6. Reactions of *Xanthomonas fragariae* in API 50 CH strips

Test†	Reaction (after six days)
D-Arabinose	Variable
Galactose	+
D-Glucose	+
D-Fructose	+
D-Mannose	+
N-acetyl glucosamine	+
Esculin	+
Sucrose	+
Trehalose	+
D-Lyxosa	+
L-Fucose	+

† The remaining sugars in the API 50 CH test strips are not utilized by *X. fragariae* (López *et al.*, 2005).

4.1.1 Fatty acid methyl ester profiling

Fatty acid methyl esters (FAMEs) associated with the cytoplasmic and outer membranes of Gram-negative bacteria are useful for bacterial identification (Sasser, 1990). Specific fatty acids that may be used to predict the genus of Gram-negative and Gram-positive bacteria are given by Dickstein *et al.* (2001). Identification is based on comparing the types and relative amounts of the fatty acids in a profile of an unknown strain with profiles from a wide variety of strains in a library database (e.g. TSBA40 library). It is critical that bacteria be grown under uniform conditions of time, temperature and nutrient media in order to obtain reproducible results. *X. fragariae* strains contain three major fatty acids: 16:1ω-7 *cis*, 15:0 *anteiso* and 15:0 *iso*. While some test strains give a good match to the library profile, other strains have differing fatty acid profiles that do not correspond well. Studies have shown that strains of *X. fragariae* show considerable diversity and fall into at least four distinct fatty acid groups (Roberts *et al.*, 1998). The method described by Roberts *et al*. (1998) is recommended for FAME profiling of *X. fragariae*. Test strains are grown on trypticase soy agar at 24 °C for 48 h, a fatty acid extraction procedure is applied and the extract is analysed using the Sherlock Microbial Identification System (MIDI) (Newark, DE, United States).

4.1.1.1 Interpretation of FAME profiling results

The FAME profiling test is positive if the profile of the test strain is identical to that of the *X. fragariae* positive control or reference strain(s). Fatty acid analysis is available from MIDI and the National Collection of Plant Pathogenic Bacteria (NCPPB) (Fera, York, United Kingdom). The composition and amounts of key FAMEs in *X. fragariae* and *X. arboricola* pv. *fragariae* are given in Janse *et al*. (2001).

4.2 Serological tests

4.2.1 Immunofluorescence

Immunofluorescence can be used for the identification of suspect *X. fragariae* strains. Prepare a suspension of approximately 10^6 cells/ml in PBS and apply the immunofluorescence procedure described in section 3.8. If performing only two identification tests for rapid diagnosis, do not use another serological test in addition to this one.

4.2.2 ELISA

Indirect ELISA or DAS-ELISA (described in sections 3.7.1 and 3.7.2, respectively) can be used for the identification of suspect *X. fragariae* strains isolated from plant material affected by suspected bacterial angular leaf spot. If performing only two identification tests for rapid diagnosis, do not use another serological test in addition to this one.

4.3 Molecular tests

4.3.1 PCR

Suspect *X. fragariae* cultures can be identified using the PCR protocols described in section 3.9.

4.3.2 REP-PCR

Specific repetitive extragenic palindromic (REP)-PCR protocols for the identification of *X. fragariae* strains are described by Opgenorth *et al*. (1996) and Pooler *et al*. (1996). Either one of these protocols can be used for the reliable identification of test strains as *X. fragariae*.

The PCR protocol described below is based on the reaction mixture and amplification conditions described by Opgenorth *et al*. (1996).

Bacterial strains to be analysed are taken from streaks or individual colonies on Pierce's disease modified medium (5.0 g sucrose, 2.5 g Phytone (BD BBL[1]), 10 g Phytagel (BD BBL[1]); distilled water to 1 litre, adjust pH to 7.5 with 2 N HCl before autoclaving;) (Opgenorth *et al.*, 1996). Different growth media can be used; however, these should be standardized before use.

The two sets of primers are:

REP1R-I: 5′-IIIICGICGICATCIGGC-3′

REP2-I: 5′-ICGICTTATCIGGCCTAC-3′

ERIC1R: 5′-ATGTAAGCTCCTGGGGATTCAC-3′

ERIC2: 5′-AAGTAAGTGACTGGGGTGAGC G-3′

The reaction buffer contains 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 6.7 µM EDTA, 30 mM 2-mercaptoethanol, 0.17 mg BSA/ml, 10% (v/v) dimethyl sulfoxide, 1.2 mM of each dNTP, 62 pmol each primer and 2 U Taq DNA polymerase. Bacteria from a representative colony of the test strain are transferred, using a sterile 10 µl pipette tip (or other suitable implement), to a PCR tube containing 25 µl of the reaction mixture. The cycling parameters are 95 °C for 6 min followed by 35 cycles at 94 °C for 1 min, 44 °C (REP primers) or 52 °C (ERIC primers) for 1 min and 65 °C for 8 min. The amplification cycles are followed by a final extension step of 68 °C for 16 min. The amplification products (5–10 µl) are electrophoresed in a 1.5% (w/v) agarose gel. Amplified DNA fragments are visualized after staining with ethidium bromide by ultraviolet transillumination.

4.3.2.1 Interpretation of REP-PCR results

Test bacterial strains are identified as *X. fragariae* if they have the same genomic fingerprints as those of the REP and ERIC genotypes of the reference strains (Pooler *et al*., 1996) amplified in the same PCR and run in the same gel. A small number of polymorphic bands may be obtained from different strains of *X. fragariae* owing to low levels of genomic variability.

4.3.3 Multilocus sequence analysis

A multilocus sequence analysis (MLSA) approach has been widely used for the specific identification of xanthomonads (Parkinson *et al.*, 2007; Almeida *et al.*, 2010; Hamza *et al.*, 2012) and could be used for the identification of *X. fragariae*, especially now that a draft genome sequence is available (Vandroemme *et al.*, 2013). However, it should be noted that this methodology has not yet been validated for the identification of *X. fragariae*. Housekeeping genes (e.g. *gyrB*, *rpoD*) are amplified using primers and conditions as described by Almeida *et al.* (2010) and Hamza *et al.* (2012). MLSA

consists of sequencing multiple loci (typically four to eight housekeeping genes) and comparing these sequences with reference sequences of *Xanthomonas* species deposited in nucleotide databases; for example, the Plant Associated and Environmental Microbes Database (PAMDB) (http://genome.ppws.vt.edu/cgi-bin/MLST/home.pl) (Almeida *et al.*, 2010), MLVAbank for microbe genotyping http://mlva.u-psud.fr/mlvav4/genotyping/ and Q-bank Bacteria database (http://www.q-bank.eu/Bacteria/).

4.4 Pathogencity tests

The identity of bacterial strains suspected of being *X. fragariae* should be confirmed by a pathogenicity test, when required. Strains selected from isolation or enrichment plates should be inoculated into attached leaves of susceptible strawberry plants (or into detached leaves as described in section 3.6). Several procedures are available: Hazel and Civerolo (1980), Civerolo *et al*. (1997a) and Hildebrand *et al.* (2005).

4.4.1 General inoculation procedure

A recommended inoculation procedure is to use *X. fragariae*-free strawberry plants of a susceptible cultivar (e.g. Camarosa, Seascape, Selva, Korona, Pajaro). If possible, plants should be held overnight in an environmental chamber at 20–25 °C with high (>90%) relative humidity and exposed to light for 4 h before inoculation to induce stomatal opening.

Prepare bacterial cell suspensions (10^6 cfu/ml) in sterile distilled water or 10 mM PBS. Apply inoculum for each strain to the abaxial surfaces of three trifoliate leaves on each of two or three plants with a low pressure spray gun, airbrush or similar device (e.g. from DeVilbiss[1]) so as not to induce water-soaking. Infection may be facilitated by wounding leaves (e.g. puncturing the abaxial surface with a needle) before applying inoculum, although it is not necessary to do this. After inoculation, incubate plants in a chamber maintained at 20–25 °C with high humidity (>90%) and a 12–14 h photoperiod. Suspensions of cells of a reference *X. fragariae* strain (prepared in the same manner as the test strain) and sterile distilled water or 10 mM PBS serve as positive and negative controls, respectively, and should be inoculated in different trays. Evaluate lesion development weekly for three weeks (21 days) post-inoculation. Re-isolate the pathogen from such lesions, as described in section 3.5, and identify by ELISA, immunofluorescence or PCR.

4.4.1.1 Interpretation of pathogenicity test results

If the bacterial cell suspension contains *X. fragariae*, initial symptoms will be dark, water-soaked (when viewed with reflected light) lesions on the lower leaf surfaces. These lesions appear translucent yellow when viewed with transmitted light. Later these lesions develop into necrotic spots surrounded by a yellow halo or marginal necrosis. The same symptoms should appear on leaves inoculated with a reference *X. fragariae* strain (positive control).

Similar symptoms should not appear on the leaves inoculated with sterile distilled water or 10 mM PBS (negative control).

4.4.2 Hypersensitive reaction

A hypersensitive reaction (HR) in tobacco leaves can be an indication of the presence of *hrp* genes and a positive reaction is induced by many plant pathogenic bacteria. A positive control, for example a strain of *Pseudomonas syringae* pv. *syringae*, can be used. Use the tobacco cultivar Samsun or Xanthi plants with more than five leaves. Prepare bacterial suspensions of 10^9 cfu/ml (OD_{600nm} = 1.0) in sterile distilled water or 10 mM PBS and infiltrate the suspension into the intercellular spaces through the abaxial surfaces of adult leaves with a syringe equipped with a 25 gauge needle.

4.4.2.1 Interpretation of HR results

Complete collapse and necrosis of the infiltrated tissue within 24–48 h post-inoculation is recorded as a positive test result. Most *X. fragariae* strains are HR positive. However, some may be HR negative,

especially after being stored for some time. Similar reactions should not appear on leaves inoculated with sterile distilled water or 10 mM PBS as a negative control.

5. Records

Records and evidence should be retained as described in section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*).

In cases where other contracting parties may be affected by the results of the diagnosis, in particular in cases of non-compliance (ISPM 13 (*Guidelines for the notification of non-compliance and emergency action*)) and where the pest is found in an area for the first time, the following records and evidence and additional material should be kept for at least one year in a manner that ensures traceability: the original sample, culture(s) of the pest, preserved or mounted specimens, or test materials (e.g. photographs of gels, printouts of ELISA results, PCR amplicons).

6. Contact Points for Further Information

Further information on this protocol can be obtained from:

United States Department of Agriculture (USDA) Agricultural Research Service (ARS) (formerly), (Edwin L. Civerolo; e-mail: emciv@comcast.net).

Plant and Environmental Bacteriology, Fera, Sand Hutton, York YO41 1LZ, United Kingdom (John Elphinstone; e-mail: john.elphinstone@fera.gsi.gov.uk).

Centro de Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias (IVIA), Carretera Moncada-Náquera km 4.5, 46113 Moncada (Valencia), Spain (María M. López; e-mail: mlopez@ivia.es; tel.: +34 963 424000; fax: +34 963 424001).

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will in turn forward it to the Technical Panel on Diagnostic Protocols (TPDP).

7. Acknowledgements

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8. References

The present annex may refer to international standards for phytosanitary measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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9. Figures

Figure 1. Symptoms of *Xanthomonas fragariae* on (A, left) an upper leaf surface and (B, right) a lower leaf surface. *Photo courtesy A.M.C. Schilder, Michigan State University, East Lansing, MI, United States.*

Figure 2. Bacterial ooze from *Xanthomonas fragariae* on a lower leaf surface.
Photo courtesy W.W. Turechek, United States Department of Agriculture Agricultural Research Service, Washington, DC, United States.

Figure 3. Symptoms of *Xanthomonas fragariae* on the calyx of fruit.
Photo courtesy A.M.C. Schilder, Michigan State University, East Lansing, MI, United States.

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ISPM 27
Diagnostic protocols for regulated pests

DP 15: *Citrus tristeza virus*

Adopted 2016; published 2016

CONTENTS

1. Pest Information

Citrus tristeza virus (CTV) causes one of the most damaging diseases of citrus, devastating epidemics of which have changed the course of the citrus industry (Moreno *et al.*, 2008). The term "tristeza", in Portuguese meaning "sadness" or "melancholy", refers to the decline seen in many citrus species when grafted on *Citrus aurantium* (sour orange) or *Citrus limon* (lemon) rootstocks. Although tristeza is predominantly a bud union disease (Román *et al.*, 2004), some CTV strains induce other syndromes, including stem pitting, stunting, reduced productivity and impaired fruit quality of many commercial cultivars, even when they are grafted on tristeza-tolerant rootstocks.

CTV probably originated in Malaysia and other countries of Southeast Asia, the putative area of origin of citrus, and it has been disseminated to almost all citrus-growing countries through the movement of infected plant material. Subsequent local spread by aphid vector species has created major epidemics.

Tree losses on sour orange rootstock were first reported in South Africa in the early twentieth century, and in Argentina and Brazil in the 1930s, likely following the introduction of CTV-infected plants probably infested with the aphid vector most efficient for transmitting the virus, *Toxoptera citricida* Kirkaldy. CTV-induced tree decline has killed or rendered unproductive trees grafted on sour orange rootstock (Bar-Joseph *et al.*, 1989; Cambra *et al.*, 2000a). CTV outbreaks have been observed in the United States, some Caribbean countries and some Mediterranean countries (especially Italy and Morocco). CTV has affected an estimated 38 million trees in the Americas (mainly Argentina, Brazil, Venezuela and California (United States)), 60 million trees in the Mediterranean Basin (especially Spain, with about 50 million trees affected) and an estimated 5 million trees elsewhere, making a total of more than 100 million trees. Tristeza disease can be managed by using citrus rootstock species that induce tolerance to the tristeza disease. Some aggressive strains of CTV cause stem pitting in certain citrus cultivars regardless of the rootstock used. This has a significant impact on fruit quality and yield in several million trees infected with these aggressive strains in most citrus industries worldwide, with the exception of those in the Mediterranean Basin where aggressive strains are not present or are not predominant. To effectively manage the stem pitting disease some citrus industries have adopted a strategy of prophylactically inoculating trees with mild strains of CTV, otherwise known as cross-protection (Broadbent *et al.*, 1991; da Graça and van Vuuren, 2010).

CTV is the largest and most complex member of the genus *Closterovirus* (Moreno *et al.*, 2008). The virions are flexuous, filamentous, 2 000 nm in length and 11 nm in diameter, and contain a non-segmented, positive-sense, single-stranded RNA genome. The CTV genome contains 12 open reading frames (ORFs), encoding at least 17 proteins, and two untranslated regions (UTRs). ORFs 7 and 8 encode proteins with estimated molecular weights of 27.4 kDa (P27) and 24.9 kDa (P25) that have been identified as the capsid proteins. CTV diversity is greater than previously thought; new genotypes have diverged from the ancestral population or have arisen through recombination with previously described strains (Harper *et al.*, 2008). CTV populations in citrus trees are quasispecies in nature, so a complex mixtures of viral genotypes and defective viral RNAs developed during the long-term vegetative propagation of virus isolates through grafting and the mixing of such isolates with aphid-vectored isolates. This results in CTV isolates containing a population of sequence variants, with one usually being predominant (Moreno *et al.*, 2008).

CTV is readily transmitted experimentally by grafting healthy citrus with virus-infected plant material. It is naturally transmitted by certain aphid species in a semi-persistent manner. The most efficient vector of CTV worldwide is *T. citricida*. *T. citricida* is well established in Asia, Australia, sub-Saharan Africa, Central and South America, the Caribbean, Florida (United States) and northern mainland Spain and Portugal as well as the Madeira Islands (Ilharco *et al.*, 2005; Moreno *et al.*, 2008). However, *Aphis gossypii* Glover is the main vector in Spain, Israel, some citrus growing areas in California (United States) and in all locations where *T. citricida* is absent (Yokomi *et al.*, 1989; Cambra *et al.*, 2000a; Marroquín *et al.*, 2004). The comparative effects of aphid vector species on the spread of CTV have been reported (Gottwald *et al.*, 1997). Other aphid species have also been described as CTV vectors (Moreno *et al.*, 2008), including *Aphis spiraecola* Patch, *Toxoptera aurantii*

(Boyer de Fonsicolombe), *Myzus persicae* (Sulzer), *Aphis craccivora* Koch and *Uroleucon jaceae* (Linnaeus). Although these listed aphid species were shown to be less efficient vectors of CTV than *T. citricida* and *A. gossypii* in experimental transmission studies, they are the predominant aphid species in some areas and are therefore likely to play a role in CTV spread, compensating for their poor transmission efficiency by their abundance (Marroquín *et al.*, 2004).

The spatial and temporal spread of CTV in citrus orchards has been studied in different parts of the world (Gottwald *et al.*, 2002). These studies provide evidence for the fact that a long period of time may elapse between the introduction of a primary source of CTV inoculum and the development of a tristeza disease epidemic (Garnsey and Lee, 1988).

2. Taxonomic Information

Name: *Citrus tristeza virus* (acronym CTV)

Synonyms: Tristeza virus

Taxonomic position: *Closteroviridae*, *Closterovirus*

Common names: Tristeza virus, citrus tristeza virus

3. Detection and Identification

Detection and identification of CTV can be achieved using biological, serological or molecular amplification tests (Figure 1 and Figure 2). The use of any one of these tests is the minimum requirement to detect and identify CTV (i.e. during routine diagnosis of the pest when it is widely established in a country). In instances where the national plant protection organization (NPPO) requires additional confidence in the identification of CTV (i.e. detection in an area where the virus is not known to occur or detection in a consignment originating from a country where the pest is declared to be absent), further tests should be done. Where the initial identification was done using a molecular amplification test, subsequent tests should be serological, and vice versa. Further tests may also be done to identify the strain of CTV present, in which case sequencing of the amplicon produced by polymerase chain reaction (PCR) may be needed. In all cases, for the tests to be considered valid, positive and negative controls must be included. The recommended techniques for the biological, serological and molecular amplification tests are described in the following sections. A flow chart for strain identification of CTV is presented in Figure 2.

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

3.1 Host range

Under natural conditions, CTV readily infects most species of *Citrus* and *Fortunella* and some species in genera known as citrus-relatives of the family Rutaceae that are also susceptible hosts of CTV; namely, *Aegle*, *Aeglopsis*, *Afraegle*, *Atalantia*, *Citropsis*, *Clausena*, *Eremocitrus*, *Hespertusa*, *Merrillia*, *Microcitrus*, *Pamburus*, *Pleiospermium* and *Swinglea* (Duran-Vila and Moreno, 2000; Timmer *et al.*, 2000). Most *Poncirus trifoliata* (trifoliate orange) clones and many of their hybrids as well as *Fortunella crassifolia* (Meiwa kumquat) and some *Citrus grandis* (pomelo) are resistant to most CTV strains (Moreno *et al.*, 2008). Consequently, CTV is absent or in very low concentration in these species. *Citrus reticulata* (mandarin), *Citrus sinensis* (sweet orange) and *Citrus latifolia* (lime) are among the cultivars most susceptible to natural CTV infection, followed by *Citrus paradisi* (grapefruit), *Citrus unshiu* (Satsuma mandarin) and *C. limon* cultivars. Among the species used as

rootstock, *Citrus macrophylla* (alemow), *Citrus volkameriana* (Volkamer lemon), *Citrus reshni* (Cleopatra mandarin) and *Citrus limonia* (Rangpur lime or lemandarin) are highly susceptible to natural CTV infection, whereas Carrizo and Troyer citranges (hybrids of sweet orange and trifoliate orange) and *C. aurantium* are rarely infected. *P. trifoliata* and *C. paradisi* × *P. trifoliata* (citrumelo) rootstocks are resistant to most CTV strains. *Passiflora gracilis* and *Passiflora coerulea* are experimental non-citrus hosts.

3.2 Symptoms

Symptom expression in CTV-infected citrus hosts is highly variable and is affected by environmental conditions, host species and the aggressiveness of the CTV strain. In addition, the virus may remain latent for several years. Some CTV strains are mild and produce no noticeable effects on most commercial citrus species, including citrus grafted on *C. aurantium*. In general, mandarins are especially tolerant to CTV infection. *C. sinensis*, *C. aurantium* (as a seedling and not as grafted rootstock), *Citrus jambhiri* (rough lemon) and *C. limonia* are usually symptomless when infected but may react to some aggressive strains. Citrus hosts that manifest symptoms are likely to include lime, grapefruit, some cultivars of pomelo, alemow and sweet orange, some citrus hybrids and some citrus-relatives of the family Rutaceae mentioned in section 3.1.

Depending on the CTV strain and citrus species or scion–rootstock combination, the virus may cause no symptoms or one of three syndromes: tristeza; stem pitting; or seedling yellows, which is mainly seen under greenhouse conditions. These three syndromes are described in the paragraphs below. Figure 1 shows the main symptoms caused by CTV.

One of the most economically significant outcomes of CTV infection is tristeza (a bud union disease), which is characterized by the decline of trees grafted on sour orange or lemon rootstocks. Sweet orange, mandarin and grapefruit scions on these rootstocks become stunted, chlorotic and often die after a period of several months or years (i.e. they experience a slow decline), while other scions experience a rapid decline or collapse some days after the first symptom is observed. The decline results from the physiological effects of the virus on the phloem of the susceptible rootstock just below the bud union. Trees that decline slowly generally have a bulge above the bud union, a brown line just at the point of bud union, and inverse pinhole pitting (honeycombing) on the inner face of sour orange rootstock bark. Stunting, leaf cupping, vein clearing, chlorotic leaves, stem pitting and reduced fruit size are common symptoms observed on susceptible hosts. However some isolates of the virus, particularly in the Mediterranean Basin citrus industry, do not induce decline symptoms until many years after infection, even in trees grafted on sour orange.

Aggressive CTV strains can severely affect trees, inducing stem pitting on the trunk and branches of lime, grapefruit and sweet orange. Stem pitting may sometimes cause a bumpy or ropy appearance of the trunks and limbs of adult trees, deep pits in the wood under depressed areas of the bark, and a reduction in fruit quality and yield. Alemow rootstocks are seriously affected by most of all CTV strains as the rootstock develops stem pitting that results in reduced tree vigour.

The seedling yellows syndrome is characterized by stunting, production of chlorotic or pale leaves, development of a reduced root system, and cessation of growth of trees grafted on sour orange, grapefruit and lemon seedlings cultivated under greenhouse conditions (20–26°C).

3.3 Biological indexing

The objective of biological indexing is to detect the presence of CTV in plant accessions or selections or in samples whose sanitary status is being assessed, and to estimate the aggressiveness of the isolate on *Citrus aurantifolia* (Mexican, key or Omani lime), *C. macrophylla* or *Citrus paradisi* Macfadyen (Duncan grapefruit) seedlings. The indicator is a graft inoculated according to conventional methods and held under standard conditions (Roistacher, 1991), with four to six replicates (or with two to three replicates if sufficient samples cannot be taken). Vein clearing in young leaves, leaf cupping or leaf distortion, short internodes, stem pitting or seedling yellows symptoms on these sensitive indicator

plants are each evidence of CTV infection after graft inoculation. Symptom onset is compared against that on positive and negative control plants. Illustrations of symptoms caused by CTV on indicator plants can be found in Roistacher (1991) and Moreno *et al.* (2008).

Biological indexing is used widely in certification schemes, as it is considered a sensitive and reliable method for the detection of a new or an unusual strain of the virus. However, it has some disadvantages: it is not a rapid test (symptom development requires three to six months post-inoculation); it can only be used to test budwood; it requires dedicated facilities such as temperature-controlled insect-proof greenhouse space; and it requires dedicated staff who can grow healthy and vigorous indicator host plants that will show appropriate symptoms and experienced staff who can accurately interpret observed disease symptoms that can be confused with symptoms of other graft-transmissible agents. Moreover, asymptomatic CTV strains that do not induce symptoms (latent strains) are not detectable on indicator plants (e.g. the CTV strain K described by Albertini *et al.* (1988)).

There are few quantitative data published on the specificity, sensitivity, other diagnostic parameters and reliability of biological assays by grafting indicator plants (indexing) for CTV detection, diagnosis or identification. Cambra *et al.* (2002) in the European Diagnostic Protocols project (DIAGPRO) and Vidal *et al.* (2012) compared Mexican lime indexing with direct tissue print-enzyme-linked immunosorbent assay (ELISA) (section 3.5.1) (using 3DF1 + 3CA5 monoclonal antibodies) and tissue print real-time reverse transcription (RT)-PCR (section 3.6.5) and concluded that either laboratory method can accurately substitute for the conventional Mexican lime biological indexing for CTV detection.

3.4 Sampling and sample preparation for serological and molecular testing

3.4.1 Sampling

General guidance on sampling methodologies is described in ISPM 31 (*Methodologies for sampling of consignments*) and in Cambra *et al.* (2002) specifically for CTV sampling. Appropriate sampling is crucial for CTV detection and identification by biological, serological or molecular amplification methods. Changes to an accepted sampling scheme could result in an effective diagnostic protocol generating false positive or false negative results. The standard sample for adult trees is five young shoots or fruit peduncles, ten fully expanded leaves, or five flowers or fruits collected around the canopy of each individual tree from each scaffold branch. Samples (shoots or fully expanded leaves and peduncles) can be taken at any time of the year from sweet orange, mandarin, lemon and grapefruit in temperate Mediterranean climates, but spring and autumn are the optimal sampling periods in tropical and subtropical climates for achieving high CTV titres. In these climates, a reduced CTV titre is observed in Satsuma mandarin during summer; consequently, the recommended period for sampling includes all vegetative seasons, with the exception of hot days (35–40 °C) in summer. Roots, however, can be sampled during hot periods if required. Flowers or fruits (when available) are also suitable materials for sampling (Cambra *et al.*, 2002). Tissue from the fruit peduncle in the region of the albedo, where the peduncle is joined to the fruit, or from the columela is the most suitable fruit sample. Standard requirements for sampling nursery plants include the collection of two young shoots or four leaves per plant. Usually chip non-budding (small pieces of bark without buds) or even leaves from infected plants are collected at any time of the year (but preferably during the vegetative period) from at least one year old shoots or tree branches for indexing according to Roistacher (1991).

Shoots, leaf petioles, fruit peduncles and flowers can be stored at approximately 4 °C for up to seven days before processing. Fruits can be stored for one month at approximately 4 °C. Use beyond these time frames may result in lower titres and the potential for false negative results in diagnostic methods

Composite samples, to be tested as a single sample, can be collected together (usually two leaves or one shoot from one to ten nursery plants or ten leaves or five shoots per adult trees collected around

the canopy) for serological or molecular amplification tests. In some circumstances (e.g. routine screening for CTV widely established in a country or an area), multiple plants may be tested simultaneously using a composite sample derived from a number of plants. The decision to test individual plant or composite plant samples by serological or molecular amplification methods depends on the virus concentration in the plants, the expected prevalence of CTV in the area (Vidal *et al.*, 2012), the limit of detection of the test method to be used, and the level of confidence required by the NPPO.

Aphids (fresh or preserved in 70% alcohol) can be individually tested for the presence of CTV. The aphids are collected directly from established colonies or caught in traps: suction traps, classic Moericke yellow water traps or the sticky shoot trap are recommended. Specimens collected are used preferably for squash real-time RT-PCR (Bertolini *et al.*, 2008) or other molecular amplification tests (Marroquín *et al.*, 2004).

3.4.2 Preparation of tissue prints

3.4.2.1 Preparation of tissue prints for serological testing

Tender shoots, leaf petioles, fruit peduncles or flower ovaries are cut cleanly. The freshly cut sections are carefully pressed against a nitrocellulose or ester of cellulose membrane (0.45 mm) and the trace or print is allowed to dry for 2–5 min. For routine serological testing, at least two prints should be made per selected shoot (one from each end of the shoot) or peduncle and one per leaf petiole or flower ovary. Printed membranes can be kept for several months in a dry and dark place

3.4.2.2 Preparation of tissue prints and aphid squashes for molecular amplification testing

Collection of plant material by hand is recommended to avoid contamination of samples by scissors. Tender shoots with fully expanded leaves or mature leaves are collected around the canopy of the tree. The leaf petiole of two leaves or shoots is pressed directly on Whatman[1] 3MM paper (0.45 mm) or positively charged nylon membrane. Several partially overlapping imprints from different leaves are made on approximately 0.5 cm^2 of the paper or membrane, according to Bertolini *et al.* (2008). The trace or print is allowed to dry for 2–5 min. For routine molecular amplification testing, one print should be made per selected leaf pedicel. Individual aphids are squashed directly onto Whatman[1] 3MM paper or positively charged nylon membrane with the aid of the round bottom of an Eppendorf[1] tube to achieve total disruption of the specimen (Bertolini *et al.*, 2008). Printed or squashed membranes can be kept for several months in a dry and dark place.

Direct methods of sample preparation (tissue print or squash) without extract preparation have been validated as an alternative to conventional extract preparation for sample processing (Vidal *et al.*, 2012).

3.4.3 Preparation of plant extracts for serological and molecular amplification testing

Fresh plant material, 0.2–0.5 g, is cut in small pieces with disposable razor blades or bleach-treated scissors to avoid sample to sample contamination and placed in a suitable tube or plastic bag. Extracts for serological testing can be prepared in tubes or in plastic bags. Samples for molecular amplification testing should only be prepared in individual plastic bags to avoid contamination among samples. The sample is homogenized thoroughly in 4–10 ml (1:20 w/v, unless otherwise stated by the manufacturer) extraction buffer using an electrical tissue homogenizer, a manual roller, a hammer or a similar tool.

[1] In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

The extraction buffer is phosphate-buffered saline (PBS), pH 7.2–7.4 ($NaCl_2$, 8 g; KCl, 0.2 g; $Na_2HPO_4 \cdot 12H_2O$, 2.9 g; KH_2PO_4, 0.2 g; distilled water, 1 litre) supplemented with 0.2% sodium diethyl dithiocarbamate (DIECA) or 0.2% mercaptoethanol, or an alternative suitably validated buffer.

3.5 Serological tests

ELISA using validated monoclonal antibodies or polyclonal antibodies is highly recommended for screening large numbers of samples for CTV detection and identification. The production of monoclonal antibodies specific to CTV (Vela *et al.*, 1986; Permar *et al.*, 1990) and others reviewed by Nikolaeva *et al.* (1996) solved the problem of the diagnostic specificity presented by polyclonal antibodies (Cambra *et al.*, 2011) and thus increased the diagnostic sensitivity of serological tests. A mixture of the two monoclonal antibodies 3DF1 and 3CA5, or their recombinant versions (Terrada *et al.*, 2000), recognizes all CTV isolates tested from different international collections (Cambra *et al.*, 1990). A detailed description, characterization and validation of these monoclonal antibodies is provided in Cambra *et al.* (2000a). A mixture of the monoclonal antibodies 4C1 and 1D12 produced in Morocco is reported to react against a broad spectrum of CTV strains (Zebzami *et al.*, 1999), but there are no validation data available

3.5.1 Direct tissue print-ELISA

Direct tissue print-ELISA, also referred to as immunoprinting ELISA or direct tissue blot immunoassay (DTBIA), is performed according to Garnsey *et al.* (1993) and Cambra *et al.* (2000b) using the method described below. A complete kit (validated in test performance and in several published studies) based on CTV-specific 3DF1 + 3CA5 monoclonal antibodies (Vela *et al.*, 1986), including preprinted membranes with positive and negative controls and all reagents, buffers and substrate, is available from Plant Print Diagnòstics SL[1]. A similar but non-validated kit based on Zebzami *et al.*, (1999) 4C1 and 1D12 antibodiesis available from Agdia[1].

Membranes that have been tissue printed (recommended size: approximately 7 × 13 cm) are placed in an appropriate container (tray, hermetic container or plastic bag), covered with a 1% solution of bovine serum albumin (BSA) in distilled water and incubated for 1 h at room temperature or overnight (about 16 h) at 4 °C (the latter is recommended). Slight agitation is beneficial during this step. The BSA solution is discarded but the membranes are kept in the same container. A conjugate solution is prepared that consists of equal concentrations of CTV-specific 3DF1 + 3CA5 monoclonal antibodies linked to alkaline phosphatase (approximately 0.1 µg/ml of each monoclonal antibody in PBS) or of 3DF1 scFv-AP/S + 3CA5 scFv-AP/S fusion proteins expressed in *Escherichia coli* (an appropriate dilution in PBS) (Terrada *et al.*, 2000). The conjugate solution is poured onto the membranes, covering them, and the membranes are incubated for 3 h at room temperature, with slight agitation. The conjugate solution is then discarded. The membranes and the container are rinsed with washing buffer (PBS, pH 7.2–7.4, with 0.05% Tween 20), and washed by shaking (manually or mechanically) for 5 min. The washing buffer is discarded and the washing process is repeated twice. The substrate for alkaline phosphatase (Sigma[1] Fast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) tablets, following the manufacturer's gives to a final concentration of 0.33 mg/ml NBT and 0.175 mg/ml BCIP) is then poured over the membranes and the membranes are incubated until a purple-violet colour appears in the positive controls (about 10–15 min). The reaction is stopped by washing the membranes with tap water. The membranes are spread on absorbent paper and allowed to dry. The prints are examined using a low-power magnification (×10 to ×20). The presence of purple-violet precipitates in the vascular region of plant material reveals the presence of CTV.

3.5.2 DAS-ELISA

Double antibody sandwich (DAS)-ELISA is performed according to Garnsey and Cambra (1991) using the method described below. Complete kits based on validated CTV-specific 3DF1 + 3CA5 monoclonal antibodies (Plant Print Diagnòstics SL[1]) and on different polyclonal antibodies (Agdia[1], Agritest[1], Bioreba[1], Loewe[1], Sediag[1]) are available.

Two wells of a microtiter plate are used for each sample and at least two wells for positive and negative controls. An appropriate dilution is prepared of the polyclonal or monoclonal (3DF1 + 3CA5) antibodies (usually 1–2 µg/ml total immunoglobulins) in carbonate buffer, pH 9.6 (Na_2CO_3, 1.59 g; $NaHCO_3$, 2.93 g; distilled water, 1 litre), and 200 µl is added to each well. The plate is incubated for 4 h at 37 °C or overnight (about 16 h) at 4 °C. The wells are washed three times with washing buffer (PBS, pH 7.2–7.4, with 0.05% Tween 20). The plant extract (section 3.4.3) is then added, 200 µl to each well. After incubation for 16 h at 4 °C, the plates are washed three times as described for direct tissue print-ELISA (section 3.5.1). Specific polyclonal or monoclonal (3DF1 + 3CA5) antibody mixtures linked with alkaline phosphatase are prepared at appropriate dilutions (approximately 0.1 µg/ml in PBS with 0.5% BSA) then 200 µl is added to each well. Incubation is carried out for 3 h at 37 °C. The plates are again washed as described for direct tissue print-ELISA (section 3.5.1). A solution of 1 mg/ml alkaline phosphatase (p-nitrophenyl phosphate) in substrate buffer (97 ml diethanolamine in 800 ml distilled water, pH adjusted to 9.8 with concentrated HCl, and the total volume then made up to 1 000 ml with distilled water) is prepared and 200 µl is added to each well. The plates are incubated at room temperature and read at 405 nm at regular intervals within 120 min, or following the instructions of the supplier of the polyclonal antibody being used.

The ELISA is considered negative if the average optical density (OD) value from each of the duplicate sample wells is <0.1 or is <2× the OD value of the negative control of healthy plant extracts. The ELISA is considered positive if the average OD value from each of the duplicate sample wells is ≥2× the OD value of the negative control of healthy plant extracts. When using polyclonal antibodies, it is essential that the negative controls are as similar as possible to the matrix tested in the same plate.

The method using 3DF1 + 3CA5 monoclonal antibodies was validated in a DIAGPRO ring test (Cambra *et al.*, 2002). A comparison of that method with other techniques and the diagnostic parameters are given in section 3.7.

While some mixtures of monoclonal antibodies detect all CTV strains specifically, sensitively and reliably, some polyclonal antibodies are not specific and have limited sensitivity (Cambra *et al.*, 2011). Therefore, the use of additional methods is recommended in situations where polyclonal antibodies have been used in an assay and the NPPO requires additional confidence in the identification of CTV.

3.6 Molecular tests

After the complete nucleotide sequence of the CTV genomic RNA became available, various diagnostic procedures based on specific detection of viral RNA were developed, including molecular hybridization with complementary (c)DNA or cRNA probes and several methods based on RT-PCR (Moreno *et al.*, 2008). These RT-PCR-based methods have greatly improved the sensitivity of detection, allowing quantification of viral RNA copies in infected citrus tissue or in CTV-viruliferous aphid species (Bertolini *et al.*, 2008). The use of a high throughput technique such as real-time RT-PCR avoids the need for any post-amplification processing (e.g. gel electrophoresis) and is therefore quicker and has less opportunity for cross-contamination than conventional PCR.

With the exception of immunocapture (IC)-RT-PCR (for which RNA isolation is not required), RNA extraction should be done using appropriately validated protocols. The samples should be placed in individual plastic bags to avoid cross-contamination during extraction. Alternatively, spotted plant extracts, printed tissue sections or squashes of plant material can be immobilized on blotting paper or nylon membranes and analysed by real-time RT-PCR (Bertolini *et al.*, 2008). It is not recommended to use spotted or tissue printed samples in conventional PCR because of its lower sensitivity compared with real-time RT-PCR, can result in false negatives.

3.6.1 RNA purification, immunocapture and cDNA synthesis

3.6.1.1 RNA purification

RNA purification should be done using appropriately validated protocols or using an RNA purification kit according to the manufacturer's instructions. The extracted RNA should be stored at –70 °C (preferably) or at –20 °C until its use as a template and for less than one year. Storage should be in small quantities to avoid degradation of RNA due to repeated freeze–thaw cycles.

3.6.1.2 Immunocapture

Immunocapture is an alternative option to RNA purification. For this procedure, a diluted antibody mixture is prepared, consisting of 1 µg/ml CTV-specific polyclonal antibodies or a dilution of monoclonal antibodies (3DF1 + 3CA5, 0.5 µg/ml + 0.5 µg/ml) in carbonate buffer, pH 9.6 (see section 3.5.2 for the composition of carbonate buffer). The antibody mixture is then dispensed into microtubes (100 µl per tube) and the tubes are incubated for 3 h at 37 °C. The coated tubes are washed twice with 150 µl sterile washing buffer (PBS, pH 7.2–7.4, with 0.05% Tween 20; see section 3.4.3 for the composition of PBS). Plant extract (100 µl) could optionally be clarified by centrifugation or filtration trough filter paper or directly used as crude extract and aliquots are dispensed into the antibody-coated microtubes. The tubes are incubated for a minimum of 2 h on ice or alternatively for 2 h at 37 °C. After this immunocapture phase, the microtubes are washed three times with 150 µl sterile washing buffer. It is in these washed tubes that cDNA synthesis and PCR amplification are performed.

3.6.1.3 cDNA synthesis

Because the preservation of RNA during storage is problematic, it is recommended to synthesize cDNA, which can be preserved for long periods with minimal temperature requirements compared with RNA. Several commercial kits are available for cDNA synthesis.

3.6.2 IC-RT-PCR

According to Olmos *et al.* (1999) the primers are:

PIN1: 5′-GGT TCA CGC ATA CGT TAA GCC TCA CTT-3′

PIN2: 5′-TAT CAC TAG ACA ATA ACC GGA TGG GTA -3′

The RT-PCR mixture consists of: ultrapure water, 14.3 µl; 10× Taq DNA polymerase buffer, 2.5 µl; 25 mM $MgCl_2$, 1.5 µl; 5 mM dNTPs, 1.25 µl; 4% Triton X-100, 2 µl; 25 µM primer PIN1, 1 µl; 25 µM primer PIN2, 1 µl; dimethyl sulfoxide (DMSO), 1.25 µl; 10 U/µl AMV reverse transcriptase, 0.1 µl; and 5 U/µl Taq DNA polymerase, 0.1 µl. Reaction mixture (25 µl) is added directly to the washed antibody-coated microtubes. The cycling parameters for RT-PCR are: 42 °C for 45 min and 92 °C for 2 min followed by 40 cycles of (92 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min), with a final elongation step at 72 °C for 10 min followed by cooling at 8 °C. The expected amplicon size is 131 base pairs (bp).

The method was validated in a DIAGPRO ring test (Cambra *et al.*, 2002). A comparison with other techniques and the diagnostic parameters are given in section 3.7.

3.6.3 IC nested RT-PCR in a single closed tube

According to Olmos *et al.* (1999) the primers are:

PEX1: 5′-TAA ACA ACA CAC ACT CTA AGG-3’

PEX2: 5′-CAT CTG ATT GAA GTG GAC-3’

PIN1: 5′-GGT TCA CGC ATA CGT TAA GCC TCA CTT-3’

PIN2: 5′-TAT CAC TAG ACA ATA ACC GGA TGG GTA-3’

The device for compartmentalization of a 0.5 ml microtube for nested RT-PCR in a single closed tube is according to Olmos *et al.* (1999). The RT-PCR master mix consists of two reaction mixtures:

A (dropped in the bottom of the microtube): ultrapure water, 15.8 µl; 10× Taq DNA polymerase buffer, 3 µl; 25 mM $MgCl_2$, 3.6 µl; 5 mM dNTPs, 2 µl; 4% Triton X-100, 2.2 µl; 25 µM primer PEX1, 0.6 µl; 25 µM primer PEX2, 0.6 µl; DMSO, 1.5 µl; 10 U/µl AMV reverse transcriptase, 0.2 µl; and 5 U/µl Taq DNA polymerase, 0.5 µl.

B (placed in the cone): ultrapure water, 2.6 µl; 10× Taq DNA polymerase buffer, 1 µl; 25 µM primer PIN1, 3.2 µl; and 25 µM primer PIN2, 3.2 µl.

The cycling parameters for RT-PCR are: 42 °C for 45 min and 92 °C for 2 min followed by 25 cycles of (92 °C for 30 s, 45 °C for 30 s and 72 °C for 1 min). After this first step, the tube is vortexed and centrifuged (6000 r.p.m. for 5 s) to mix B with the products of the first amplification. The tube is then placed in the thermal cycler again and the reaction proceeds as follows: 40 cycles of (92 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min), with a final elongation step at 72 °C for 10 min followed by cooling at 8 °C. The expected amplicon size is 131 bp.

The method was validated in a DIAGPRO ring test (Cambra *et al.*, 2002). A comparison with other techniques and the diagnostic parameters are given in section 3.7.

3.6.4 General considerations for RT-PCR and nested RT-PCR

The RT-PCR protocols may need to be modified and optimized when using different reagents or thermocycler platforms.

If conventional RT-PCR is used for the detection of CTV, IC-RT-PCR is recommended. Conventional RT-PCR without IC is not sensitive, and may give false negative results. It is possible that the presence of inhibitors affects the sensitivity of conventional RT-PCR.

The test on a sample is negative if the CTV-specific amplicon of the expected size is not detected in the sample in question but is detected in all positive controls. The test on a sample is positive if the CTV-specific amplicon of the expected size is detected in the sample in question, providing that there is no amplification in any of the negative controls.

3.6.5 Real-time RT-PCR

Two real-time RT-PCR assays have been described, one by Bertolini *et al.* (2008) and the other by Saponari *et al.* (2008).

According to Bertolini *et al.* (2008) the primers and probe are:

3′UTR1: 5′-CGT ATC CTC TCG TTG GTC TAA GC-3′

3′UTR2: 5′-ACA ACA CAC ACT CTA AGG AGA ACT TCT T-3′

181T: FAM-TGG TTC ACG CAT ACG TTA AGC CTC ACT TG-TAMRA

The reaction is carried out in a final volume of 25 µl. The real-time RT-PCR mixture consists of: ultrapure water, 0.95 µl; 2× AgPath-ID One-Step RT-PCR Master Mix (Applied Biosystems[1]), 12.5 µl; 25× RT-PCR enzyme mix, 1 µl; 10 µM primer 3′UTR1, 2.4 µl; 10 µM primer 3′UTR2, 2.4 µl; 5 µM probe FAM-labelled 181T, 0.75 µl; and 5 µl of RNA extracted or released from a membrane added to 20 µl of the real-time RT-PCR mix. The cycling parameters are: 45 °C for 10 min and 95 °C for 10 min followed by 45 cycles of (95 °C for 15 s and 60 °C for 1 min). The expected amplicon size is 95 bp.

For the tissue print real-time RT-PCR, a diagnostic sensitivity of 0.98, a specificity of 0.85, and a positive and negative likelihood ratio of 6.63 and 0.021, respectively, were estimated (Vidal *et al.*,

2012). These diagnostic parameters show that tissue print real-time RT-PCR was the most sensitive technique when compared with direct tissue print-ELISA, validating its use for routine CTV detection and diagnosis, and highly recommending it for assessing the CTV-free status of any plant material. The high sensitivity of this technique allows the accurate analysis of composite samples (up to ten batched trees or nursery plants) as one diagnostic sample when tested in any season of the year, and it also allows analysis of aphid species to detect low concentrations of CTV. For additional diagnostic parameters of validation of tissue print real-time RT-PCR, see section 3.7.

According to Saponari *et al.* (2008) the primers and probe are:

P25F: 5′-AGC RGT TAA GAG TTC ATC ATT RC-3′

P25R: 5′-TCR GTC CAA AGT TTG TCA GA-3′

CTV-CY5: CY5-CRC CAC GGG YAT AAC GTA CAC TCG G

The reaction is carried out in a final volume of 25 µl. The real-time RT-PCR mixture consists of: ultrapure water, 6.6 µl; 2× iScript One-Step RT-PCR Kit for Probes (Bio-Rad1), 12.5 µl; iScript reverse transcriptase supermix, 0.5 µl; 10 µM primer P25F, 1 µl; 10 µM primer P25R, 2 µl; 5 µM probe CTV-CY5, 0.4 µl; and 2 µl of RNA extracted or released from a membrane added to 23 µl of the real-time RT-PCR mix. The cycling parameters are: 55 °C for 2 min and 95 °C for 5 min followed by 40 cycles of (95 °C for 15 s and 59 °C for 30 s). The expected amplicon size is 101 bp.

Diagnostic parameters (i.e. sensitivity, specificity, accuracy, positive and negative likelihood ratios and post-test probability of disease) are not reported for this real-time RT-PCR protocol.

3.6.7 Interpretation of results from conventional and real-time RT-PCR

3.6.1 Controls for molecular tests

For the test result obtained to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid isolation and amplification of the target pest or target nucleic acid. For RT-PCR a positive nucleic acid control and a negative amplification control (no template control) are the minimum controls that should be used

Positive nucleic acid control. This control is used to monitor the efficiency of the test method (apart from the extraction) and in RT-PCR, the amplification. Pre-prepared (stored) RNA or CTV-infected plant material printed on a membrane may be used. The stored RNA or CTV preparations should be verified periodically to determine the quality of the control with increased storage time.

Internal control. For the real-time RT-PCR described by Saponari *et al.* (2008), mRNA of the mitochondrial gene *NADH dehydrogenase* 5 (*nad5*) could be incorporated into the RT-PCR protocol as an internal control to eliminate the possibility of RT-PCR false negatives due to nucleic acid extraction failure or degradation or the presence of RT-PCR inhibitors. Because this is a host target, care should be taken not to contaminate the laboratory with *nad5* DNA, which would result in false confidence in the internal control reaction.

Negative amplification control (no template control). This control is necessary for conventional and real-time RT-PCR to rule out false positives due to contamination during the preparation of the reaction mixture. RNase-free PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage.

Positive extraction control. This control is used to ensure that target nucleic acid extracted is of sufficient quantity and quality for RT-PCR and that the target virus is detectable. Nucleic acid is extracted from infected host tissue or healthy plant or insect tissues that have been spiked with CTV.

For RT-PCR, care needs to be taken to avoid cross-contamination due to aerosols from the positive control or from positive samples.

Negative extraction control. This control is used to monitor contamination during nucleic acid extraction and/or cross-reaction with the host tissue. The control comprises nucleic acid that is extracted from uninfected host tissue and subsequently amplified. Multiple controls are recommended to be included when large numbers of positive samples are expected.

3.6.7.1 Conventional RT-PCR and IC-RT-PCR

The pathogen-specific RT-PCR will be considered valid only if:

(1) the positive control produces the correct size amplicon for the virus; and

(2) the negative extraction control and the negative amplification control do not produce amplicons of the correct size for the virus.

If the mRNA mitochondrial gene *nad5* internal control primers are also used (forward: 5′-GAT GCT TCT TGG GGC TTC TTG TT-3′, reverse: 5′-CTC CAG TCA CCA ACA TTG GCA TAA-3′; 181 bp product), then the negative extraction control (healthy plant tissue) (if used), positive control and each of the test samples must produce a 115 bp amplicon. Failure of the samples to amplify with the internal control primers suggests for example that the RNA extraction has failed, RNA has not been included in the reaction mix, compounds inhibitory to RT-PCR are present in the RNA extract or the RNA has degraded.

The test on a sample will be considered positive if it produces an amplicon of the correct size.

3.6.7.2 Real-time RT-PCR

The pathogen-specific real-time RT-PCR will be considered valid only if:

(1) the positive control produces an amplification curve with the virus-specific primers; and

(2) the negative extraction control and the negative amplification control do not produce amplification curves with the virus-specific primers.

The test on a sample will be considered positive if it produces a typical amplification curve in an exponential manner. The cycle threshold (Ct) value needs to be verified in each laboratory when implementing the test for the first time.

3.7 Validation by a test performance study

In a DIAGPRO ring test (Cambra *et al.*, 2002) conducted by ten laboratories using a set of ten coded samples including CTV-infected and healthy tissue samples from the Valencian Institute of Agrarian Research (IVIA) collection, direct tissue print-ELISA using 3DF1 + 3CA5 monoclonal antibodies was 99% accurate (the number of true positives and true negatives diagnosed by the technique/number of samples tested). This accuracy was greater than that achieved with DAS-ELISA (98% accurate), IC-RT-PCR (94% accurate) and IC nested RT-PCR in a single closed tube (89% accurate). The sensitivity of direct tissue print-ELISA was 0.98 while the sensitivity of the other above-mentioned techniques was 0.96, 0.96 and 0.93, respectively (Vidal *et al.*, 2012). The diagnostic specificity of direct tissue print-ELISA was 1.0 while the diagnostic specificity of the other techniques was 1.0, 0.91 and 0.82, respectively. The positive predictive value (positive tests that actually have the disease; Sackett *et al.*, 1991) of direct tissue print-ELISA was 1.0 while the positive predictive value of the other techniques was 1.0, 0.94 and 0.89, respectively. The negative predictive value (Sackett *et al.*, 1991) of direct tissue print-ELISA was 0.97 while the negative predictive value of the other techniques was 0.95, 0.94 and 0.88, respectively (Harju *et al.*, 2000).

Direct tissue print-ELISA using 3DF1 + 3CA52 monoclonal antibodies was found to be the most reliable, simple and economical method for routine analysis of plant material when compared with biological indexing on Mexican lime, ELISA, IC-RT-PCR and IC nested RT-PCR for CTV detection (Cambra *et al.*, 2002). Direct tissue print-ELISA was also validated by Ruiz-García *et al.* (2005) and analysed by them to show that it was as sensitive as DAS-ELISA (the system detected 97% of positive

trees using four petioles) but was more user-friendly and less expensive. Direct tissue print-ELISA using 3DF1 + 3CA52 monoclonal antibodies was compared with biological indexing on Mexican lime and tissue print real-time RT-PCR for CTV detection (Vidal *et al.*, 2012). Various diagnostic parameters were evaluated and direct tissue print-ELISA was determined to be the most specific and accurate method, with the highest post-test probability of detecting the disease at any level of CTV prevalence.

4. Identification of Aggressive CTV Strains

The identification of CTV strains requires a biological, serological or molecular amplification test.

There are no nucleic acid-based methods allowing reliable typing of CTV strains according to their aggressiveness because CTV is a phenotype. The genetic basis of the high biological variability of CTV is still largely unknown (Moreno *et al.*, 2008). Little is also known about the biological role of its diversity and particularly about the effects of recombination. Additionally, genotype grouping has not been standardized (Harper, 2013). A wide range of molecular methods have been used to differentiate between different CTV strains, including molecular hybridization, double-stranded (ds)RNA patterns, restriction fragment analyses of amplified CTV cDNA, amplification by PCR of different genome regions, real-time PCR (Moreno *et al.*, 2008; Yokomi *et al.*, 2010), genome sequencing, and re-sequencing microarrays. More recently, sequential analyses of enzyme immunoassays and capillary electrophoresis-single-strand conformation polymorphism have been attempted (Licciardello *et al.*, 2012). However, none of these technologies is practical for the reliable categorization of naturally spreading CTV strains, and none has been validated yet, their application being limited to research purposes.

Given the genetic and biological variability of CTV, techniques other than sequencing may provide erroneous results when attempting to identify CTV strains. The use of deep sequencing, also referred to as next generation sequencing, could rapidly supply information about the genomic sequence. However, the nucleotide sequence of CTV cannot yet be related to the biological properties and behaviour of the strain (i.e. aggressiveness and transmissibility). Even though CTV strains have been classified and grouped by their phenotype, virulence, host range, epitope composition and, more recently, by sequence identity of one or more genes (Moreno *et al.*, 2008), no clear correlation with biological behaviour has been found (Harper, 2013).

The recommended methods to obtain information related to the biological properties of a specific CTV strain are (Figure 2):

(1) Biological indexing using a range of indicator plants such as C. aurantifolia, C. macrophylla, C. sinensis or C. paradisi (Duncan cultivar) for stem pitting evaluation; and C. aurantium or C. limon seedlings for seedling yellows evaluation (Roistacher, 1991; Ballester-Olmos *et al.*, 1993).

(2) Reactivity against the monoclonal antibody MCA13 (Permar *et al.*, 1990), which recognizes an epitope that is well conserved in severe (aggressive) CTV strains but lacking in mild (less aggressive) strains (Pappu, *et al.*, 1993). The reaction with MCA13 is strongly associated with the capacity to induce the decline of trees grafted on sour orange or lemon rootstocks. The majority of CTV strains that produce stem pitting in grapefruit or in sweet orange are MCA13-positive.

4.1 Biological indexing

Biological indexing of aggressive CTV strains follows the procedures set out in section 3.3.

4.2 Serological tests using MCA13

4.2.1 Direct tissue print-ELISA

A complete kit based on the CTV-specific MCA13 monoclonal antibody, including preprinted membranes with positive and negative controls and all reagents, buffers and substrate, is available from Plant Print Diagnòstics SL[1]. The method is as follows.

The membranes are tissue printed and blocked as in section 3.5.1. A solution of CTV-specific MCA13 monoclonal antibody linked to alkaline phosphatase (approximately 0.1 µg/ml in PBS) is prepared and poured onto the membranes, covering them, and the membranes are incubated for 3 h at room temperature, with slight agitation. Washing and development of the membranes and reading and interpretation of the results is as in section 3.5.1. The presence of usually small purple-violet precipitates in the vascular region of plant material reveals the presence of a CTV strain of increased aggressiveness.

4.2.2 DAS-ELISA

DAS-ELISA is performed according to Garnsey and Cambra (1991) using the method described below. A kit based on the CTV-specific MCA13 monoclonal antibody is available from Plant Print Diagnòstics SL[1].

Coating is performed as described in section 3.5.2. The CTV-specific MCA13 monoclonal antibody linked with alkaline phosphatase is added as conjugate at an appropriate dilution (approximately 0.1 µg/ml in PBS with 0.5% BSA). Incubation, washing, substrate addition and interpretation of results is according to section 3.5.2.

5. Records

Records and evidence should be retained as described in section 2.5 of ISPM 27 (Diagnostic protocols for regulated pests).

In cases where other contracting parties may be affected by the results of the diagnosis, in particular in cases of non-compliance and where the virus is found in an area for the first time, the following additional material, if relevant, should be kept in a manner that ensures traceability:

- The original sample should be kept at −80 °C or freeze-dried and kept at room temperature.
- RNA extractions should be kept at −80 °C and/or printed tissue sections and/or spotted plant extracts on paper or nylon membranes should be kept at room temperature.
- RT-PCR amplification products should be kept at −20 °C.

6. Contact Points for Further Information

Further information on this protocol can be obtained from:

Centro de Protección Vegetal, Instituto Valenciano de Investigaciones Agrarias (IVIA), Carretera Moncada-Náquera km 4.5, 46113 Moncada (Valencia), Spain (Mariano Cambra; e-mail: mcambra@ivia.es or mcambra@mcambra.es).

Departamento de Fitossanidade, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul (UFRGS), Avenida Bento Gonçalves 7712, 91540-000 Porto Alegre, Brazil (Edson Bertolini; e-mail: edson.bertolini@ufrgs.br; tel.: +55 (51) 3308 8100).

APHIS-USDA-PPQ-CPHST, 4700 River Road, Riverdale, MD 20737, United States (Laurene Levy; e-mail: laurene.levy@aphis.usda.gov; tel.: +1 301 851 2078; fax: +1 301 734 8724).

Citrus Research International (CRI), PO Box 28, 1200 Nelspruit, Mpumalanga, South Africa (S.P. Fanie van Vuuren; e-mail: faniev@cri.co.za).

Alico, Inc., Suite 100, 10070 Daniels Interstate Court, Fort Myers, FL 33913, United States (Marta Isabel Francis; e-mail: mfrancis@alicoinc.com; tel.: +1 863 673 4774).

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will in turn forward it to the Technical Panel on Diagnostic Protocols (TPDP).

7. Acknowledgements

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8. References

The present annex refers to ISPMs. ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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9. Figures

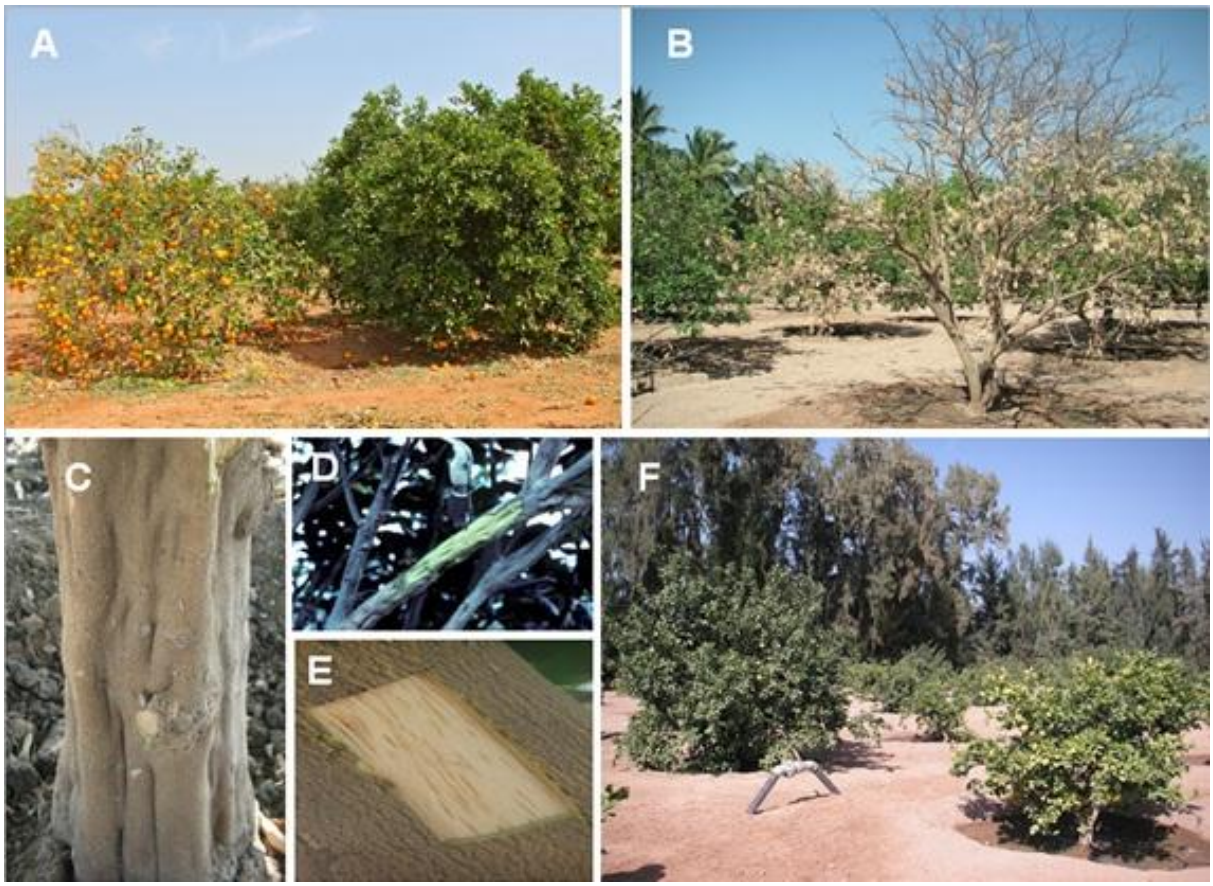

Figure 1. Symptoms of *Citrus tristeza virus* (CTV) infection: (A) tristeza syndrome or decline of sweet orange grafted on sour orange infected with CTV (left) and symptomless tree (right); (B) collapse or quick decline of grapefruit grafted on sour orange; (C) stem pitting on the trunk of grapefruit grafted on Troyer citrange caused by an aggressive CTV strain; (D) severe stem pitting on the branches of grapefruit; (E) stem pitting on the trunk of sweet orange grafted on Cleopatra mandarin; and (F) pronounced stunting of CTV-infected sweet orange trees grafted on Carrizo citrange (right) compared with a healthy tree (left).
Photo courtesy (A) P. Moreno; (B, C, E) M. Cambra; (D) L. Navarro; and (F) M. Cambra and J.A. Pina. All from Instituto Valenciano de Investigaciones Agrarias, IVIA, Moncada, Spain.

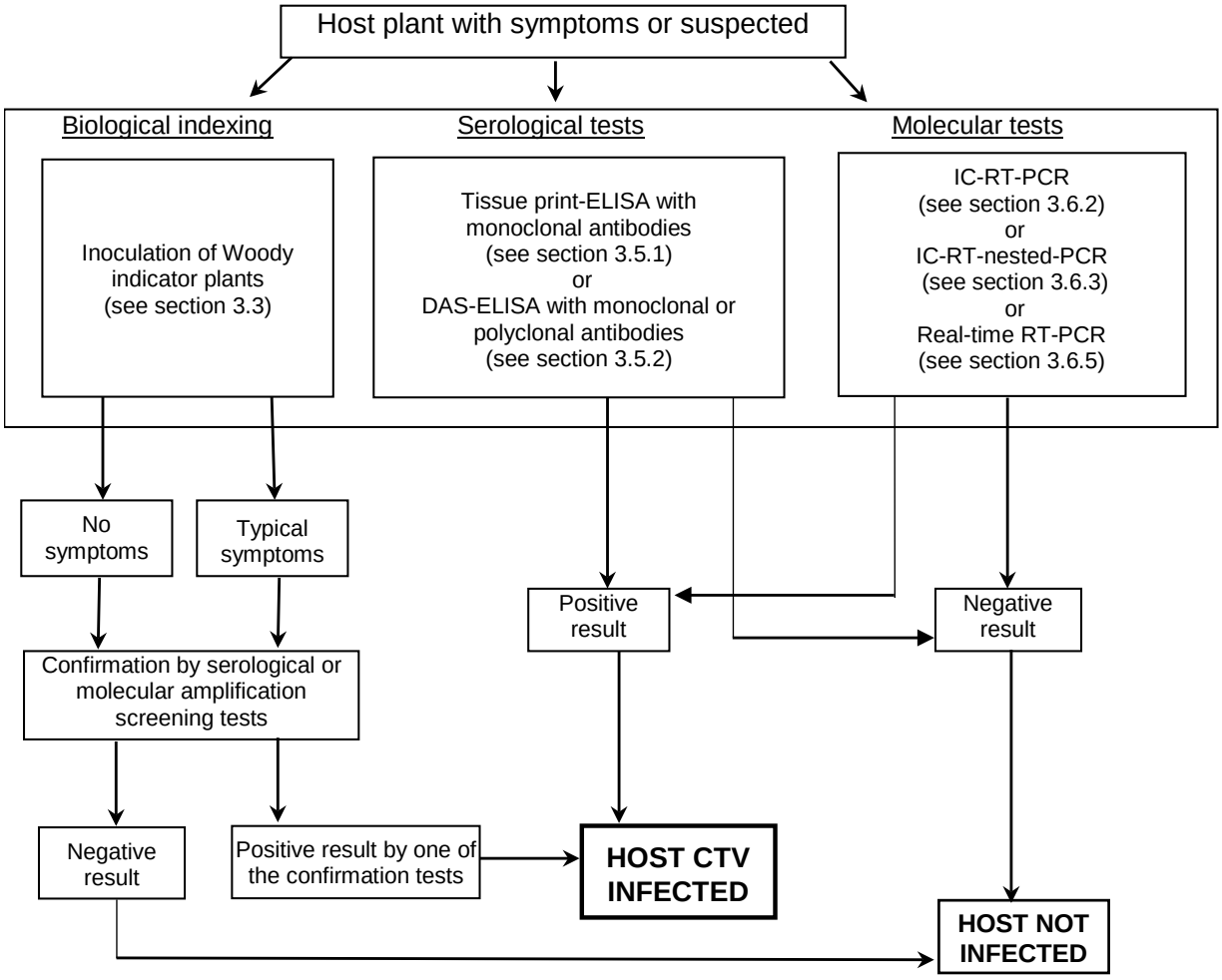

Figure 2. Flow chart for the detection and identification of *Citrus tristeza virus* (CTV).
DAS, double antibody sandwich; ELISA, enzyme-linked immunosorbent assay; IC, immunocapture; PCR, polymerase chain reaction; RT, reverse transcription.

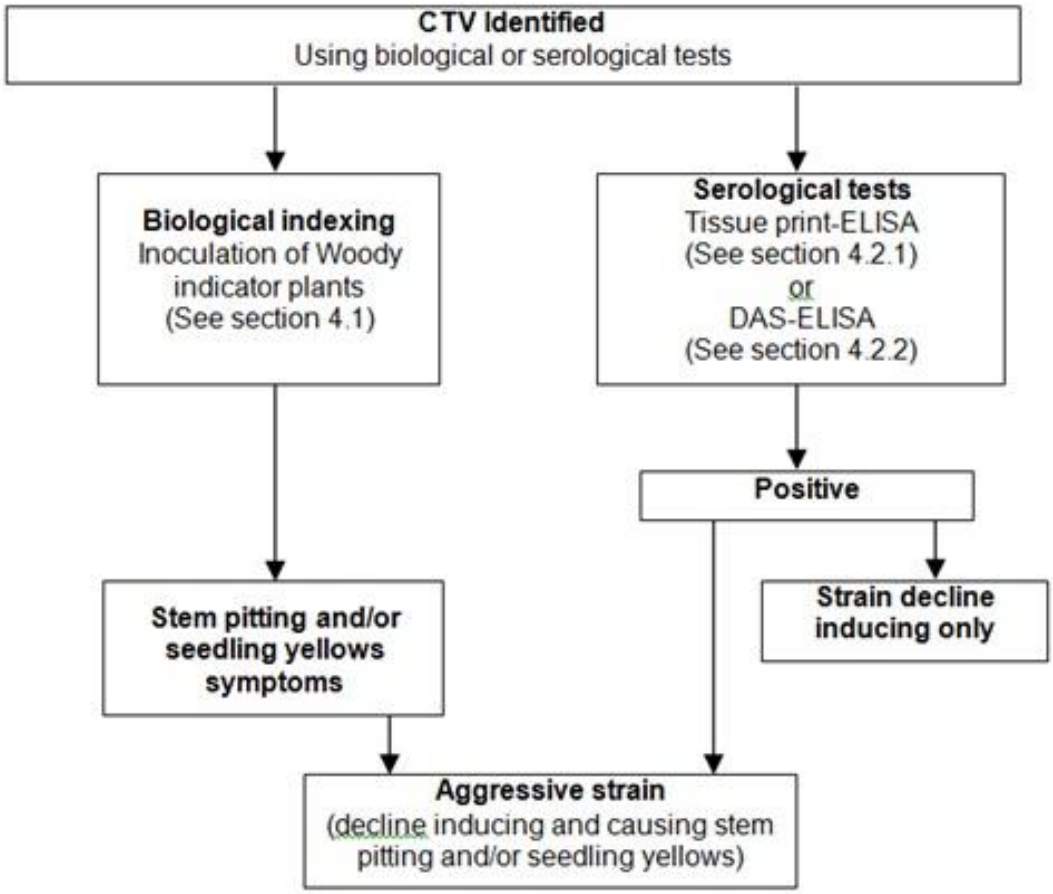

Figure 3. Flow chart for the identification of aggressive strains of *Citrus tristeza virus* (CTV). DAS, double antibody sandwich; ELISA, enzyme-linked immunosorbent assay.

Publication history

This is not an official part of the standard.

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2006-04 CPM-1 (2006) added work programme topic: Nematodes (2006-008).

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2015-02 Member consultation.

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2015-11 Submitted to SC for approved for DP notification period (2016_eTPDP_Feb_02).

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ISPM 27
Diagnostic protocols for regulated pests

DP 16: Genus *Liriomyza*

Adopted 2016; published 2016

CONTENTS

1. Pest Information

Agromyzidae is a family of small flies whose larvae feed on the internal tissue of plants, often as leafminers and stem miners. The majority of agromyzid species are either host-specific or restricted to a small group of plants that are related to each other. However, a few highly polyphagous species have become agricultural and horticultural pests in many parts of the world. These include four species of *Liriomyza* that are listed in plant quarantine legislation in various countries: *L. bryoniae*, *L. huidobrensis*, *L. sativae* and *L. trifolii*. These are all polyphagous pests of both ornamental and vegetable crops. The species level identification in this protocol is restricted to these four species.

Liriomyza is predominantly found in the north temperate zone but species are also found in the Afrotropical, Neotropical and Oriental regions. The adult flies of the 300-plus species of *Liriomyza* look very similar: they are all small (1–3 mm in length) and, from above, are seen to be largely black with, in most species, a yellow frons and scutellum (e.g. Figure 1). As a result, separating the species of the genus can be difficult. Furthermore, in order to identify the four species of quarantine concern a diagnostician not only has to distinguish between these four species, but also has to distinguish them from the relevant background fauna of indigenous *Liriomyza* species.

L. bryoniae is essentially a Palaearctic species with records from across Europe and Asia, and from Egypt and Morocco in North Africa (CABI, 2013). It is highly polyphagous and has been recorded from 16 plant families (Spencer, 1990). It is a pest of tomatoes, cucurbits (particularly melons, watermelon and cucumber) and glasshouse-grown lettuce, beans and lupins (Spencer, 1989, 1990).

L. huidobrensis is thought to have originated in South America and has now spread throughout much of the world, including parts of North America, Europe, Africa, Asia and the Pacific (Lonsdale, 2011; CABI, 2013). However, the species as formerly taxonomically defined was recently split into two morphocryptic species – *L. huidobrensis* and *L. langei* – and there is some uncertainty about the precise delineation of their relative distribution. Currently, *L. langei* has been confirmed only from the United States and is highly likely that all invasive populations outside the United States are *L. huidobrensis* as now taxonomically defined (Scheffer and Lewis, 2001; Scheffer *et al*., 2001; Takano *et al*., 2008; Lonsdale, 2011). *L. huidobrensis* is highly polyphagous and has been recorded from 14 plant families (Spencer, 1990). The most economically important crops it attacks are sugar beets, spinach, peas, beans, potatoes and ornamental plants (most commonly *Gypsophila*, rarely carnations and chrysanthemums) (Spencer, 1989).

L. sativae originated in North, Central and South America and has now been spread to many parts of Asia, Africa and the Pacific, but not to Europe or Australia (Lonsdale, 2011; CABI, 2013). However, distributional notes on *L. sativae* are likely to be incomplete as there is evidence to indicate that the species is continuing to expand its range rapidly. It is another highly polyphagous pest of many vegetable and flower crops (Spencer, 1973, 1990). It has been recorded from nine plant families, though it is mainly found on hosts in the Cucurbitaceae, Fabaceae and Solanaceae (Spencer, 1973, 1990).

L. trifolii, also originally from North, Central and South America, has been spread to large parts of Europe, Africa, Asia and the Pacific, most likely as the result of trade in chrysanthemums cuttings (Martinez and Etienne, 2002; Lonsdale, 2011; CABI, 2013). It is highly polyphagous and has been recorded from 25 plant families (Spencer, 1990). The most economically important crops it attacks are beans, celery, chrysanthemums, cucumbers, gerberas, *Gypsophila*, lettuce, onions, potatoes and tomatoes (Spencer, 1989).

A further (fifth) species, *L. strigata*, is included in the diagnostic protocol because it is closely related to both *L. bryoniae* and *L. huidobrensis*, and is as such a species that a diagnostician must be able to eliminate when seeking to positively identify the four quarantine species. *L. strigata* is an Eurasian species (Pitkin *et al.* (n.d.) quoting Spencer (1976), Dempewolf (2001), Ellis (2013) and Pape *et al.* (2013). The eastern borders of its distribution are not clearly defined, but the range extends beyond the Ural Mountains (Spencer, 1976) and it has been doubtfully recorded in Southeast Asia (Dempewolf,

2004). It is highly polyphagous, having been recorded from 29 plant families worldwide (Spencer, 1990).

2. Taxonomic Information

Name: *Liriomyza* Mik, 1894

Synonyms: *Agrophila* Lioy, 1864; *Antineura* Melander, 1913; *Haplomyza* Hendel, 1914; *Praspedomyza* Hendel, 1931; *Craspedomyza* Enderlein, 1936; *Triticomyza* Blanchard, 1938

Taxonomic position: Insecta, Diptera, Agromyzidae, Phytomyzinae

Name: *Liriomyza bryoniae* (Kaltenbach, 1858)

Synonyms: *Liriomyza solani* Hering, 1927; *Liriomyza hydrocotylae* Hering, 1930; *Liriomyza mercurialis* Hering, 1932; *Liriomyza triton* Frey, 1945; *Liriomyza citrulli* Rohdendorf, 1950; *Liriomyza nipponallia* Sasakawa, 1961

Common name: Tomato leafminer

Name: *Liriomyza huidobrensis* (Blanchard, 1926)

Synonyms: *Liriomyza cucumifoliae* Blanchard, 1938; *Liriomyza decora* Blanchard, 1954; *Liriomyza dianthi* Frick, 1958

The taxonomic relationship between *L. huidobrensis* (Blanchard) and *L. langei* Frick is complex. *L. huidobrensis* was originally described from specimens taken from *Cineraria* in Argentina by Blanchard (1926). Frick (1951) described *L. langei* from California as a species that he noted was primarily a pest of peas although it had also damaged *Aster*. In 1973, Spencer then synonymized the two species as they were (and de facto remain) morphologically indistinguishable. Following a study of their mitochondrial and nuclear DNA sequences (Scheffer, 2000; Scheffer and Lewis, 2001), and supported by later rearing experiments (Takano *et al*., 2008), the two species were formally separated as two cryptic species (Lonsdale, 2011). The name *L. langei* Frick was resurrected and applied to the cryptic species from California, and the name *L. huidobrensis* (Blanchard) was applied to the cryptic species from South and Central America.

Lonsdale (2011) attempted to delineate diagnostic morphological characters that could differentiate "most" specimens of the two species, but found the characters "subtle and sometimes overlapping" so he recommended the use of molecular data to support identification whenever possible. Scheffer and her collaborators consider that the ranges of the two species do not overlap (although Lonsdale (2011) recorded *L. huidobrensis* from California, once in 1968 and once in 2008, he states that it is unknown if the populations established), and that all of the invasive populations that they had studied were *L. huidobrensis* as so defined (Scheffer and Lewis, 2001; Scheffer *et al.*, 2001). This means that reports from California in the literature predating Scheffer's papers should almost certainly be considered as applying to *L. langei*. *L. langei* is predominantly a Californian species although it has apparently been introduced into Hawaii, Oregon and Washington; populations found in Florida, Utah and Virginia in the mid-1990s did not establish (Lonsdale, 2011). Only *L. huidobrensis* has been confirmed in Mexico (Lonsdale, 2011), but Takano *et al*. (2005) reported that specimens of *L. langei* (described as the Californian clade) were intercepted at a Japanese inspection site on fresh vegetables originating from Mexico.

Common names: Serpentine leafminer, pea leafminer, South American leafminer, potato leafminer fly

Name: *Liriomyza sativae* Blanchard, 1938

Synonyms:	*Agromyza subpusilla* Frost, 1943; *Liriomyza verbenicola* Hering, 1951; *Liriomyza pullata* Frick, 1952; *Liriomyza canomarginis* Frick, 1952; *Liriomyza minutiseta* Frick, 1952; *Liriomyza propepusilla* Frost, 1954; *Liriomyza munda* Frick, 1957; *Liriomyza guytona* Freeman, 1958; *Lemurimyza lycopersicae* Pla and de la Cruz, 1981.
Common names:	Vegetable leafminer, American leafminer, chrysanthemum leafminer, serpentine vegetable leafminer, melon leafminer
Name:	*Liriomyza trifolii* (Burgess, 1880)
Synonyms:	*Agromyza phaseolunulata* Frost, 1943; *Liriomyza alliovora* Frick, 1955
Common names:	American serpentine leafminer, serpentine leaf miner, broad bean leafminer, Californian leafminer, celery leafminer, chrysanthemum leaf miner

3. Detection

Feeding punctures and leaf mines are usually the first and most obvious signs of the presence of *Liriomyza*. While fully formed mines should be readily visible to quarantine officials, early signs of infestation are much less obvious and are easily overlooked (Spencer, 1989). Mines remain intact and relatively unchanged over a period of weeks. Mine configuration is often considered a reliable guide to the identification of agromyzid species (as in many such cases the species are host-specific). However, with the polyphagous pest species, mine configuration is affected by the host, by the physical and physiological condition of each leaf, and by the number of larvae mining the same leaf. This wider variability means that identification from mine configuration alone should be treated with caution (EPPO, 2005). Examples of mine configuration for the four quarantine species and *L. strigata* are provided in Figures 2 to 4.

Female flies use their ovipositor to puncture the leaves of the host plants, causing wounds that serve as sites for feeding (by both female and male flies) or for oviposition. Feeding punctures of *Liriomyza* species are rounded, usually about 0.2 mm in diameter, and appear as white speckles on the upper surface of the leaf. Oviposition punctures are usually smaller (0.05 mm) and more uniformly round. Feeding punctures made by the polyphagous agromyzid pest species *Chromatomyia horticola* and *Chromatomyia syngenesiae* are distinctly larger and more oval than those made by *Liriomyza* flies. The appearance of feeding and oviposition punctures does not differ among *Liriomyza* species, and the pattern of their distribution on the leaf cannot be used to identify species. Feeding punctures cause the destruction of a large number of cells and are clearly visible to the naked eye (EPPO, 2005).

The larvae feed mostly in the upper part of the leaf, mining through the green palisade tissue. Mines are usually off-white, with trails of frass appearing as broken black lines along the length of the leaf. Repeated convolutions in the same small area of the leaf will often result in discoloration of the mine, with dampened black and dried brown areas appearing, usually as the result of plant-induced reactions to the leafminer (EPPO, 2005).

There are three larval stages, all of which feed within the leaves. The larvae predominantly feed on the plant in which the eggs are laid. The larvae of *Liriomyza* spp. leave the leaf when ready to pupariate (Parrella and Bethke, 1984), and their exit hole characteristically takes the form of a semicircular slit; in contrast, the larvae of *C. horticola* and *C. syngenesiae* pupate inside the leaf at the end of the larval mine, with the anterior spiracles usually projecting out from the lower surface of the leaf. *Liriomyza* puparia, therefore, may be found in crop debris, in the soil or sometimes on the leaf surface.

Species may be found in different locations of the plant and surrounds depending on the life stages present, as follows:

- eggs: inserted just below the leaf surface
- larvae: inside mines on leaves
- pupae: in crop debris, in the soil or sometimes on the leaf surface
- adult: free-flying, or on leaf surfaces while producing feeding and oviposition punctures.

3.1 Collection and preservation of specimens

Liriomyza flies can be collected as immature life stages in association with mined leaf samples or as adults. Because the morphological characters used to diagnose species are based on male genitalia, adult males are needed in order to confirm species identification. Adult females are often identifiable with certainty only to genus level. Collecting multiple specimens from a plant or a location will increase the likelihood of obtaining male flies, which is important unless molecular tests are to be used for diagnosis of immature life stages.

3.1.1 Collecting adults

Adult flies are normally found on the foliage, and can be collected by hand or swept from the foliage with a hand net into glass vials, or collected with a vacuum sampler. Alternatively, they can be collected by using yellow sticky traps, particularly in glasshouses. However, the most practical and reliable method for collecting leafminer flies such as *Liriomyza* species is to collect mined leaves containing live larvae. These can be placed in a large jar for rearing to adult flies in the laboratory. Techniques for rearing agromyzids are described in Griffiths (1962) and Fisher *et al.* (2005).

Adults and larvae can be placed in 70% ethanol and stored indefinitely, although their colour fades gradually with time. Vials of specimens in ethanol should be sealed to avoid leakage and packed with cushioning material in a strong box. Dry storage of adults, for example as pinned specimens, is also possible.

Specimens required for molecular diagnostic work should be killed and preserved in 96–100% ethanol, stored frozen (at about –20 or–4.0 °C) or preserved on FTA cards (Whatman)[1] (Blacket *et al.*, 2015).

3.1.2 Collecting immature life stages

If the intention is to collect and preserve plant samples, leaves with suspect feeding punctures or mines should be picked and placed between sheets of newspaper to permit slow drying.

Leaves with occupied mines from which it is intended to rear individuals in the laboratory in order to obtain life stages, particularly adults, for identification need to be packed in slightly damp, but not overly wet, laboratory tissue, and mailed in padded and sealed bags. In the laboratory, the mined leaves with live larvae can be placed in sealed Petri dishes with damp filter paper inserts and stored in an incubator at about 23 °C (checking every two or three days to remove leaves that are developing fungus, bacteria, etc.).

[1] In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

4. Identification

Identification of leafminer species by morphological examination is restricted to adult male specimens because there are no adequate keys for the species-level identification of adult females or for eggs, larvae or pupae. Identification of adult material is possible by examination of morphological characters, in particular the genitalia of the male fly. The morphological characters of the male genitalia are examined under a high-power microscope (at about 100× magnification). Using this protocol with good quality preparations should allow adults of the four quarantine species of *Liriomyza* to be identified with certainty by morphological examination alone (with the exception of *L. huidobrensis* and *L. langei* for the reasons discussed in section 1).

Molecular tests for identification can be applied to all life stages, including the immature stages for which morphological identification to species level is not possible. Additionally, in cases where adult specimens are atypical or damaged, molecular tests may provide further relevant information about identity. However, the specificity of molecular tests may be limited as they will have been developed for a purpose and evaluated against a restricted number of species, using samples from different geographic regions. Therefore, the results from molecular tests need to be carefully interpreted.

4.1 Morphological identification of the adult *Liriomyza*

Examination of the male genitalia (in particular, the distiphallus (Figure 5)), is necessary in order to obtain a positive identification for any of the four target species of *Liriomyza*. A brief account of a satisfactory method of preparing specimens (based on Malipatil and Ridland, 2008) is outlined below. More details on or variations to the method are provided by Spencer (1981, 1992), Spencer and Steyskal (1986) and EPPO (2005). Evidence of distiphallic structure should be compared with characters of external morphology (Table 1) in order to confirm the species identification.

4.1.1 Preparation of the genitalia of adult male *Liriomyza* for microscopic examination

4.1.1.1 Determining the sex of flies

In the male fly, the lobes of the epandrium, which are dark and pubescent and not as heavily sclerotized as the female tube, curve around and down at the rear of the abdomen, from the dorsal to the ventral sides (Figure 6(a)). A slit-like opening is seen between the lobes, triangular when more fully open, through which the rest of the male genitalia can be viewed. The lobes barely extend beyond the last tergite. In the female fly, the abdominal segments beyond segment 6 form a black, heavily sclerotized tube that extends beyond the 6th tergite (Figure 6(b)), with a circular opening visible in posterior view at the end of the tube. The 6th tergite covers the basal half of the tube from above, though it is visible in lateral and ventral views.

4.1.1.2 Preparation of the male distiphallus for examination

The abdomen should be removed from the body to enable clearing of tissues and observation. This can be accomplished by using fine dissecting needles (which can be made by gluing the blunt end of pointed micro pins into the end of a wooden matchstick, first making a shallow hole with a normal pin), to carefully separate the abdomen from the rest of the fly. The abdomen can be boiled in 10% potassium hydroxide (KOH) or sodium hydroxide (NaOH) for 2–4 min or, alternatively, left in cold 10% KOH or NaOH overnight to clear the tissues. Transferring the treated abdomen to a bath of distilled water will neutralize the KOH or NaOH. The abdomen is then ready for transfer to a drop of glycerol on a cavity slide.

Under a binocular stereomicroscope and using the fine dissecting needles, the genital complex is carefully dissected out from the surrounding membranes, cuticle and associated musculature. Using the fine dissecting needles, the genital complex is positioned for lateral viewing under a compound microscope at up to 400× magnification. The genital complex is repositioned for ventral viewing of the distiphallus at 400× magnification, without the addition of a cover. The distiphallus needs to be

viewed in different orientations (e.g. lateral, dorsal, ventral), which requires repositioning under a lower magnification.

To make semi-permanent slides (e.g. for routine identification), the genital complex should be transferred to a drop of glycerol on a clean flat slide. The genitalia are immersed gently in the mountant, and a round coverslip is lowered carefully over it to evenly spread the mountant.

If permanent slide mounts are required, the abdomen should be cleared in KOH and neutralized in cold glacial acetic acid as described above. Then, the abdomen can be transferred to 70% ethanol and, using the fine dissecting needles under a binocular stereomicroscope, the genital complex carefully dissected from the surrounding membranes, cuticle and associated musculature. The dissected genitalia should be transferred first to absolute ethanol for 2–4 min, then to clove oil (in which, if necessary, they can be stored for any length of time). The genitalia are transferred to 70% ethanol (for approximately 10 min), then to 95% ethanol (for approximately 10 min) and finally to clove oil (for at least 5 min). The genitalia can then be permanently mounted on a slide in a drop of Canada balsam under a coverslip. All slide mounts must be labelled with adequate data detailing locality, host, date of collection, name of collector (if known), species name, name of identifier, and a code label to cross-reference to the remaining specimen.

The remainder of the fly specimen should be mounted on a card point with an appropriate label cross-referenced to its genitalia mounted on the slide.

4.1.2 Identification of the family Agromyzidae

Worldwide, the family Agromyzidae comprises about 2 500 species (Spencer, 1989, 1990). Detailed descriptions of agromyzid morphology are given by Spencer (1972, 1973, 1987), Dempewolf (2004) and Boucher (2010).

Morphological nomenclature here follows Yeates *et al*. (2004). This online resource can also be consulted for clear illustrations of the anatomy of a typical acalyptrate fly (such as Agromyzidae).

The following combination of characters define the family Agromyzidae (Hennig, 1958; Spencer, 1987; Boucher 2010) (Figure 7):

- small in size, up to 1–6 mm, but usually 1–3 mm
- vibrissae present
- one to seven frontal setae present
- wing with costal break present at the apex of Sc
- wing cell cup small; wing veins A_1+CuA_2 not reaching wing margin
- male with pregenital sclerites with a fused tergal complex of tergites 6–8, with only two spiracles between tergite 5 and the genital segment
- female with the anterior part of abdominal segment 7 forming an oviscape.

Generally the larvae (Figure 8(a)) are cylindrical in shape, tapering anteriorly, with projections bearing the anterior and posterior spiracles (Figure 8(b) and (d)), the former located on the dorsal surface of the prothorax, the latter posteriorly directed at the rear. The larvae also possess strongly sclerotized mouthparts; the mandibles with their longitudinal axis are at about right angles to the rest of the cephalopharyngeal skeleton (Figure 8(c)) and usually bear two or more pairs of equally sized anteriorly directed teeth, with the ventral cornua (the posteriorly directed paired “arms”) commonly shorter than the dorsal ones.

In practice, agromyzids are recognizable because their larvae feed in the living tissue of plants (three-quarters of them are leafminers). However, there are leafminers in other Dipteran families such as Anthomyiidae and Drosophilidae. For a summary of information on the morphology and biology of the immature stages of agromyzids, with an extensive bibliography and illustrations of the cephalopharyngeal skeleton and posterior spiracles for a number of species, see Ferrar (1987).

4.1.3 Identification of the genus *Liriomyza*

Adult flies of the genus *Liriomyza* have the following morphological characters (EPPO, 2005; Spencer, 1976):

- fronto-orbital setulae reclinate (backward pointing)
- dark pre-scutellar area concolorous with the scutum in most species, rarely yellow
- scutellum yellow in most species, rarely dark
- subcosta becomes a fold distally and ends in costa separately
- costa extends to vein M_{1+2}
- discal cell (dm) small
- second (outer) crossvein (dm-cu) present in most species
- stridulating organ present in males (a "scraper", a chitinized ridge on the hind femora; and a "file", a line of low chitinized scales on the connecting membrane between the abdominal tergites and sternites).

In practice, most species of *Liriomyza* (including the four target species included in this diagnostic protocol) are seen from above to be mostly black with a yellow frons and a bright yellow scutellum. The legs are variably yellow. The target species possess the typical wing venation (Figure 9) and the generalized male genitalia of the genus.

There are several genera that may be confused with *Liriomyza*. The closely related genera *Phytomyza*, *Chromatomyia* and *Phytoliriomyza* can generally be separated from *Liriomyza* by their proclinate (forward pointing) fronto-orbital setulae (always reclinate or occasionally upright or missing in *Liriomyza*), and by the scutellum, which is generally grey or black but occasionally slightly yellowish centrally (entirely yellow in most *Liriomyza*). In *Phytomyza* and *Chromatomyia*, the costa extends only to R_{4+5}, whereas in *Phytoliriomyza* and *Liriomyza* it extends to vein M_{1+2} (Spencer, 1977). *Phytoliriomyza* species are gall-forming (on a stem or leaf) internal feeders, whereas *Chromatomyia*, *Phytomyza* and *Liriomyza* species are typically leafminers.

4.1.4 Identification of *Liriomyza* species

4.1.4.1 Morphological characters of adult *Liriomyza* spp.

A simplified summary of the main diagnostic characters for *L. bryoniae*, *L. huidobrensis*, *L. sativae* and *L. trifolii* (as well as for *L. strigata* for the purposes of elimination) is given in Table 1. This is accompanied by illustrative images (photomicrographs) of the distiphallus in Figures 10 and 11.

More detailed descriptions and illustrations of the morphology of these species are provided by Spencer (1965, 1973), Dempewolf (2004), Malipatil *et al.* (2004) and Shiao (2004). Key diagnostic features are shown in the Pest and Disease Image Library (PaDIL) (Malipatil 2007a, 2007b, 2007c).

Identification of the adults can also be carried out with keys. Malipatil and Ridland (2008) provide a key to 17 species of economic importance, including a few species endemic to Australia. In addition, an identification system for pest species from around the world based on photomicrographs is available in Dempewolf (2004). With particular reference to keys for *Liriomyza* species, there are some extensive regional back catalogues and keys available through the works of Spencer. These cover the regional background fauna, which obviously differs from region to region, and by doing so differentially affects the positive process of eliminating non-target taxa. A full list of these works is listed in Spencer (1973).In addition, considering the host plant on which the suspected quarantine *Liriomyza* species has been detected can help by narrowing down the other potential agromyzid species that may occur in the same biological context and which may need to be eliminated from consideration (e.g. for Europe, see Ellis (n.d.).

Table 1. Adult morphological characters of selected *Liriomyza* species[†]

	L. bryoniae	***L. huidobrensis*[‡]**	***L. sativae***	***L. strigata***	***L. trifolii***
Male distiphallus	Two distal bulbs; bulb rims circular	Two distal bulbs, meeting only at their rims; bulb rims drawn out antero-ventrally	One distal bulb with a slight constriction between upper and lower halves in dorso-ventral view; bulb appears more strongly sclerotized with a shorter basal stem	Two distal bulbs, meeting from their rims to their bases; bulb rims drawn out antero-ventrally	One distal bulb with marked constriction between lower and upper halves in dorso-ventral view; bulb appears less distinctly sclerotized with a longer basal stem
Vertical setae	Both vertical setae on yellow ground	Both vertical setae on black ground	Outer vertical setae on black ground that may just reach inner vertical setae, which are otherwise on yellow ground	Black coloration behind the eyes extending to at least the outer vertical setae, but inner vertical setae on yellow ground	Both vertical setae on yellow ground
Anepisternum	Predominantly yellow, small black mark at front lower margin	Yellow with variable black patch generally across the lower three-quarters	Predominantly yellow, with dark area varying in size from a small bar along the lower margin to a patch along the entire lower margin, well up the front margin and narrowly up the hind margin	Yellow, but with black patch variable on lower and front margins, and this can extend along the lower half	Yellow, small blackish grey mark at front lower margin
Vein Cu 1A	*a* twice length of *b*	*a* 2–2.5 times length of *b*	*a* 3–4 times length of *b*	*a* 2–3 times length of *b*	*a* 3–4 times length of *b*
Third antennal segment	Small, yellow	Slightly enlarged, usually darkened	Small, yellow	Small, yellow	Small, yellow
Frons and orbits	Frons bright yellow, orbits slightly paler	Frons yellow, generally more orange than pale lemon-yellow; upper orbits slightly darkened at least to upper orbital setae	Frons and orbits bright yellow	Frons and orbits yellow	Frons and orbits yellow

	L. bryoniae	*L. huidobrensis*‡	*L. sativae*	*L. strigata*	*L. trifolii*
Femur	Bright yellow with some brownish striations	Yellow, variably darkened with black striations	Bright yellow	Yellow with some brownish striations	Yellow, occasional slight brownish striations
Mesonotum	Black, largely shining but with distinct matt undertone	Black, matt	Black, shining	Black, shining but slightly matt	Matt black with grey undertone
Male abdominal tergites	Second and third visible tergites divided by a yellow medial furrow	Only the second visible tergite divided by a yellow medial furrow	Only the second visible tergite divided by a yellow medial furrow	–	Second to fifth visible tergites divided by a yellow medial furrow
Wing length	1.75–2.1 mm	1.7–2.25 mm	1.3–1.7 mm	1.8–2.1 mm	1.3–1.7 mm

Source: Compiled from Spencer (1973, 1976), with information on the distiphallus from EPPO (2005) and information on the male abdominal tergites from Shiao (2004) (who did not include *L. strigata* in his analysis).

† See also Figures 7 to 11.

‡ *L. langei* is morphologically indistinguishable from *L. huidobrensis.*

4.1.4.2 Distiphallic structure of adult male *Liriomyza* spp.

The *Liriomyza* species considered here separate into two distinct natural groups based on the structure of the male genitalia (particularly the distiphallus) as well as the body colour and the structure of the posterior spiracles of the larvae:

- group 1: *L. bryoniae*, *L. huidobrensis* and *L. strigata*
- group 2: *L. sativae* and *L. trifolii*.

However, the external characters of the adult flies useful for identification (Table 1), particularly those based on colour, do not fall neatly into these two groupings.

The distiphallus is a very small, fragile structure enclosed by membranes. It is the terminal part of the aedeagus (the intromittent organ, part of the male genitalia) (Figure 5) and its complex three-dimensional structure is of considerable diagnostic value. Indeed, the distiphallus provides a single character by which all four target species can be identified reliably. The basic structure of the distiphallus differs in the two natural species groups: in group 1, there are two distal bulbs side by side (Figure 10), while in group 2, there is only one distal bulb, which has a medial constriction dividing it into distinct lower and upper sections (Figure 11). A key that facilitates identification of the four target species using the distiphallus is provided below. For convenience, the key also includes *L. strigata*, which is closely related to *L. bryoniae* and *L. huidobrensis* and is also polyphagous and therefore to be found on similar host plants.

However, the differences between some of the species pairs are subtle and the evidence of the distiphallic structure should be cross-checked with the evidence of external morphology (Table 1) in order to ensure that the distiphallic structure has not been misinterpreted. If all the evidence correlates, all other species of *Liriomyza*, including those not discussed here, can be eliminated.

Diagnostic key for identification of *Liriomyza* spp. using the male distiphallus

This key is to be used in conjunction with Figures 10 and 11.

1. With one distal bulb (Figure 11(e), (f))……**2**

– With a pair of distal bulbs (Figure 10(a)–(c), (g)–(k))……**3**

2. With marked constriction between the apical and basal parts of the bulb: basal section strongly curved (Figure 11(f))…….....***L. trifolii***

– With slight constriction only between the apical and basal parts of the bulb: basal section not strongly curved (Figure 11(e))……***L. sativae***

3. With bulb rims circular (not drawn out antero-ventrally); evenly sclerotized (Figure 10(a)) …….....***L. bryoniae***

– With bulb rims spiralled (drawn out antero-ventrally) (Figure 10(b), (c))……**4**

4. With bulbs meeting in the midline only at their rims (Figure 10(h))……***L. huidobrensis****

– With bulbs meeting in the midline from their rims to their bases (Figure 10(i))……***L. strigata***

* *L. langei* is morphologically indistinguishable from *L. huidobrensis.*

4.1.4.3 Morphological characteristics of the immature stages of the four target species of Liriomyza

Of the four life stages (egg, larva, pupa and adult) only the adult male flies can be positively identified to species level using morphological features (the shape of the male genitalia). The morphological characteristics of larvae and pupae can be used to distinguish between the members of the two natural species groups described in section 4.1.4.2. This information can contribute towards species identification but is insufficient by itself to allow species identification. To complement morphological identification, molecular assays can be used to distinguish between the species included in the protocol (section 4.2).

Eggs

The eggs are laid into the leaf tissue. They are white and oval, about 0.25 mm in length. Neither genus nor species identification is possible.

Larvae and pupae

There are three larval instars, which feed as they tunnel through the leaf tissue. The newly emerged larvae are about 0.5 mm long but reach 3.0 mm when fully grown. They are typical of agromyzids in their gross form (see section 4.1.2). Pupae (Figure 12) are oval cylinders in shape, about 2.0 mm in length, very slightly flattened ventrally, with projecting anterior and posterior spiracles. In practice, for larvae and pupae, the two natural groups can be distinguished from each other (but not the species within the groups) morphologically as follows.

Group 1 larvae

Larvae of *L. bryoniae*, *L. huidobrensis* and *L. strigata* are cream-coloured but in the final instar develop a yellow–orange patch dorsally at the anterior end, which can extend around to the ventral surface (Figure 13)). Each posterior spiracle consists of an ellipse with pores along the margin. It can be difficult to observe the number of pores, which according to Spencer (1973) are: *L. bryoniae*, 7–12 pores; *L. huidobrensis*, about 6–9 pores; and *L. strigata*, 10–12 pores. Puparia are variable in colour, from yellow–orange to dark brown. In *L. bryoniae* and *L. strigata*, puparia are mostly, but not exclusively, at the lighter end of the colour range. The colour of *L. huidobrensis* puparia mostly tends to anthracite. The form of the larval spiracles is retained in the puparium although the pores are less clearly discernible.

Group 2 larvae

Larvae of *L. sativae* and *L. trifolii* are translucent when newly emerged and yellow–orange over the entire body later. Each posterior spiracle is tricorn-shaped with three pores, each on a distinct projection, the outer two elongate. Puparia are yellowish orange, sometimes a darker golden brown. The form of the larval spiracles is retained in the puparium but the detail is less obvious.

4.2 Molecular identification of *Liriomyza* species

Various polymerase chain reaction (PCR)-based molecular tests have been used to identify *Liriomyza* species, including PCR-restriction fragment length polymorphism (RFLP), end-point PCR using species-specific primers, real-time PCR, and DNA sequence comparison. Of these tests, the ones that can be used to distinguish between the four target species (i.e. *L. bryoniae*, *L. huidobrensis*, *L. sativae* and *L. trifolii*) or between *L. huidobrensis* and *L. langei* are described below.

In this diagnostic protocol, tests (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. No method reported for these species has been formally validated for analytical sensitivity and reproducibility.

The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

The specificity of each method is described below. This indicates the *Liriomyza* species against which each method was evaluated and the original use for which the assay was designed. Considering the specific limitations of molecular tests, a negative molecular test result does not exclude the possibility of positive identification by morphological tests.

4.2.1 Controls for molecular tests

For the test result obtained to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid isolation and amplification of the target pest nucleic acid. For PCR a positive nucleic acid control, a negative amplification control (no template control) and, when relevant, a negative extraction control are the minimum controls that should be used.

4.2.2 DNA Extraction

DNA suitable for PCR applications can be successfully extracted from a single larva, pupa or adult *Liriomyza* specimen using various commercial DNA extraction kits and following manufacturer instructions (Scheffer *et al.*, 2001, 2006; Kox *et al.*, 2005; Nakamura *et al.*, 2013). For additional information on the kits used for each of the tests described below, refer to the source paper. Laboratories may find that alternative extraction techniques work equally well; DNA may be extracted using any DNA extraction method suitable for insects. The treated tissue is crushed or ground using a sterile micropestle or similar apparatus in all published protocols.

Positive nucleic acid control. This control is used to monitor whether or not the test performed as expected under the experimental conditions and parameters. A positive control can be any nucleic acid that contains the target sequence (i.e. *Liriomyza* nucleic acid that has been analysed previously).

Negative amplification control (no template control). This control is necessary for PCR to rule out false positives due to contamination during preparation of the reaction mixture or non-specific amplification. PCR-grade water that was used to prepare the reaction mixture is added in place of the DNA volume at the amplification stage.

Negative extraction control. This control is used to monitor contamination during nucleic acid extraction and/or cross-reaction with the host tissue. The control comprises an extraction reaction without tissue sample added.

4.2.3 PCR-RFLP identification of the four target species

Kox *et al.* (2005) report a PCR-RFLP assay of a region on the *Cytochrome oxidase* II (*COII*) gene that can be used to distinguish the four target species. The specificity of the assay was further investigated by analysing four additional *Liriomyza* species: *L. strigata, L. langei, L. chinensis* and *L. scorzonerae*. The *L. langei* and *L. huidobrensis* specimens could not be distinguished with this assay. The other three species were separated successfully.

4.2.3.1 Amplification of the COII gene

According to Kox *et al.* (2005), samples are amplified in a 50 µl reaction mixture composed of the following final concentrations of reagents: 0.6 µM each primer, 0.2 mM dNTPs, 1 U HotStarTaq[1] DNA polymerase, 1× PCR buffer and 1.5 mM $MgCl_2$. Each reaction includes either 1–5 µl DNA as a template or PCR-grade water as a negative control. PCR is performed using the following primer pair:

TL2-J-3037-forward (F): 5´-ATGGCAGATTAGTGCAATGG-3´ (Simon *et al.*, 1994)

K-N-3785Lir-reverse (R): 5´-GTT(A/T)AAGAGACCATT(A/G)CTTG-3´ (Kox *et al.*, 2005)

The thermal cycling parameters for PCR are a 15 min denaturation step at 95 °C followed by 35 cycles of (15 s at 94 °C, 1 min at 55 °C and 45 s at 72 °C) and a final extension step for 10 min at 72 °C before cooling to room temperature. After PCR amplification, 5 µl of the PCR product is subjected to electrophoresis on a 1.5% agarose gel in Tris-acetate-ethylenediaminetetraacetic acid (EDTA) (TAE) buffer with a 100 base pair (bp) DNA ladder to confirm the presence of PCR products before RFLP analysis.

The *COII* PCR is considered valid only if:

- the positive control produces an amplification product of the expected size for the target *COII* gene
- the negative extraction control and the negative amplification control do not produce an amplification product of the expected size for the target *COII* gene.

4.2.3.2 Restriction digestion and separation of products

For each sample, 5 µl of PCR product is digested with restriction enzymes *Dde*I, *Hinf*I, *Ssp*I and *Taq*I, each in a separate reaction, according to the manufacturer's instructions. Digested PCR product is then separated by electrophoresis on a 3% agarose gel in TAE buffer along with a 100 bp DNA ladder to allow the size of the fragments to be determined.

It is not possible to determine the exact fragment size of digested products separated under the electrophoretic conditions described, but relative separation values are used to compare results with expected RFLP profiles for the species. Positive control samples with known fragment sizes and patterns can be run alongside test samples to enable comparison of sizes more precisely. A positive control should be included for each digestion enzyme tested to ensure that the enzyme digests the DNA as expected. The RFLP test is considered valid only if the positive control produces fragments of the expected size for the target *COII* gene. The RFLP patterns observed on the agarose gel allow differentiation of the four target species of *Liriomyza*. Diagnostic profiles for the species are provided in Table 2 by enzyme. If the composite fragment profile of a sample matches the known fragment profile of one of the five species in the table, the sample can be identified as that species based on the assay. If the fragment profile does not match one of the known species fragment profiles, the sample is not diagnosed to species based on the assay. If a sample is diagnosed as *L. huidobrensis*, further testing may be needed to confirm it is not the cryptic species *L. langei* (section 4.2.5).

Table 2. Restriction fragment length polymorphism profiles for *Liriomyza* species

	Predicted fragment sizes (base pairs) for restriction enzymes			
Species	*Dde*I	*Hinf*I	*Ssp*I	*Taq*I
L. bryoniae	790	421, 369	392, 326, 72	486, 163, 111, 30
L. huidobrensis†	790	421, 369	399, 391	306, 163, 159, 111, 30, 21
L. sativae "USA"‡	567, 223	421, 282, 59, 27	399, 391	306, 210, 163, 81, 30
L. sativae "Asia"‡	790	421, 310, 59	717, 73	306, 210, 163, 81, 30
L. strigata	790	421, 342, 27	399, 391	267, 219, 141, 72, 67
L. trifolii	619, 171 or 386, 223, 171	421, 310, 59	391, 326, 73	306, 163, 159, 141, 21 or 306, 163, 159, 111, 30, 21

Source: Data from Kox *et al.* (2005).

† Including cryptic species *L. langei.*

‡ USA and Asia are known alternative variants; both of these are *L. sativae.*

4.2.4 Species-specific PCR primers for identification of the four target species

A multiplex PCR assay to distinguish the four target species without the need for a post-PCR restriction digestion procedure was reported by Nakamura *et al.* (2013). This assay uses six primers that target the *Cytochrome oxidase* I (*COI*) gene. Five of these each bind to a sequence unique to a *Liriomyza* species, and are used as forward primers. The sixth primer binds to a segment of the *COI* gene conserved in all *Liriomyza* species, and is used as a reverse primer, to complete primer pairing. The size of the PCR products can be used to discriminate among *L. bryoniae, L. huidobrensis, L. sativae, L. trifolii* and *L. chinensis*. Unlike the PCR-RFLP assay of Kox *et al.* (2005) (section 4.2.3), the specificity of this assay against *L. strigata* has not been verified.

4.2.4.1 Amplification of the COI gene

According to Nakamura *et al.* (2013), samples are amplified in a 10 µl reaction mixture composed of the following final concentrations of reagents: 0.5 µM of each of the six primers, 0.2 mM dNTPs, 1 U TaKaRa[1] Ex Taq DNA polymerase, 1× TaKaRa[1] Ex Taq PCR buffer and 2 mM $MgCl_2$. Each reaction includes either 0.5 µl DNA as a template or PCR-grade water as a negative control. PCR is performed using the following six primers designed by Nakamura *et al.* (2013):

Lb600-F: 5′-CTAGGAATGATTTATGCAATG-3′

Lc920-F: 5′-CATGACACTTATTATGTTGTTGCA-3′

Lh1150-F: 5′-CAATCGGATCTTCAATTTCCCTTC-3′

Ls1040-F: 5′-TTATTGGTGTAAATTTAACC-3′

Lt780-F: 5′-TTATACACCAACTACTTTGTGAA-3′

L1250-R: 5′-GAATWGGRWAAATYACTTGACGTTG-3′

The thermal cycling parameters for PCR are a 1 min denaturation step at 94 °C followed by 32 cycles of (30 s at 94 °C, 30 s at 55 °C and 2 min at 72 °C). PCR products are visualized by electrophoresis on a 1.8% agarose gel with a 100 bp DNA ladder to allow the product size to be determined.

The multiplex *COI* PCR is considered valid only if:

- the positive control produces an amplification product of the expected size for the target *COI* gene
- the negative extraction control and the negative amplification control do not produce an amplification product of the expected size for the target COI gene.

The expected PCR product sizes for the five species are 649 bp (*L. bryoniae*), 359 bp (*L. chinensis*), 107 bp (*L. huidobrensis*/*L. langei*), 207 bp (*L. sativae*) and 461 bp (*L. trifolii*). It is not possible to determine the exact fragment size of PCR products separated under the electrophoretic conditions described, but relative separation values are used to compare results with expected species-specific primer profiles for the species. Positive control samples with known band size for the species can be run alongside test samples to enable comparison of sizes more precisely.

A sample is identified as one of the five species if it produces a single PCR product of the expected size for that species. This assay is not able to distinguish *L. huidobrensis* from *L. langei*. If a sample is suspected as *L. huidobrensis*, further testing may be needed to confirm it is not the cryptic species *L. langei* (section 4.2.5). This assay was developed for *Liriomyza* identification in Japan and specificity has been directed to that purpose. As a result, cross-reactivity with *L. strigata* and populations of *L. trifolii* outside Japan has not been verified.

4.2.5 Distinguishing the cryptic species *L. langei* and *L. huidobrensis*

4.2.5.1 PCR-RFLP

Scheffer *et al.* (2001) described a PCR-RFLP assay for distinguishing *L. huidobrensis* and *L. langei* based on variation at a mitochondrial locus including part of the *COI* gene, the leucine tRNA and all of the *COII* gene. This 1 031 bp region is amplified using primers reported in Simon *et al.* (1994):

C1-J-2797-F: 5′-CCTC-GACGTTATTCAGATTACC-3′

TK-N-3785-R: 5′- GTTTAAGAGACCAGTACTTG-3′

The thermal cycling parameters for PCR are a 2 min denaturation step at 92 °C followed by 35 cycles of (1 min 30 s at 92 °C, 1 min 30 s at 50 °C and 2 min 30 s at 72 °C) and a final extension step for 7 min at 72 °C. After PCR amplification, the PCR product is subjected to electrophoresis with a DNA ladder to check PCR success before RFLP analysis.

The *COI–COII* PCR is considered valid only if:

- the positive control produces an amplification product of the expected size for the target *COII* gene
- the negative extraction control and the negative amplification control do not produce an amplification product of the expected size for the target *COII* gene.

For each sample, PCR product is digested with restriction enzymes *Spe*I and *Eco*RV, each in a separate reaction, according to the manufacturer's instructions. Digested PCR product is then separated by electrophoresis on a 1.5% agarose gel along with a 100 bp DNA ladder to allow the size of the fragments to be determined.

It is not possible to determine the exact fragment size of digested products separated under the electrophoretic conditions described, but relative separation values are used to compare results with expected RFLP profiles for the species. Positive control samples with known fragment sizes and patterns can be run alongside test samples to enable comparison of sizes more precisely. A positive control should be included for each digestion enzyme tested to ensure that the enzyme digests the

DNA as expected. The RFLP test is considered valid only if the positive control produces fragments of the expected size for the target gene.

L. huidobrensis samples produce a single uncut (1 031 bp) fragment when digested with *Spe*I and two cut (175 bp and 856 bp) fragments when digested with *Eco*RV. In contrast, *L. langei* samples produce two cut (420 bp and 611 bp) fragments when digested with *Spe*I and a single uncut (1 031 bp) fragment when digested with *Eco*RV. If the composite fragment profile of a sample matches these known fragment profiles the sample can be identified as that species based on the assay.

4.2.5.2 DNA sequence comparison

Scheffer (2000) reported PCR and DNA sequence information for a mitochondrial DNA locus including partial sequences of the *COI* and *COII* genes that can distinguish the two cryptic species *L. huidobrensis* and *L. langei*. A subsequent publication by Scheffer *et al.* (2006) included additional sequences of the 3' end of the *COI* gene for investigation of species diversity. These data were analysed using molecular phylogenetic techniques but were not developed into diagnostic protocols.

4.2.6 DNA barcoding

Efforts to generate a more taxonomically comprehensive resource of DNA sequence records for the 5' region of the *Liriomyza COI* gene used in animal DNA barcode studies are ongoing (e.g. Bhuiya *et al.*, 2011; Maharjan *et al.*, 2014). There are currently DNA barcode records for 31 species of *Liriomyza* (including the four target species) available on the Barcode of Life Data System (BOLD) (http://www.boldsystems.org). Alternative barcodes and procedures are provided on Q-bank (www.q-bank.eu), a curated database including sequences obtained from reference material. A recent study (Maharjan *et al.*, 2014) included details for the separation of *L. huidobrensis, L. trifolii, L. sativae, L. bryoniae* and *L. chinensis*. Despite these advances in DNA sequencing resources, the methodology is not described in detail here for *Liriomyza* species identification because interpretation rules for the resources have not yet been published in the scientific literature. DNA barcoding identification results should be interpreted carefully for possible issues such as: (1) potential preferential PCR amplification of parasitoids or nuclear mitochondrial copies of the *COI* gene (i.e. nuclear mitochondrial pseudogenes (numt); (2) the possibility of misidentification with closely related sister species (i.e. species complexes); and (3) a different scope of geographic coverage of the reference specimens in the sequence databases.

5. Records

Records and evidence should be retained as described in section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*).

In cases where other contracting parties may be adversely affected by the results of the diagnosis, the following records and evidence and additional material should be kept for at least one year in a manner that ensures traceability: preserved or slide-mounted specimens, photographs of distinctive taxonomic structures, DNA extracts and photographs of gels.

6. Contact Points for Further Information

Further information on this protocol can be obtained from:

State Government of Victoria Department of Economic Development, Jobs, Transport and Resources, AgriBio, 5 Ring Road, Bundoora, Vic. 3083, Australia (Mallik Malipatil; e-mail: mallik.malipatil@ecodev.vic.gov.au; tel.: +61 3 9032 7302; fax: +61 3 9032 7604).

Fera Science Ltd (Fera), National Agri-Food Innovation Campus, Sand Hutton, York, YO41 1LZ, United Kingdom (Dominique Collins; e-mail: dom.collins@fera.co.uk; tel.: +44 1904 462215; fax: +44 1904 462111).

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will in turn forward it to the Technical Panel on Diagnostic Protocols (TPDP).

7. Acknowledgements

The first draft of this protocol was written by Mallik B. Malipatil (State Government of Victoria Department of Economic Development, Jobs, Transport and Resources, Australia), Dominique W. Collins (Fera, United Kingdom) and Mark Blacket (State Government of Victoria Department of Economic Development, Jobs, Transport and Resources, Australia), Norman Barr (United States Department of Agriculture – Animal and Plant Health Inspection Service, United States) drafted the section on molecular identification.

The following reviewers provided comments on the draft version of this document: Stephen Gaimari (California Department of Food and Agriculture, United States), Anthony Rice (Department of Agriculture and Water Resources, Australia), Ren Iwaizumi (Yokohama Plant Protection Station, Ministry of Agriculture, Forestry and Fisheries, Japan) and Ramona Vaitkevica (State Plant Protection Service of Latvia).

8. References

The present annex refers to ISPMs. ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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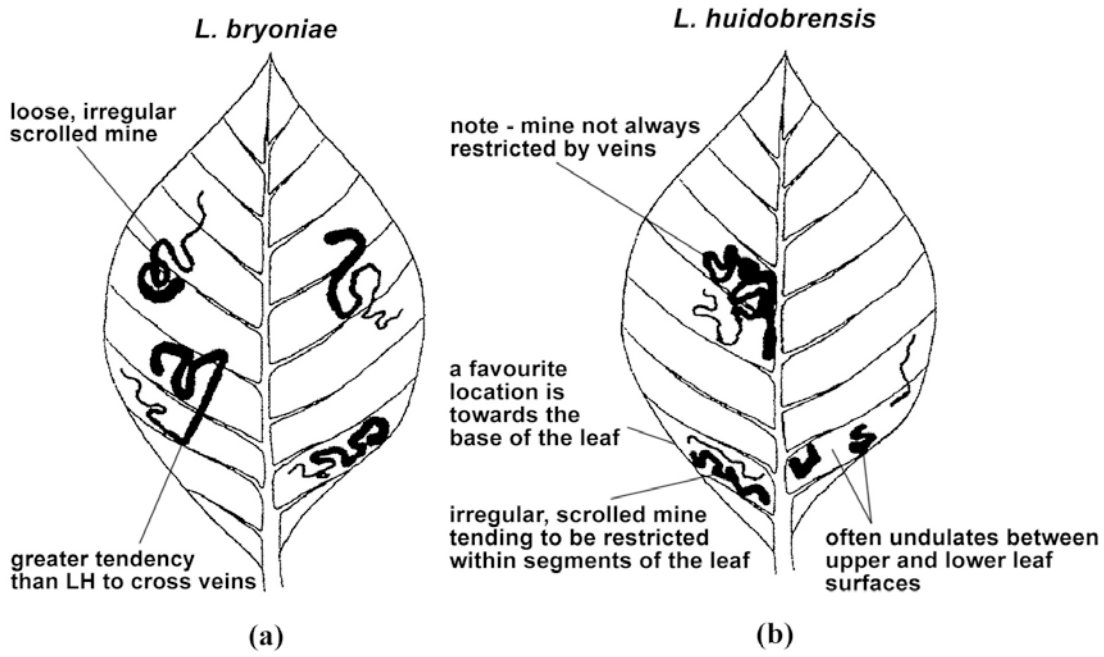
9. Figures

Figure 1. Adult of *Liriomyza bryoniae*.
Photo courtesy Department of Environment, Food and Rural Affairs, United Kingdom



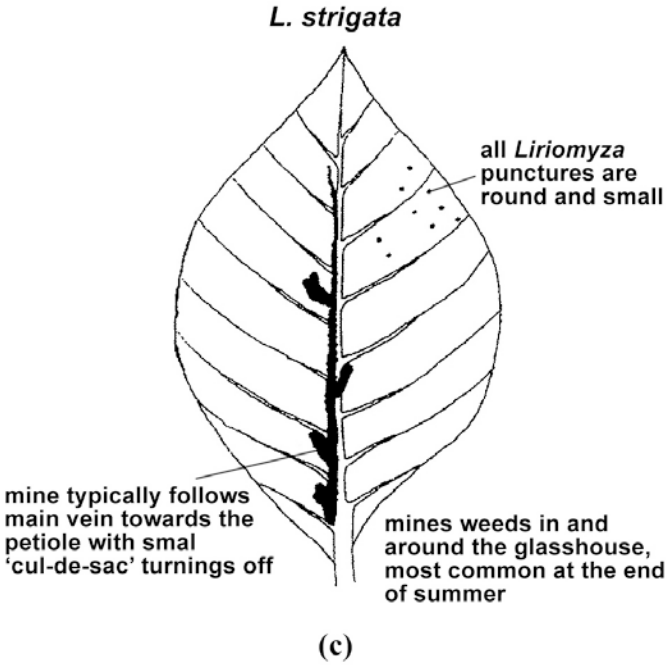


Figure 2. Typical characteristics of mines of (a) *Liriomyza bryoniae*, (b) *Liriomyza huidobrensis* and (c) *Liriomyza strigata.*
Source: EPPO (2005).

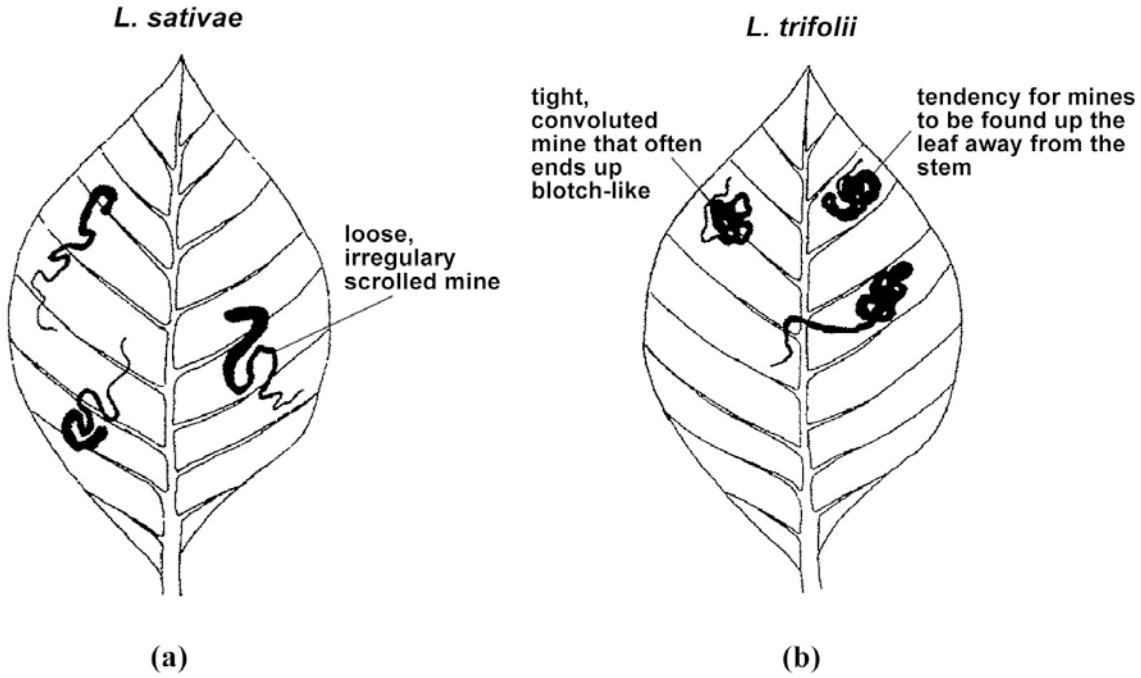


Figure 3. Typical characteristics of mines of (a) *Liriomyza sativae* and (b) *Liriomyza trifolii.*
Source: EPPO (2005).

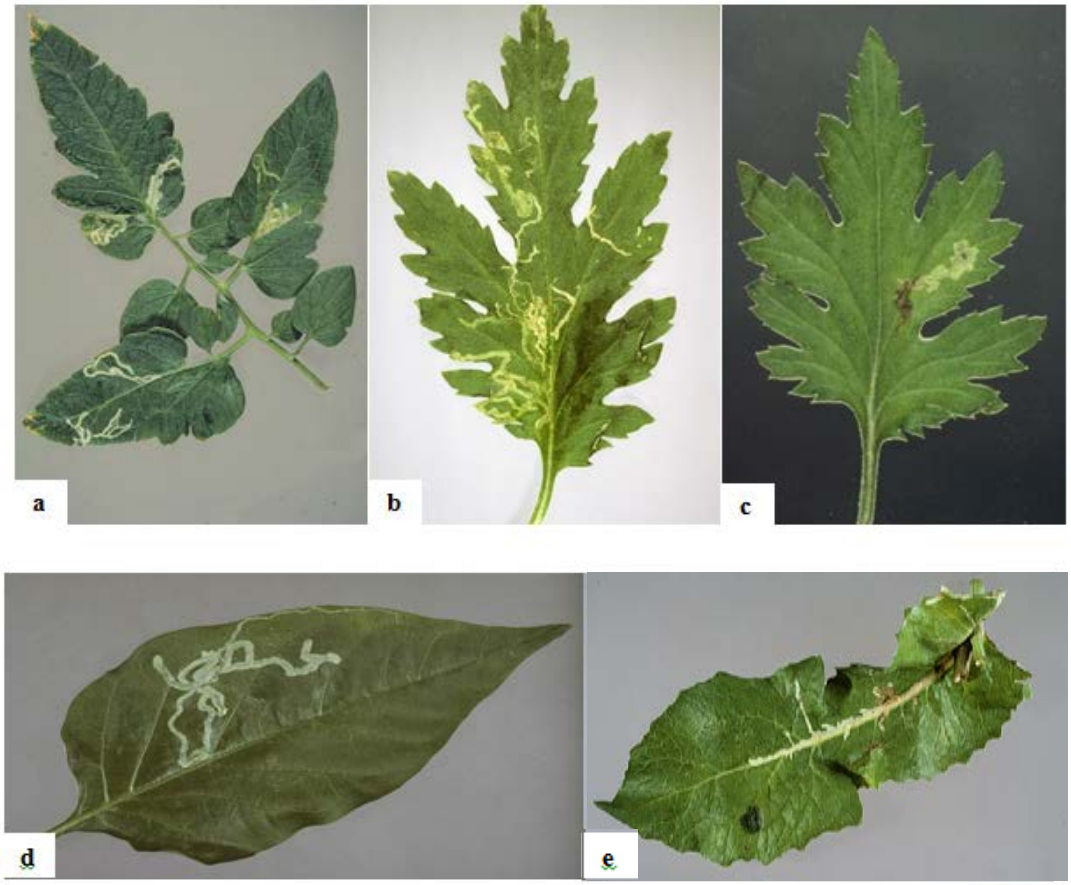


Figure 4. Typical mines of *Liriomyza* spp.: (a) *L. bryoniae* on tomato; (b) *L. huidobrensis* on chrysanthemum; (c) *L. trifolii* on chrysanthemum; (d) *L. sativae* on pepper; and (e) *L. strigata* on an unidentified host.
Photo courtesy Department of Environment, Food and Rural Affairs, United Kingdom

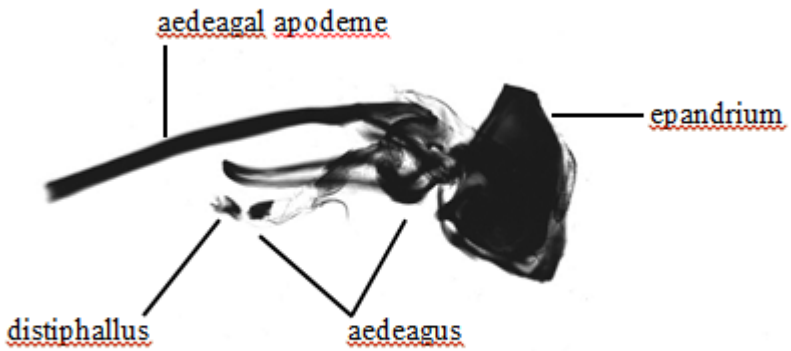


Figure 5. Male genitalia of *Liriomyza huidobrensis* (lateral view).
Photo courtesy Department of Environment, Food and Rural Affairs, United Kingdom.

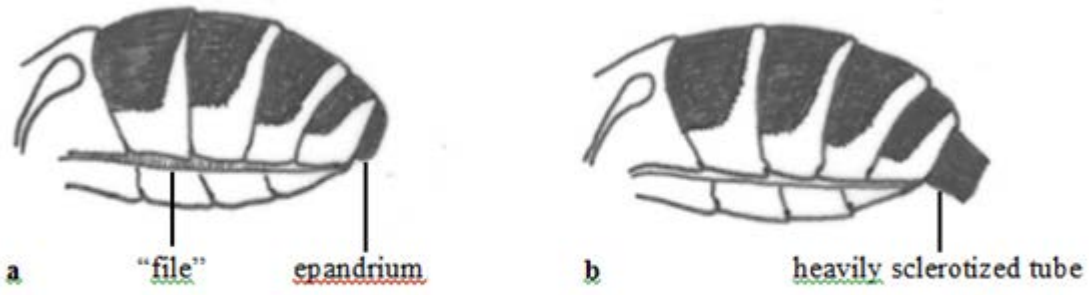
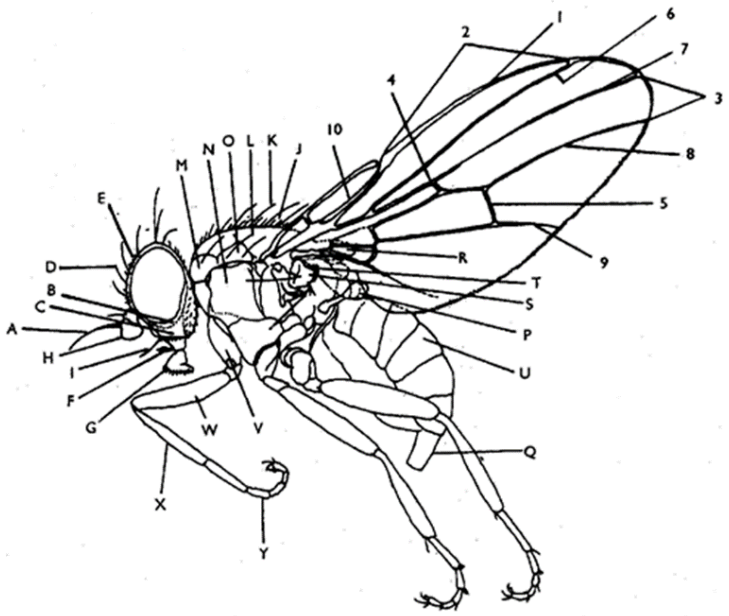


Figure 6. Abdomen in (a) male and (b) female *Liriomyza*.
Photo courtesy Department of Environment, Food and Rural Affairs, United Kingdom United Kingdom.



Side view of typical *Agromyza* sp. (after SASAKAWA): A = arista, B = cheek, C = jowl, D = orbital bristles, E = orbital setulae, F = palp, G = proboscis, H = third antennal segment, I = vibrissa, J = acrostichals, K = dorso-central bristles, L = mesonotum, M = humerus, N = mesopleural area, O = notopleural area, P = haltere, Q = ovipositor sheath, R = scutellum, S = squama, T = squamal fringe, U = tergites, V = coxa, W = femur, X = tibia, Y = tarsi.
1 = costa, 2 = second costal section, 3 = fourth costal section, 4 = first cross-vein, 5 = second cross-vein, 6 = R_1, 7 = R_{4+5}, 8 = M_{1+2}, 9 = M_{3+4}, 10 = sub-costa.

Figure 7. Adult morphology of Agromyzidae.
Source: Spencer (1973).

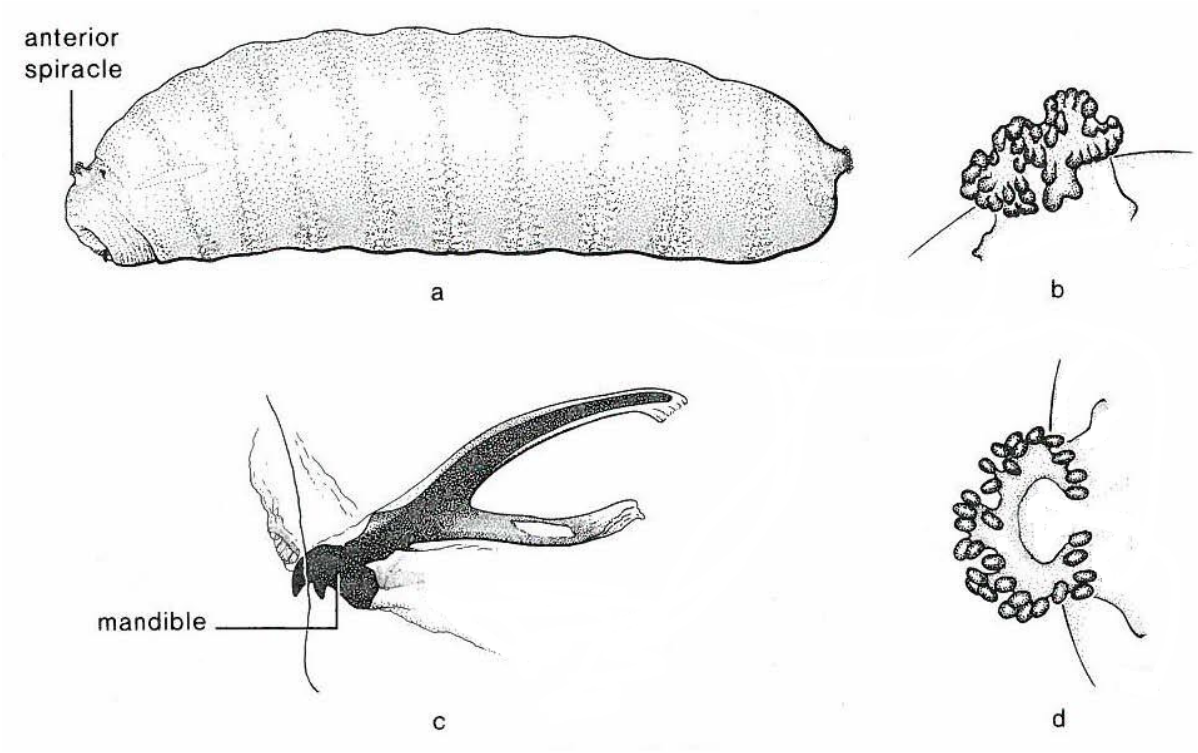


Figure 8. Larval morphology of Agromyzidae (*Phytomyza chelonei*): (a) lateral view; (b) anterior spiracle; (c) cephalopharyngeal skeleton; and (d) posterior spiracle.
Source: Stehr (1991).

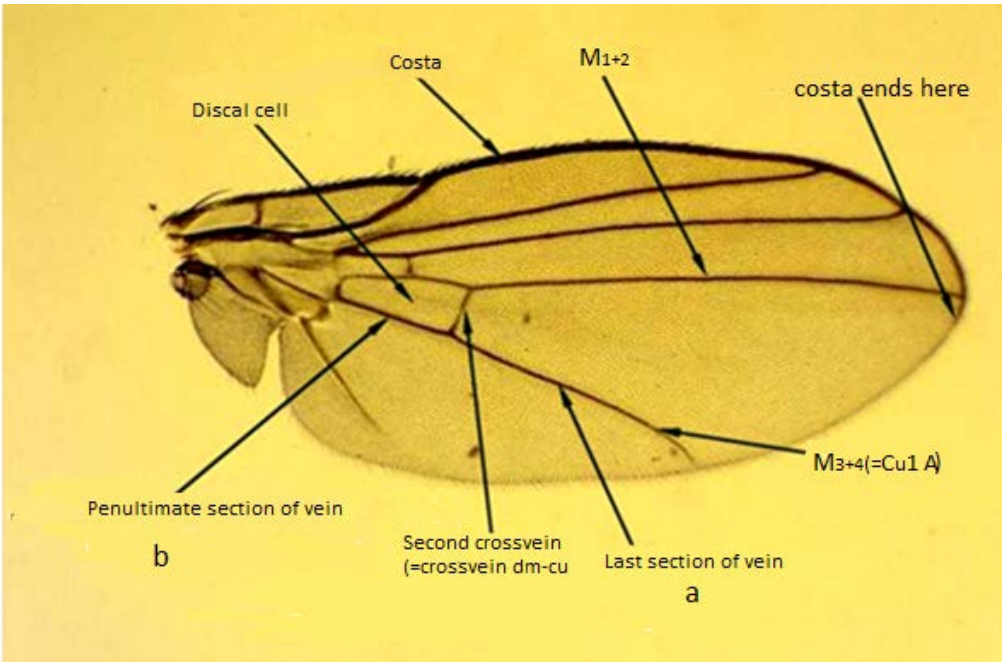


Figure 9. Wing venation of *Liriomyza.*
Photo courtesy Victorian State Government Department of Environment, Land, Water and Planning, Australia.

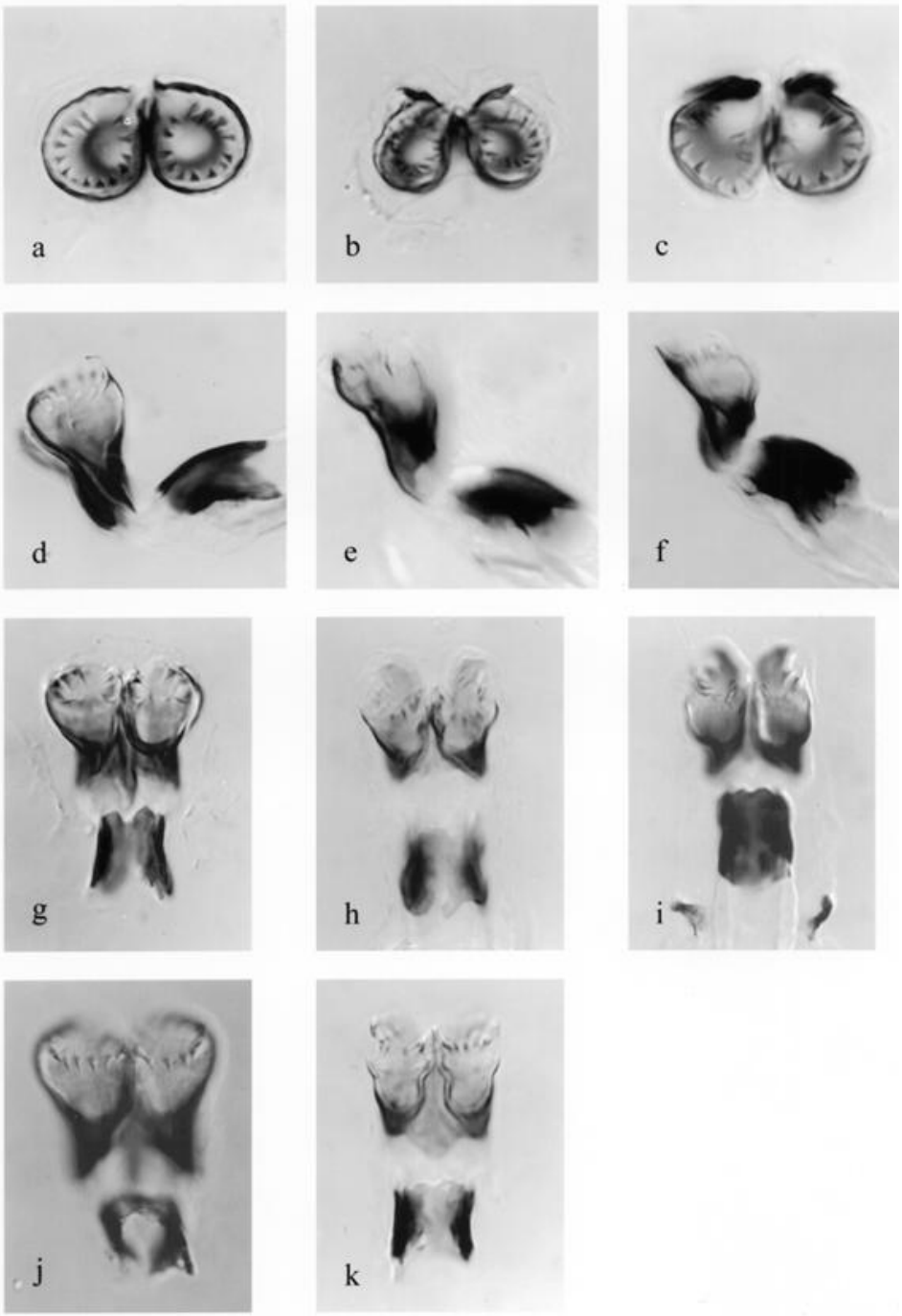


Figure 10. Distiphallus of *Liriomyza* spp. (×400 magnification): (a) *L. bryoniae*, anterior view; (b) *L. huidobrensis*, anterior view; (c) *L. strigata*, anterior view; (d) *L. bryoniae*, lateral view; (e) *L. huidobrensis*, lateral view; (f) *L. strigata*, lateral view; (g) *L. bryoniae*, dorso-ventral view; (h) *L. huidobrensis*, dorso-ventral view; (i) *L. strigata*, dorso-ventral view; (j) *L. bryoniae*, dorso-ventral view (in a different plane from (g)); and (k) *L. huidobrensis*, dorso-ventral view (in a different plane from (h)).
Photo courtesy Department of Environment, Food and Rural Affairs, United Kingdom.

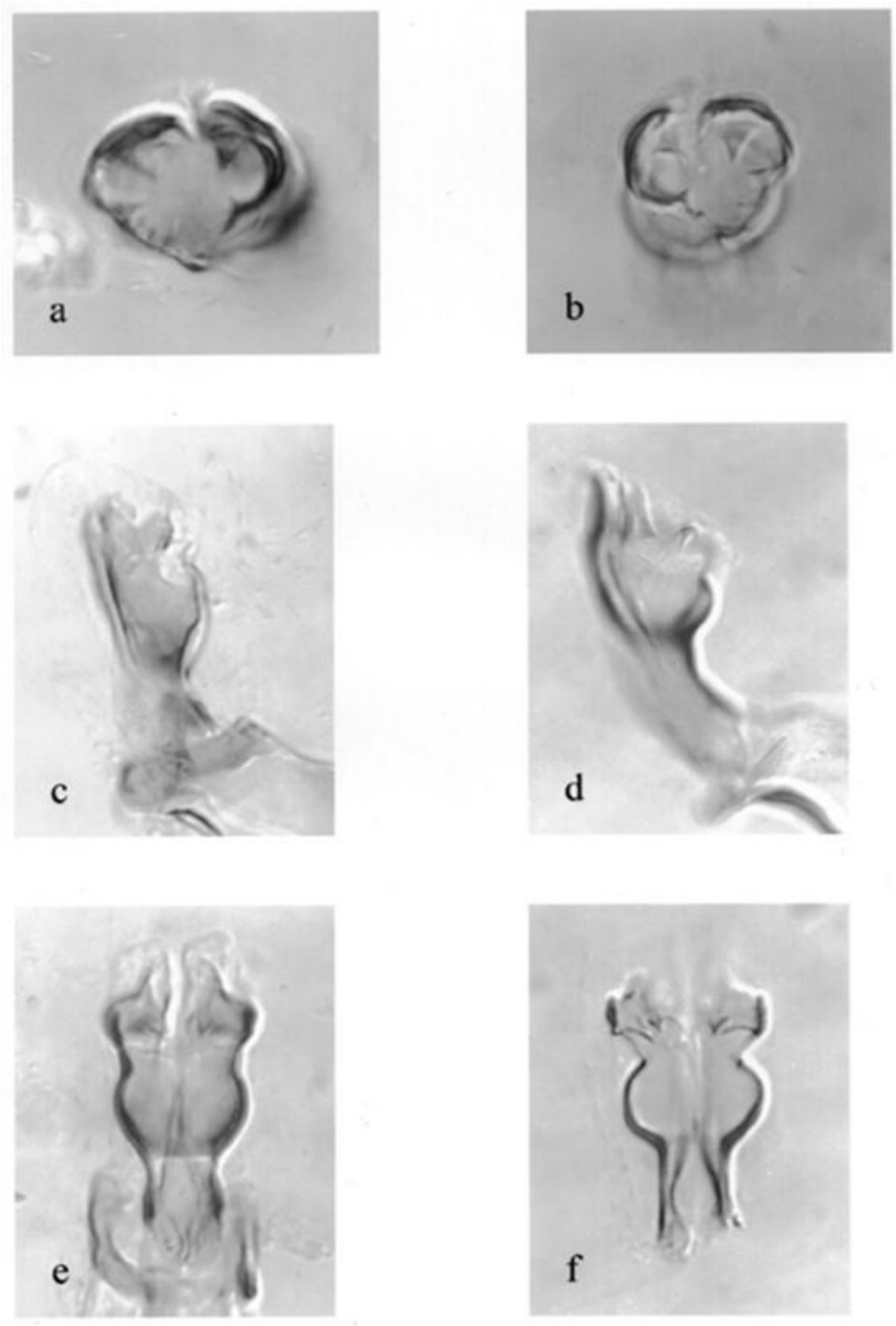


Figure 11. Distiphallus of *Liriomyza* spp. (×400 magnification): (a) *L. sativae*, anterior view; (b) *L. trifolii*, anterior view; (c) *L. sativae*, lateral view; (d) *L. trifolii*, lateral view; (e) *L. sativae*, dorso-ventral view; and (f) *L. trifolii*, dorso-ventral view.
Photo courtesy Department of Environment, Food and Rural Affairs, United Kingdom.

Figure 12. Pupa of *Liriomyza* sp
Photo courtesy Victorian State Government Department of Environment, Land, Water and Planning, Australia.

Figure 13. Third larval instar of *L. bryoniae*
Photo courtesy Department of Environment, Food and Rural Affairs, United Kingdom.

Publication history

This is not an official part of the standard.

2006-11 SC added original subject: *Liriomyza* spp. (2006-017).

2007-03 CPM-2 added topic to the work programme (Insects and mites).

2014-07 (TPDP) reviewed and approved the draft for SC e-decision for approval for member consultation.

2014-10 SC e-decision for approval for member consultation (2014_eSC_Nov_12).

2015-02 Member consultation.

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ISPM 27
Diagnostic protocols for regulated pests

DP 17: *Aphelenchoides besseyi, A. fragariae* and *A. ritzemabosi*

Adopted 2016; published 2016

CONTENTS

2. Pest Information

Aphelenchoides species occur worldwide (Fortuner and Williams, 1975; CABI, n.d.). The majority of species within the genus *Aphelenchoides* Fischer, 1894 are mycetophagous, but a small group including *A. besseyi* (Christie, 1894), *A. fragariae* (Ritzema Bos, 1891) and *A. ritzemabosi* (Schwartz, 1911) also feed on higher plants. To date, 180 species of *Aphelenchoides* (and 19 of uncertain status) have been described (Sánchez-Monge *et al*., 2015). Plant-feeding *Aphelenchoides* species have the ability to survive unfavourable conditions in a quiescent stage. The members of this group are called foliar,leaf or bud nematodes because they are common and widespread parasites on these parts of plants. They are migratory ectoparasites and endoparasites of leaves, buds, stems and very occasionally corms, causing crinkling, blotching and reduced size of the leaves, resulting in a reduction of quality and yield of many ornamental and crop plants such as *Oryza sativa* (rice), *Fragaria* spp. (strawberry) and *Chrysanthemum* spp. (Hunt, 1993). It is important to identify the particular species in the infestation as the life cycle of each species is slightly different.

A. besseyi is known as the causal agent of white tip disease on its major host, *O. sativa*, wherever this host occurs worldwide. However, the nematode also infests *Fragaria* spp., where it is a cause of crimp disease recorded from Australia, the United States and more recently Europe. Other crops recorded as infested include grasses (*Panicum, Pennisetum* and *Setaria*), ornamentals (e.g. *Begonia* and *Chrysanthemum*) and vegetables (e.g. *Allium* and *Dioscorea*) (CABI, n.d.). The nematode was recently also identified as the causal agent of black spot disease on *Phaseolus vulgaris* (bean) (Chaves *et al*., 2013).

Plants of *O. sativa* susceptible to *A. besseyi* can be symptomless, but yield or quality loss occurs when symptoms are visible. Seed infested with *A. besseyi* has lowered viability and delayed germination (Tamura and Kegasawa, 1959), and diseased plants have reduced vigour and height (Todd and Atkins, 1958). The nematode is capable of withstanding desiccation and may be found in an anhydrobiotic state beneath the hulls of grains of *O. sativa*.

As with some other *Aphelenchoides* species, *A. besseyi* may be found between leaves and buds in *Fragaria* spp. and may cause distortion of the leaves, which is more noticeable on newly formed leaves after growth resumes in spring (Brown *et al.*, 1993). On *Fragaria* spp., *A. besseyi* appears in summer and is called the "summer crimp nematode" (Esser, 1966). It is a parasite of warm regions; according to EPPO/CABI (1997), *A. besseyi* is not found beyond 43° north latitude on rice or beyond 40° north on strawberries grown outdoors.

A. fragariae is an endoparasite and ectoparasite of the aerial parts of plants, and it has an extensive host range – more than 250 plant species in 47 families – and it is widely distributed in temperate and tropical regions throughout the world (EPPO, n.d.).

A. fragariae is a causal agent of crimp or spring dwarf disease on *Fragaria* spp. and can also cause serious damage to many other agricultural and ornamental crops, including ferns, foliage and flowering plants, and herbaceous and woody perennials (Kohl, 2011). *A. fragariae* is commonly found in the aerial parts of plants, corms and soil or growing media associated with host plants. It can be detected on leaves showing discoloured mosaic or angular spots. *A. fragariae* is responsible for an economic loss of millions of dollars each year in the ornamental nursery industry (Jagdale and Grewal, 2006). This nematode feeds on the epidermis, mesophyll and parenchyma tissues of leaves or fronds, resulting in chlorosis or vein-delimited lesions that turn necrotic, and in defoliation over time. In the absence of plant residues or wild host plants, *A. fragariae* can survive a few months in the soil (Ambrogioni and Greco, 2014). The nematode can be distributed over long distances in shipments of asymptomatic infested plants.

A. ritzemabosi is an obligate plant parasite, inhabiting leaves, buds and growing points. It may feed endoparasitically on mesophyll cells of leaves or ectoparasitically on buds and growing points (Siddiqi, 1974). Like *A. fragariae*, these nematodes do not enter the stem tissue but move within a water film on the surface to reach the leaves and buds. The leaves are invaded through the stomata. The nematodes

feed on and destroy the mesophyll cells, resulting in angular leaf spot in several hosts, and also causing dwarfing and leaf wilt. The cells in infested areas die and the leaves develop brown lesions delimited by the veins (Franklin, 1978). The nematodes exit the leaves through the stomata and once again migrate in a water film to infect flower buds (Southey, 1993).

A. ritzemabosi is rarely encountered in soil, where it cannot complete its life cycle or survive the winter. The nematode overwinters in dormant buds and growing points of *Chrysanthemum* spp. stools, which serve as a source of infestation (Hesling and Wallace, 1960). *A. ritzemabosi* survives unfavourable conditions through anhydrobiosis and can retain viability for some months in dried plant material. Like other *Aphelenchoides* species, *A. ritzemabosi* can reproduce on fungi, and soil fungi may therefore contribute to its survival in the absence of a host (Hooper and Cowland, 1986).

A. ritzemabosi was found in association with *Phytophthora cryptogea* on diseased *Gloxinia* plants (Stokes and Alfieri, 1969) and is linked with *Corynebacterium fascians* in the onset of "cauliflower disease" in strawberries (Crosse and Pitcher, 1952). Madej *et al.* (2000) found several plant-parasitic fungi in association with *A. ritzemabosi* on *Chrysanthemum* and *Zinnia* plants affected by this nematode, which increased the necrotic symptoms observed, i.e the number of necrosis.

A. ritzemabosi is a major pest of *Chrysanthemum* spp. in Australia, Europe, New Zealand and North America, and it has been reported on this host from several other countries (CABI/EPPO, 2000; EPPO, n.d.). Both *A. ritzemabosi* and *A. fragariae* cause damage to *Fragaria* spp. in several European countries as well as in Mexico (CABI/EPPO, 2000; EPPO, n.d.). The nematode has been recorded on a wide range of ornamental and other hosts from Asia, Europe, North America, Oceania and South America (CABI/EPPO, 2000; EPPO, n.d.) – about 200 host plant species according to Escuer and Bello (2000) and McCuiston (2007). *A. ritzemabosi* was reported as occurring in South Africa by Wager in 1972, but these records were made on the basis of symptoms only and the nematodes were not positively identified taxonomically. The first report of *A. ritzemabosi* in South Africa that was morphologically identified was on *Nerine* bulbs in nurseries (Swart *et al.*, 2007).

2. Taxonomic Information

Name:	*Aphelenchoides besseyi* Christie, 1942
Synonyms:	*Aphelenchoides oryzae* Yokoo, 1948; *Asteroaphelenchoides besseyi* (Christie, 1942) Drozdovsky, 1967
Taxonomic position:	Nematoda, Rhabditida, Tylenchina, Aphelenchoidea, Aphelenchoididae, Aphelenchoidinae, *Aphelenchoides*
Common names:	Preferred common name: rice leaf nematode (CABI, n.d.); other common names: summer crimp nematode, white tip, white tip nematode of rice (CABI, n.d.)
Name:	*Aphelenchoides fragariae* (Ritzema Bos, 1890) Christie, 1932
Synonyms:	*Aphelenchus fragariae* Ritzema Bos, 1891; *Aphelenchus olesistus* Ritzema Bos, 1893; *Aphelenchoides olesistus* (Ritzema Bos, 1893) Steiner, 1932; *Aphelenchus olesistus* var. *longicollis* Schwartz, 1911; *Aphelenchoides olesistus* var. *longicollis* (Schwartz, 1911) Goodey, 1933; *Aphelenchus pseudolesistus* Goodey, 1928; *Aphelenchoides pseudolesistus* (Goodey, 1928) Goodey, 1933; *Aphelenchus ormerodis* Jegen, 1920 (nec. Ritzema Bos, 1891)
Taxonomic position:	Nematoda, Rhabditida, Tylenchina, Aphelenchoidea, Aphelenchoididae, Aphelenchoidinae, *Aphelenchoides*
Common names:	Strawberry spring dwarf nematode, strawberry crimp nematode

Name: *Aphelenchoides ritzemabosi* (Schwartz, 1911) Steiner and Buhrer, 1932

Synonyms: *Aphelenchoides ribes* (Taylor, 1917) Goodey, 1933; *Aphelenchus phyllophagus* Stewart, 1921; *Aphelenchus ribes* (Taylor, 1917) Goodey, 1923; *Aphelenchus ritzema-bosi* (Schwartz, 1911); *Pathoaphelenchus ritzemabosi* (Schwartz, 1911) Steiner, 1932; *Pseudaphelenchoides ritzemabosi* (Schwartz, 1911) Drozdovski, 1967; *Tylenchus ribes* Taylor, 1917

Taxonomic position: Nematoda, Rhabditida, Tylenchina, Aphelenchoidea, Aphelenchoididae, Aphelenchoidinae, *Aphelenchoides*

Common names: Chrysanthemum foliar nematode, leaf and bud nematode

3. Detection

3.1 Symptoms produced by the nematodes on host plants

A. besseyi, *A. fragariae* and *A. ritzemabosi* may occasionally be found in the growing media of infested hosts, but are most commonly found in infested plant foliage, including leaves, flowers, buds, and seed heads or pods. Symptoms of infestation by these nematodes vary according to the host.

3.1.1 Symptoms of *Aphelenchoides besseyi*

During early growth of *O. sativa*, the most conspicuous symptom caused by this nematode is the emergence of the chlorotic tips of new leaves from the leaf sheath (Figure 1(A)). These tips later dry and curl, while the rest of the leaf may appear normal. The young leaves of infested tillers can be speckled with a white splash pattern or have distinct chlorotic areas. Leaf margins may be distorted and wrinkled but leaf sheaths are symptomless. The flag leaf enclosing the panicle crinkles and distorts, and the panicle is reduced in size, as are the grains. Symptoms may be confused with calcium and magnesium deficiency. Infested panicles are shorter than normal panicles, with fewer spikelets and a smaller proportion of filled grain (Dastur, 1936; Yoshii and Yamamoto, 1951; Todd and Atkins, 1958). In severe infestations, the shortened flag leaf is twisted and can prevent the complete extrusion of the panicle from the boot (Yoshii and Yamamoto, 1950; Todd and Atkins, 1958). The panicles also often stay erect (Liu *et al.*, 2008) and discoloration can be observed on them (CABI, n.d.). The grain is small and distorted (Todd and Atkins, 1958) and the kernel may be discoloured and cracked (Uebayashi *et al.*, 1976) (Figure 1(B)). Infested plants mature late and have sterile panicles borne on tillers produced from high nodes.

On *Fragaria* spp., *A. besseyi* is the causal agent of "summer dwarf disease" (Perry and Moens, 2006). Symptoms include leaf crinkling and distortion, and dwarfing of the plant with an associated reduction in flowering (Figure 1(C)). Symptoms may be similar to and therefore confused with those caused by other *Aphelenchoides* species, emphasizing the importance of correct identification.

In *O. sativa* and *Fragaria* spp., *A. besseyi* feeds ectoparasitically, but the nematode may also be endoparasitic, as in *Ficus elastica* and *Polianthes tuberosa*, in which it causes leaf drop and leaf lesions, respectively. On *Capsicum annum* var. *longum* the infestation appears to result in rotting of the pods and premature pod drop, similar to some fungal diseases (Hockland and Eng, 1997). In the grass *Sporobolus poirettii*, this nematode stimulates growth, resulting in increased flowering.

3.1.2 Symptoms of *Aphelenchoides fragariae*

Common symptoms of plants damaged by *A. fragariae* are chlorosis; necrosis; distortion, deformation and dwarfing of the leaves, stems, flowers or bulbs; leaf tattering; and defoliation. The symptoms are often confused with symptoms caused by powdery mildew. Symptoms typically manifest as vein-delimited lesions or blotches that start as lightly chlorotic and then turn brown to black or necrotic and dry (Figure 2). Symptom expression, however, may be highly variable as a result of the different

characteristics of host plant species and the influence of environmental conditions. Infested plants sometimes do not exhibit symptoms until the plant is heavily infested with nematodes.

The shape and pattern of the blotches is closely related to the venation pattern of the leaf, the infested leaves appearing pale green to tan in colour or showing dark brown mosaic spots or angular necrotic lesions (Figure 3) (Knight *et al*., 2002; Khan *et al*., 2008; Kohl, 2011). On *Hosta* spp., leaf blotch symptoms appear as long, narrow necrotic patches bounded by longer veins, and in severe cases, the entire leaf dries and dies (Figure 4) (Zhen *et al.*, 2012). The leaf blotch symptoms on ferns appear as narrow, linear patches perpendicular to the midrib of the frond, corresponding to closely spaced lateral veins, as chevron-like stripes (Figure 5) (Cobon and O'Neill, 2011). On *Cyclamen* spp., *Begonia* spp. and *Andrographis paniculata*, infested leaves show water-soaked irregular patches that later turn brown (Figure 6) (Southey, 1993; dan Supriadi, 2008). In general, the blotches form more or less angular chlorotic areas in ternate or palmate leaves with reticulate venation or with main veins radiating from the petiole–lamina junction, while infected thicker and succulent leaves initially show water-soaked irregular patches that subsequently become necrotic without defined margins; ultimately, the entire leaf dies (Richardson and Grewal, 1993; Southey, 1993). On *Fragaria* spp., the initial symptoms of infestation are stunted plant growth and deformation of buds, leaves and flowers; infested plants show malformations including twisting and puckering of leaves, discoloured areas with hard and rough surfaces, undersized leaves with crinkled edges, tight aggregation or death of crowns, reddened and stunted petioles, and flower stalks with aborted or partly aborted flowers (Figure 7). Heavily infested plants do not produce fruit (Siddiqi, 1975).

For plants in general, *A. fragariae* infests buds or the crown, causing buds to decay, flowers to shrivel, and leaves, petioles and stems expanding from the infested buds to become misshapen, crinkled and stunted and to develop brown scars (Richardson and Grewal, 1993; Southey, 1993).

3.1.3 Symptoms of *Aphelenchoides ritzemabosi*

On *Chrysanthemum* spp., infestation from the soil, dead leaves or weed hosts progresses from the base of the plant upwards under moist conditions. Infested leaves show characteristic angular blotches delimited by the principal veins. The discoloration progresses from translucent yellowish and brownish green to dark brown. At a late stage, dead shrivelled leaves, hanging down, extend to the top of the plant (Figure 8). Although some stems of a given plant may bear dead leaves, other stems may be symptomless. The nematodes also invade and feed within the buds, sometimes killing the growing point and preventing flowering or producing malformed leaves with surface irregularities and rough brown scars.

On *Fragaria* spp., damage is most noticeable on newly formed leaves, which become puckered and distorted and may show rough, greyish feeding areas near the base of the main veins. The cauliflower disease of *Fragaria* spp., resulting in the continued production of axillary buds on affected crowns, was experimentally induced in *Fragaria* spp. runners through co-inoculation of *A. ritzemabosi* and *C. fascians* by Crosse and Pitcher (1952).

A. ritzemabosi causes polygonal blotches that are bound by veins on leaves of infested plants of *Nicotiana* spp., similar to symptoms on *Chrysanthemum* spp. (Shepherd and Barker, 1990; Johnson, 1998).

This nematode also infests many herbaceous plants; most show the typical interveinal leaf blotches and distortions of the upper leaves resulting from bud infestation. *A. ritzemabosi* is also associated with the death of lower leaves and buds and malformed growth of shoots in *Lavandula* spp. Woody plants such as *Buddleia* are also attacked, in which the nematode causes the death of buds and leaf distortions. Attacked *Viola* spp. are stunted, and affected leaves curl downwards, wither and die, while the undersides of leaves show typical water-soaked blotches (Thomas, 1968; Southey, 1993). Stunting and shoot blindness occurs on attacked *Crassula coccinia* (Atkinson, 1964). Combined infestation of *A. ritzemabosi* with *Ditylenchus dipsaci* in stem tissues shows as discoloration caused by feeding of the nematode after cell separation by *D. dipsaci*.

3.2 Extraction of the nematodes from plant material

3.2.1 Direct examination

In leaves infested with *A. besseyi, A. fragariae* or *A. ritzemabosi*, nematodes can be detected by inspecting cut leaves, especially small and young ones, immersed in tap water in a Petri dish under a stereomicroscope (the nematodes will swim into the water within 30 min if there is a heavy infestation).

3.2.2 Extraction methods

A. besseyi, A. fragariae and *A. ritzemabosi* can be extracted from plant material, soil or growing medium with suspected infestation using the Baermann funnel technique (Baermann, 1917), modified Baermann-tray method (Hooper and Evans, 1993), adapted sugar-flotation method (Coolen and D'Herde, 1972) or mistifier technique (Hooper *et al*., 2005). These extraction methods should be conducted for 48 h at room temperature to detect low levels of infestation. In heavily infested plant material, nematodes can be isolated by soaking plant material in water for 1 h. Any plant material to be tested should be cut into small pieces or sliced before extraction to increase the efficacy of extraction. Complementary information on extraction methods, including advantages and drawbacks, can be found in EPPO (2013a).

For the Baermann funnel technique (Hooper and Evans, 1993), a piece of rubber tubing is attached to a glass or plastic funnel stem and closed with a spring or screw clip. The funnel is placed in a suitable support and almost filled with water. Plant material containing nematodes is cut into small pieces, placed in a square of butter muslin, which is folded to enclose the material, and gently submerged in the water in the funnel. Nematodes emerge from the tissues and sink to the bottom of the funnel stem. After some hours, or preferably overnight, some of the water can be run off and examined for nematodes.

The modified Baermann-tray method for nematode extraction (Hooper and Evans, 1993) avoids the possibility that oxygen becomes limiting to the nematodes or that they lodge on the sloping funnel sides by using a shallow dish instead of a funnel and by supporting the material to be extracted on a sieve. The sieve is made from a plastic ring (cut from a polyethylene or Perspex[1] cylinder or a vinyl drainpipe) about 6–8 cm in diameter and 2 cm deep, with a piece of butter muslin stretched over one end and held by a rubber band or secured between two closely fitting rings; alternatively, nylon gauze can be stuck on or fused to the plastic ring. A milk filter or paper tissue is placed in the sieve and the chopped plant material is put on it. The sieve is then placed in water in a Petri dish or similar container. Small supports (e.g. glass rods or small feet attached to the sieve ring) are used to create a space of about 2 mm between the base of the sieve and the collecting dish. The material should be almost awash, and when it is not, more water should be added carefully between the outside of the sieve and the edge of the collecting dish. After a few hours, or overnight, the sieve is gently removed and the contents of the dish are examined for nematodes. The sieve can be re-immersed in fresh water for further extraction from the same sample.

The adapted sugar-flotation method (Coolen and D'Herde, 1972) follows instructions for "mobile stages". Nematodes are released from plant material by means of a mixer that has two running speeds (e.g. Waring[1] blender). A container with a capacity of 0.5 litre is half filled with water. The sample is mixed with the water at low speed. The suspension is poured through a 1 000 µm sieve placed on a homogenization jar and rinsed with a fine, powerful, fan-shaped water jet produced by a low-volume fog spray nozzle until the jar contains 0.5 litre. After homogenization of the suspension by compressed air (about 1 min), a 100 ml aliquot is tapped off from the bubbling mixture into a centrifuge tube. Kaolin powder (1 ml) is added and the tube contents are thoroughly mixed by a mechanical stirrer (which is carefully cleaned after each operation). The mixture is centrifuged for 5 min at 1 800 *g*, after which the

[1] In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

supernatant is poured off. The residue is mixed with a sugar solution (∂ = 1.15) by mechanical stirring for at least 30 s. The suspension is centrifuged again for 4 min at 1 800 *g*. The sugar solution is poured into a sieve of 5–20 µm aperture sieve, which is placed in a small dish previously filled with the same liquid, until the meshes of the sieve are just covered. After about 1 min the dish is gently emptied sideways. The mobile stages on the sieve are washed with a spray atomizer into 100 ml water, ready for identification.

The mistifier technique, as described by Hooper *et al*. (2005), results in recovery of nematodes that are more active than the Baermann methods because oxygenation is better, and sap and decomposition products from the plant material, especially from bulbs such as *Narcissus* spp., which inactivate the nematodes, are washed away. A fine mist of water is sprayed over the plant material. A spray nozzle, passing about 4.5 litre water per hour, is used. Most systems use an intermittent spray of, for example, 1 min in every 10 min. Oil burner nozzles or gas jets can sometimes be adapted, and a water pressure of about 2.8 kg/cm^2 is usually required to produce a suitable mist. The plant material to be treated is cut into pieces 3–4 mm long and placed in a support in the funnel as described for the modified Baermann-tray method. Optimum sample size depends on the sieve diameter and water flow rate; increasing the sample size can decrease the efficacy of extraction. Nematodes collected in the tube attached to the funnel stem can be released in a beaker for further examination. Compared with the modified Baermann techniques, plant material will decompose much more slowly, thus allowing prolonged extraction times of up to two weeks. Several funnels can be set up on a rack and one or two nozzles can supply all of them. The whole apparatus can be set up on a bench if enclosed with a polyethylene cover and left to stand on a drainage tray.

More refined methods are required for detection of *A. besseyi* in *O. sativa* seeds. Moretti *et al.* (1999) recommend the use of *O. sativa* chaff or hull as an alternative testing material.

More recently, laboratories wishing to use universally tested methods have adopted the Seed Health Testing Method 7-025 for detection of *A. besseyi* on *O. sativa* produced by the International Seed Testing Association (see Remeeus and Pelazza, 2014). The seeds are dehulled using a mill with a 1 mm distance between the rolls (Critical Control Points) (e.g. TR120 rice husker[1] (Kett Electric Laboratory)). The kernels or hulls are transferred in a nylon sieve with a mesh of 0.25 mm to a beaker of 45 mm diameter. The beaker is filled with approximately 20 ml water and is left undisturbed for 24 h at 25 ± 2 °C. The sieve is removed from the beaker and squeezed gently. The contents of the beaker are examined for the presence of nematodes.

Under a stereomicroscope, stylet-bearing nematodes with a well-demarcated large metacorpus are separated from other nematodes present in the Petri dish and transferred with a pipette or a needle to a glass slide for microscopic examination.

4. Identification

The morphological terms used in this section are defined in EPPO (2013b).

Although *Aphelenchoides* can be identified to species level based on morphological examination, this method is possible only for adult specimens, as is the case for most other plant-parasitic nematode species. For precise species-level identification, the morphological characters of *Aphelenchoides* species need to be carefully examined under a high power microscope with at least ×1 000 magnification for use with immersion oil or by using a scanning electron microscope.

Because *Aphelenchoides* is very difficult to identify to species level using morphological characters alone, molecular diagnostic tools have been developed to support the morphological identification of *Aphelenchoides* species (Ibrahim *et al.*, 1994a, 1994b). Molecular methods can be applied to identification of all life stages, including the immature stages, and may be particularly helpful when there is a low level of infestation or when adult specimens are atypical or damaged. However, the specificity of currently available molecular tests may be limited as they have generally been developed and evaluated using a restricted number of species and populations from different geographic regions.

4.1 Morphological identification of aphelenchs

4.1.1 Preparation of aphelenchs for morphological identification

Individual nematodes of *Aphelenchoides* species can be picked from the extract produced by any of the extraction methods described in section 3.2.2 and collected in a drop of water on a slide. The nematodes are slowly heated (to approximately 60 °C) until they become immobile (Hooper *et al*., 2005). The habitus of nematodes killed by gentle heating is almost straight. The nematodes can be sealed on the slide with wax or they can be placed in a drop of fixative before sealing with wax. There are some differences in the appearance of water and fixed specimens, with the former being preferable, but in fixed preparations some features such as the stylet are more distinct.

4.1.2 Identification of the family Aphelenchoididae

The family Aphelenchoididae is characterized by a large metacorpus and pharyngeal glands usually not enclosed in a bulb (overlapping). The dorsal pharyngeal gland opens into the metacorpus. Males have caudal papillae.

A. besseyi, *A. fragariae* and *A. ritzemabosi* belong to the family Aphelenchoididae, members of which share the morphological characteristics outlined in Table 1.

Table 1. Main morphological characteristics shared by the family Aphelenchoididae

Body part	Characteristic
Body form	Vermiform, not swollen
Lateral field	Usually with four or fewer incisures (two to four, rarely six)
Stylet	Slender, with narrow lumen and usually with small basal knobs or swellings
Pharynx	Isthmus rudimentary or absent, nerve ring circumpharyngeal to circumintestinal, pharyngeal glands lobe-like and long, dorsally overlapping intestine
Post-uterine sac	Usually present
Spicule	Rose thorn-shaped or derived therefrom
Adanal bursa	Rarely present (reported to date only from *Pseudoaphelenchus*)
Gubernaculum	Absent
Tail shape	Both sexes similar, conoid, with pointed or rounded, often mucronate, terminus

4.1.3 Identification of the genus *Aphelenchoides*

As with many other nematode genera, *Aphelenchoides* species are morphologically very similar; these nematodes are vermiform, with most species appearing stout and slow-moving. However, the few economically important species such as *A. besseyi*, *A. fragariae* and *A. ritzemabosi* tend to be slim, pale and relatively long when compared with most other *Aphelenchoides* species. They are also good swimmers (with a serpentine motion).

The members of the genus can be diagnosed by the following morphological characteristics (Figure 9):

- body length from 0.2 to 1.3 mm, but most commonly from 0.4 to 0.8 mm
- heat relaxed females become straight to ventrally arcuate (Figure 9(A))

- heat relaxed males assume a walking-stick shape with the tail region curled ventrally (Figure 9(B))
- cuticle finely annulated, lateral field with two to four (rarely six) incisures (Figure 9(D))
- stylet very difficult to see under low power microscopy; under high power, the stylet varies from clearly discernible to very faint. Generally about 10–12 µm long. Similarly, basal knobs or swellings are sometimes clear but often indistinct.
- pharynx: pharyngeal procorpus long and slender; metacorpus well developed, spherical to rounded-rectangular, with central valve plates; oesophageal gland lobe long, with dorsal overlap of the intestine (Figure 9(C))
- vulva typically post-median, usually between 60 and 75 percent of the body length
- ovary monoprodelphic, typically outstretched, but may be flexed
- post-vulval sac almost always present
- tail shape conoid to variable (Figure 9(E, F)), in male more strongly curved ventrally and papillae variable
- tail terminus without or with one or more mucros (Figure 9(G, H)) (a mucro is defined as a structure at the end of the tail terminus). Mucros can be definitively discerned only at ×1 000 with oil immersion. The presence or absence of mucros, and the shapes they assume, can be used to distinguish species, and are a key element in the identification of *A. besseyi*, *A. fragariae* and *A. ritzemabosi*.
- spicules well-developed, thorn-shaped, paired and separate
- bursa absent.

Aphelenchoides species can be distinguished from species of other genera encountered in soil and plant material by using the key in Table 2.

Table 2. Key to distinguish *Aphelenchoides* species from species of other genera in soil and plant material

1	Stylet present	**2**
	– Stylet absent	**NAS**
2	Four-part pharynx with a cylindrical procorpus followed by a valvulated metacorpus, slender isthmus and glandular basal bulb	**3**
	– Two-part pahrynx, anterior part slender, posterior part expanded, glandular and muscular (Note that the cylindrical procorpus and the valvulated metacorpus are considered separate parts)	**NAS**
3	Dorsal pharyngeal gland outlet in metacorpus; metacorpus very large, often nearly as wide as the diameter of the body	**4**
	– Dorsal pharyngeal gland outlet in procorpus behind stylet knobs; metacorpus moderate to reduced in size (less than three-fourths body width)	**NAS**
4	Pharyngeal glands lobe-like, long dorsal overlap of intestine	**5**
	–Pharyngeal glands pyriform, no overlap of intestine; or pharyngeal glands lobe-like, ventral overlap of intestine	**NAS**
5	Lateral fields with four or fewer incisures; stylet with basal knobs or swellings; female tail conoid, elongate conoid, convex conoid or subcylindroid to a pointed or narrowly rounded terminus; male spicules robust, thorn-shaped; adanal bursa absent	**6**

	– Lateral fields with six or more incisures; stylet without basal knobs; female tail short, subcylindroid and with broadly rounded terminus; male spicules slender, tylenchoid; adanal bursa present	**NAS**
6	Tail of both sexes short, usually less than four times anal body width	**7**
	– Tail of both sexes elongate to filiform, usually more than four times anal body width	**NAS**
7	Stylet slender, often about 10–12 µm and usually less than 20 µm; vulval flap absent; male without small bursa-like flap at tail tip	***Aphelenchoides***
	– Not with the above combination of characteristics	**NAS**

NAS, not *Aphelenchoides* spp.

4.1.4 Identification of *Aphelenchoides* to species level

The identification of species in the *Aphelenchoides* genus is complex and requires a systematic approach. It is generally agreed that a combination of morphological and molecular methods is required for the most reliable identification. The first step in diagnosis is to record and measure the critical morphological features of as many female specimens as are available, ideally 20. In practice, far fewer adult specimens are usually available, and in such cases, the nematologist should prepare the specimen(s) with great care to avoid damaging the few features available, leaving sufficient juveniles or females for analysis by molecular tools. Males are not included in the keys presented in this diagnostic protocol, but the shape and size of their spicules may assist in confirming the final identification.

The plant-infesting *Aphelenchoides* species include *A. besseyi*, *A. blastophthorus*, *A. fragariae*, *A. ritzemabosi* and *A. subtenuis*, which live in the above-ground parts of plants. They can be distinguished from other species of the genus by their slender body and the more posterior position of the hemizonid: six to ten annules behind the excretory pore (versus one to three annules) (Thorne, 1961).

Aphelenchoides is a large genus. Allen (1952) provided a key to the four species of bud and leaf nematodes (*A. besseyi*, *A. fragariae*, *A. ritzemabosi* and *A. subtenuis*). Sanwal (1961) produced a dichotomous key to the 35 *Aphelenchoides* species that were recognized at the time. Fortuner (1970) devised a dichotomous key to 11 *Aphelenchoides* species with star-shaped mucros. Baranovskaya (1981) provided a dichotomus key to 97 species with descriptions of 105 species. Shahina (1996) provided a compendium to 141 *Aphelenchoides* species and used tail terminus to divide these species into four groups: (1) tail simple without any outgrowth or mucronate structure; (2) tail terminus with one or sometimes two mucronate structures; (3) tail with tetramucronate spine or star-shaped; and (4) tail outgrowth other than with a spine or star-shaped. EPPO (2004) devised a polytomous key to 17 *Aphelenchoides* species including 14 species with star-shaped mucros and 3 species of bud and leaf nematodes without star-shaped mucros (*A. blastophthorus*, *A. fragariae* and *A. ritzemabosi*), and divided the tail terminus of *Aphelenchoides* species into five groups: (1) with star-shaped mucro; (2) with a single mucro; (3) bifurcate; (4) mucro shape belonging to other type at tail tip; and (5) without mucro.

A. besseyi differs from other plant-parasitic species of the genus by having a star-shaped mucro, although non-pathogenic species of *Aphelenchoides* also have star-shaped mucros. *A. besseyi* is the most common plant-parasitic species with a star-shaped mucro although plant-parasitic species can be found in strawberries (*A. blastophthorus*, *A. fragariae* and *A. ritzemabosi*) as follows: *A. besseyi* has a post-vulval sac that is always less than one-third of the distance from the vulva to the anus, whereas sacs of the other species are longer than this; the tail of *A. besseyi* has a conoid shape, similar to *A. blastophthorus*, but shorter than that of *A. fragariae* and *A. ritzemabosi*, which tend to be elongate conoid; the excretory pore is usually positioned near the anterior edge of the nerve ring in *A. besseyi*, whereas in the other species it is either level with or posterior to the nerve ring; and the spicules of *A. besseyi* are distinctive in that the proximal ends have an indistinct dorsal process (or apex) and have only a moderately developed ventral one (rostrum), while spicules of *A. blastophthorus* are

comparatively large for the genus, have a rather stout dorsal limb that is characteristically flattened about midway along its arch, with its distal end curved ventrally to give it a hooked or knobbed appearance, and the apex and rostrum are pronounced structures, spicules of *A. fragariae* have a moderately developed apex and rostrum, and the smoothly curved spicules of *A. ritzemabosi* seem to lack a dorsal or ventral process.

A. besseyi, *A. blastophthorus*, *A. fragariae*, *A. ritzemabosi* and *A. subtenuis* live as parasites in buds and leaves of plants. *A. saprophilus*, a fungivorous species, is also often found in damaged or diseased plant material, including bulbs and corms. Andrássy (2007) provided a key to 47 *Aphelenchoides* species found in Europe, including the six species encountered in buds and leaves. A short dichotomous key to *Aphelenchoides besseyi*, *A. fragariae* and *A. ritzemabosi* is given in Table 3.

For this diagnostic protocol, which concentrates on the three leaf and bud nematode species *A. besseyi*, *A. fragariae* and *A. ritzemabosi*, a dichotomous polytomous key is offered that should allow the reader to proceed reliably to the relevant specialist section. It is frequently not possible to determine a singular character in a short key that is required for identification, while a polytomous key allows the combination of a range of characters for a provisional identification.

As *A. besseyi*, *A. fragariae* and *A. ritzemabosi* can all occur in a wide range of habitats, including occasionally in planting media, all *Aphelenchoides* nematodes that may be found in these habitats need to be considered in a diagnosis. Unfortunately, many of these nematodes are difficult to identify because there is little to distinguish them, a problem not alleviated by the poor descriptions of the species themselves. However, several authors have improved the original descriptions for the three targeted species. In addition, studies on *Aphelenchoides* species have shown the degree of variation in measurements made on populations from different hosts.

As with all identifications involving the use of morphological characters, the combination of several key features is crucial to a positive diagnosis. In the polytomous key there is some overlap of codes, and users are advised to refer to original descriptions if in doubt about a diagnosis or to refer to Table 6 for further guidance and proceed to molecular testing to confirm.

A flow diagram of the identification process is provided in Figure 10.

Reference material can be found through various resources; for example, Q-bank (see http://www.q-bank.eu/Nematodes) or Nematode Collection Europe (NCE) (see http://www.nce.nu).

Table 3. Simplified key to distinguish *Aphelenchoides besseyi*, *A. fragariae* and *A. ritzemabosi* from other species

1	Post-vulval sac length more than one-third the distance between the vulva and the anus	**2**
	– Post-vulval sac length less than one-third the distance between the vulva and the anus; star-shaped mucro present	***A. besseyi***
2	Lateral field with three or four incisures	**3**
	– Lateral field with two incisures, body slender (a = 45–63), cephalic region almost continuous with body contour	***A. fragariae***
3	Tail terminus with a single mucro	**Other species**
	– Tail terminus with two to four processes pointing posteriorly giving it a paintbrush-like appearance, usually four incisures, stylet about 12 µm long, post-vulval sac usually more than half the distance between the vulva and the anus	***A. ritzemabosi***

4.2 Morphological identification of *Aphelenchoides besseyi*

4.2.1 Morphological characteristics

Details and views are provided in Figure 11.

Female. Body slender, straight to slightly arcuate ventrally when relaxed. Cephalic region rounded, unstriated, slightly offset and wider than body at lip base. Lateral fields about one-fourth as wide as body, with four incisures. Metacorpus oval, with a distinct valvular apparatus slightly behind its centre. Excretory pore usually near anterior edge of nerve ring. Post-vulval sac narrow, inconspicuous, not containing sperm, 2.5–3.5 times anal body width but less than one-third the distance from the vulva to the anus. Tail conoid, 3.5–5 anal body widths long. Terminus bearing a mucro of diverse shape with three to four pointed processes.

Male. Often as numerous as females. Posterior end of body curved by about 180 degrees in relaxed specimens. Tail conoid, with terminal mucro with two to four pointed processes. Spicules typical of the genus except that the proximal ends lack a distinct apex and have only a moderately developed rostrum. The dorsal limb spicules measure 18–21 µm (mean 19.2 µm).

4.2.2 Identification using morphological keys

In this diagnostic protocol an attempt has been made to reduce the number of comparisons required by selecting only those *Aphelenchoides* species that have a star-shaped mucro, together with those pest species that might also be encountered in foliage. It should be noted that the following procedure relies heavily on the original descriptions and drawings of species, which are sometimes contradictory. For example, the tail shape for *A. aligarhiensis* is described as elongate conoid, but the accompanying drawing does not show this. There is also no accompanying value for c´, which is an indicator of tail shape (the value of tail length divided by body width at anus). Similarly, the excretory pore for *A. jonesi* is said to be opposite the nerve ring, but the accompanying drawing shows it to be posterior to the nerve ring. In such cases the written description is the one included in this diagnostic protocol. Where possible, original data have been supplemented by additional published information for the most commonly encountered species.

4.2.2.1 Dichotomous key for *Aphelenchoides besseyi*

A short dichotomous key is provided in this diagnostic protocol as an added value for identification. Only characters from female nematodes have been considered. The key is complemented by Figure 11, showing critical features, and by Table 2 and Table 6, which provide more details of those *Aphelenchoides* species that have a star-shaped mucro together with those pest species that might also be encountered in foliage. After the key has been consulted, a check on the probable identity of the nematode should be made with reference to Table 3 and the relevant species description.

Dichotomous key to distinguish *A. besseyi* from other related species of *Aphelenchoides*

1	Star-shaped mucro present	**2**
	– Star-shaped mucro absent	**not *A. besseyi***
2	Post-vulval sac up to one-third of the length of the distance from the vulva to the anus	**3**
	– Post-vulval sac more than one-third of the length of the distance from the vulva to the anus	***A. aligarhiensis*, *A. brevistylus*, *A. fujianensis*, *A. lichenicola***
3	Tail shape is conoid or elongate conoid	**4**
	– Tail shape is subcylindroid	***A. siddiqii***

4	Stylet length in the range 10–12.5 µm – Stylet length outside the range 10–12.5 µm	**5** ***A. asteromucronatus, A. hylurgi, A. wallacei***
5	Four lateral lines – Fewer or more than four lateral lines	**6** ***A. andrassyi, A. asterocaudatus, A. unisexus***
6	Excretory pore anterior to, or level with, the anterior level of the nerve ring – Excretory pore level with the nerve ring	***A. besseyi*** ***A. goodeyi, A. jonesi, A. silvester***

4.2.2.2 Polytomous key for *Aphelenchoides* species

Polytomous keys allow the easy addition of new species into an identification procedure. For this diagnostic protocol several important features of species related to *A. besseyi* have been selected to produce a small polytomous key (Table 4). The selected features have been given codes. It has been feasible to convert only six morphological characters into codes. Each specimen for identification should be examined and assigned a set of these codes (A–F) according to the following categorization (Figure 12).

A. Tail terminus or mucro shape

1 = star (Figure 12(A) (a–f))

2 = single terminal mucro (Figure 12(A) (g–m))

3 = bifurcate (Figure 12(A) (n))

4 = other (Figure 12(A) (o–t))

5 = no mucro (Figure 12(A) (u–v))

B. Length of the post-vulval sac

1 = one-third or less the distance between the vulva and the anus

2 = more than one-third the distance between the vulva and the anus

3 = no post-vulval sac

C. Tail shape

1 = conoid: cone-shaped, with both sides of the tail surface tapering at an equal angle to the tail tip. Total length not exceeding five times the anal body width (Figure 12(B) (a)).

2 = elongate conoid: an elongated cone, with a length five times or more the anal body width (Figure 12(B) (b-c))

3 = dorsally convex conoid: at first appearance this tail shape is curved ventrally. The dorsal side of the tail is curved in a convex manner before it joins the ventral surface. The ventral surface is usually concave, but from some viewpoints may appear straight. The tail may be any length (Figure 12(B) (d-e)).

4 = subcylindroid: both sides of the tail appear to run parallel for most of its length, and they end in a hemispherical or subhemispherical tail tip (Figure 12(B) (f))

D. Stylet length (µm)

1 = 10–13

2 = less than 10

3 = more than 13

E. Lateral lines (number of)

1 = 4 lines

2 = 3 lines

3 = 2 lines

4 = unknown

F. Relative position of the excretory pore and nerve ring

1 = excretory pore anterior to or level with the anterior level of the nerve ring (Figure 12(C) (a))

2 = excretory pore level with the nerve ring (Figure 12(C) (b))

3 = excretory pore posterior to or opposite the posterior level of the nerve ring (Figure 12(C) (c))

The set of codes obtained should be compared with those set out in Table 4, which will allow a provisional diagnosis to be made. A positive diagnosis is made when the value of most of the codes matches the reference species.

Table 4. Polytomous codes of selected *Aphelenchoides* species

Species	A	B	C	D	E	F
A. besseyi	1	1	1	1	1	1
A. hylurgi	1	1	1	1	4	3
A. unisexus	1	1	1/3	1	3	3
A. asteromucronatus	1	1	1/3	2	1	3
A. siddiqii	1	1	3/4	1	1	1/2/3
A. asterocaudatus	1	1/2	1	1	3	3
A. andrassyi	1	1/2	2/3	1/2	2	4
A. wallacei	1	1/2	3	3	1	1
A. goodeyi	1	2	1	1	1	3
A. lichenicola	1	2	1	1/2	1	2/3

A. silvester	1	2	1	1/2	1	4
A. fujianensis†	1	2	1	1/3	1	1/2
A. jonesi	1	2	1	1/3	1	2
A. brevistylus	1	2	1/2	2	3	1
A. aligarhiensis	1	2	2/3	1	1	1
A. blastophthorus	2	2	1/2	3	1	2
A. subtenuis	2	2	4	1	2	2/3
A. ritzemabosi	4	2	2	1	1	3
A. fragariae	2/4	2	2	1	3	2/3

Source: EPPO (2004).

Notes:

A. nonveilleri and *A. saprophilus*, mentioned in previous editions of this key, are now considered by the author to be species indeterminate.

The assignment of more than one code for a particular feature for a species is based on variation noted in published data or seen in practice.

† The codes for *A. fujianensis* were assigned by Zuo *et al.* (2010).

4.3 Morphological identification of *Aphelenchoides fragariae*

4.3.1 Morphological characteristics

Allen (1952), Siddiqi (1975) and Hunt (1993) all provide detailed descriptions of *A. fragariae* (see Figure 13). This description was modified from Hunt (1993).

Female. Body slender (a = 45–70), straight to arcuate ventrally when relaxed. Cuticle finely annulated, lateral field with two incisures. Cephalic region almost continuous with body, appears smooth under the microscope, and four to five annuli visible by scanning electron microscopy (Khan *et al*., 2007, 2008). Stylet slender, about 8–14 µm long, often 10–11 µm; conus and shaft nearly equal in length; basal knobs minute but distinct. Pharynx typical of the genus, metacorpus oval and highly muscular with central valve plates, pharyngeal gland lobe with dorsal overlap of the intestine, two to four body widths long. Nerve ring encircling isthmus near its base, about one body width behind metacorpus. Excretory pore level with or close behind nerve ring. Genital tract monoprodelphic, outstretched, with oocytes in a single row, never reaching pharynx. Post-vulval sac long, extending more than half the distance between the vulva and the anus. Tail elongate conoid with a single simple spike or minute mucro at tail tip.

Male. Abundant. Essentially similar to female in general morphology. Tail arcuate through 45 to 90 degrees when relaxed, not sharply curved like a hook, with a simple terminal spine. Three pairs of caudal papillae present. Spicules rose thorn-shaped with moderately developed apex and rostrum, dorsal limb 10–19 µm long.

The key diagnostic features to distinguish *A. fragariae* from the other known species of *Aphelenchoides* are:

- body 0.4–1.0 mm long, very slender (a = 45–70) (Figure 13(D–F))
- stylet slender, about 8–14 µm long, with distinct basal knobs (Figure 13(A, B))
- tail elongate conoid with a single simple spike or mucro at tail tip (Figure 13(G, I, M, P, Q))

- post-vulval sac extending more than half the distance between the vulva and the anus (Figure 13(F))
- excretory pore level with or close behind nerve ring
- lateral field with generally two incisures (Figure 13(H, O))
- males common, spicules on dorsal limb 14–17 µm long (Figure 13(J–L)).

4.3.2 Comparison with similar species

A. fragariae is similar to *A. arachidis*, *A. helophilus*, *A. resinosi* and *A. rhytium*, but can be distinguished from all other species described in *Aphelenchoides* by its more slender body (a = 45–70), lateral field with generally two incisures and tail terminus with a single mucro. *A. fragariae* can be distinguished from these similar species using the key given in Table 6. A diagnostic compendium of *A. fragariae* and similar species and bud and leaf nematodes of the genus is presented in Table 6, which provides details to help to determine the identity of these similar species.

Table 5. Dichotomous key to distinguish *Aphelenchoides fragariae* from morphologically similar species

1	Female tail more than 30 µm long, conoid to elongate conoid	**2**
	– Female tail shorter, 22–28 µm long, subcylindroid with bluntly rounded tip	***A. arachidis***
2	Female body length less than 1.0 mm, tail mucro offset; male spicules less than 25 µm long	**3**
	– Female body 0.8–1.3 mm, tail mucro not offset; male spicules 26 µm long	***A. helophilus***
3	Post-vulval sac length less than half the distance between the vulva and the anus	**4**
	– Post-vulval sac length more than half the distance between the vulva and the anus	***A. fragariae***
4	Female tail less than 40 µm long, two lateral incisures; spicules 13–15 µm long	***A. resinosi***
	– Female tail 56.2 µm long, lateral incisures absent; spicules 22.9 µm long	***A. rhytium***

Table 6. Morphological characteristics of *Aphelenchoides fragariae* compared with similar species

Species	L (mm)	A	B	C	c´	Tail (µm)	Tail shape[†]	Terminal mucro shape[1]	V	PVS/VA[‡]	Stylet (µm)	LL[§]	Spicules (µm)	References
A. arachidis	0.51–1.0	39–50	11–18	25–42	2–3	22–28	Subcylindroid	Single central spine	67–74	Approximately half	11–12	2	15–25	Bridge and Hunt (1985)
A. besseyi	0.66–0.75	32–42	10.2–11.4	17–21	3.5–5.0	36–42	Conoid	Star	68–70	Less than one-third	10–12	4	18–21	Franklin and Siddiqi (1972); Andrássy (2007)
A. blastophthorus	0.68–0.95	28–50	9.0–12.8	15–28	2.3–5.0	42–48	Conoid	Single central spine	62–74	Approximately half	15–19.5	4	24–32	Hooper (1975); Shahina (1996)
A. fragariae	0.45–0.80	36–63	8–15	12–20	4.9	38–42	Elongate conoid	Single central spine	64–71	More than half	10–11	2 [¶]	14–17	Siddiqi (1975); Shahina (1996)
A. helophilus	0.80–1.30	43–78	12–14	14–20	5.5	>40	Elongate conoid	Single central spine	65–79	Unknown	12	Unknown	26	Shahina (1996); Andrássy (2007)
A. resinosi	0.40–0.80	29–53	7–13	12–19	3–4	33.7	Conoid	Single central spine	66–79	Less than half	10–11	2	13–15	Kaisa *et al*. (1995)
A. rhytium	0.78–0.94	43–48	11.7–13.4	16–21		56.2	Elongate conoid	Single central spine	67	Less than half	11	Absent	22.9	Massey (1974); Shahina (1996)
A. ritzemabosi	0.77–1.20	40–45	10–13	18–24	4–5	47	Elongate conoid	Peg with two to four minute processes	66–75	More than half	12	4	20–22	Allen (1952); Siddiqi (1974); Andrássy (2007)
A. saprophilus	0.45–0.62	26–33	8–12	12–18	2.5–3.0	32	Conoid	Ventral peg	66–70	Approximately half	11	4	22–23	Shahina (1996); Andrássy (2007)
A. subtenuis	0.87–1.15	44–57	12–17	24–28	2.78–3.27	42.4	Subcylindroid	Single ventral spine	69–71	More than half	11	3 or 4	18–23	Allen (1952); Deimi *et al.* (2006)

Source: Adapted from EPPO (2004).

[†] Shape of female tail and terminal mucro are presented in Figure 14.

[‡] Post-vulval sac (PVS) length divided by the distance between the vulva and the anus (VA).

[§] Number of lateral lines (LL).

[¶] Specific populations of *A. fragariae* can have more than two lateral fields.

4.4 Morphological identification of *Aphelenchoides ritzemabosi*

4.4.1 Morphological characteristics

Details and views are provided in Figure 15. Morphological characteristics are from Siddiqi (1974).

Female. Body slender, 0.77–1.20 mm long; annules 0.9–1.0 µm wide, distinct; lateral fields one-sixth to one-fifth as wide as body, with four incisures. Lip region hemispherical, set off by a constriction, slightly wider than adjacent body, no annulations visible under a light microscope; framework hexaradiate, weakly sclerotized. Stylet about 12 µm long, with distinct basal knobs and sharply pointed anterior. Procorpus slender; metacorpus large, somewhat oval in shape, highly muscular, with prominent internal cuticular thickening and orifices of dorsal and subventral pharyngeal glands. Nerve ring in neotype 1.5 body widths behind bulb. Excretory pore 0.5–2 body widths posterior to nerve ring. Three pharyngeal glands forming a lobe extending about four body widths over intestine dorsally. Pharyngo-intestinal junction about 8 µm behind metacorpus, indistinct and valve not discernible. Intestine with small spherical granules and a distinct lumen throughout. Vulva slightly protruding, transverse slit. Post-vulval uterine sac extending for more than half the distance between the vulva and the anus, often containing sperm. Ovary single anteriorly outstretched, oocytes in multiple rows. Tail elongate conoid, bearing a terminal peg which has two to four minute processes pointing posteriorly giving it a paintbrush-like appearance.

Male. Common. Posterior end of body usually curved through 180 degrees upon relaxation. Lip region, stylet and pharynx similar to that in female. Testis single, outstretched. Three pairs of ventro-submedian caudal papillae: first pair adanal, second midway on tail, third near tail end. Spicules smoothly curved, rose thorn-shaped, lacking a dorsal or ventral process at the proximal end; dorsal limb 20–22 µm long. Tail peg with two to four processes, of variable shape.

Measurements. ♀♀: L = 0.77–1.20 mm; a = 40–45; b = 10–13; c = 18–24; V = $^{48–33}$66– 75$^{14–17}$. Neotype ♀: L = 0.85 mm; a = 42; b = 12; c = 18; V = ^{35}68^{17}; stylet = 12 µm. ♂♂: L = 0.70–0.93 mm; a = 31–50; b = 10–14; c = 16–30; T = 35–64.

4.4.2 Comparison with similar species

Aphelenchoides species are morphologically very similar and can be easily confused. Molecular identification (section 4.5) may contribute to identification when there is any uncertainty with morphological identification. More information for comparison with similar species can be found in Table 6.

4.5 Molecular identification of *Aphelenchoides* species

Several molecular tests for the identification of *Aphelenchoides* species have been developed and are now in use (McCuiston *et al.*, 2007; Rybarczyk-Mydłowska *et al.*, 2012). Polymerase chain reaction (PCR) with species-specific primers can be used for diagnosis of nematodes isolated from plant material (section 3.2.2). Any development stage can be subjected to the molecular tests.

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

The final volume of the PCR is based on the descriptions in the source papers. PCR could also be carried out in a volume that accords with the instructions of the Taq DNA polymerase enzyme being used.

4.5.1 DNA extraction

DNA may be extracted from single or mixed sexes and life stages (adults, juveniles and eggs) of nematodes. There is a risk of false negatives if DNA is not extracted properly, so a negative molecular

test result does not exclude the possibility of positive identification by morphological methods. For each PCR test described below, the DNA extraction methods refer to the source paper for the original specific procedure used. DNA may also be extracted using other methods suitable for nematodes. Alternatively, commercial kits for DNA isolation are available.

4.5.2 Real-time PCR for four foliar nematode species

Rybarczyk-Mydłowska *et al.* (2012) designed a small subunit (SSU) ribosomal (r)DNA-based species-specific PCR for four foliar nematode species: *A. besseyi*, *A. fragariae*, *A. ritzemabosi* and *A. subtenuis.* The species-specific primers were designed based on the full-length SSU rDNA sequences of these four *Aphelenchoides* species, and they were used for real-time PCR to rapidly identify one or more foliar nematode species isolated from plant material and soil.

The primer pair (designed with locked nucleic acid (LNA), designated by * below) recommended for the detection of *A. besseyi* is:

1770: 5′-GCG GGA TTC GTG GTT C*T-3′
1772: 5′-CGA CAT GCC GAA ACA GAG-3′

The primers specific for *A. fragariae* (located in the SSU rDNA sequence) used in this PCR are:

1469: 5′-CTT ATC GCA CGA CTT TAC G-3'
1472: 5′-TCA AAG TAA TCC GCA TCC AAT-3'

The primer pair (designed with LNA, designated by * below) recommended for the detection of *A. ritzemabosi* is:

1496: 5′-CGC TGG TGG GTT TCG A-3′
1499: 5′-CCC GCT AAG AAA TGA TCA C*C-3′

The exact melting temperature of the specific fragment produced by each primer pair is not provided in Rybarczyk-Mydłowska *et al.* (2012) but can be estimated from the graphs included as approximately 85 °C for *A. besseyi* (primers 1770/1772), approximately 84 °C for *A. fragariae* (primers 1469/1472) and approximately 87 °C for *A. ritzemabosi* (primers 1496/1499). Nevertheless, these values should be confirmed under the specific conditions of each laboratory.

Each real-time PCR is performed according to the conditions described in Table 7.

Table 7. Real-time PCR master mix composition, cycling parameters and amplicons (Rybarczyk-Mydłowska *et al.*, 2012)

Reagent	Final concentration
PCR-grade water	–[†]
PCR buffer Absolute qPCR mix SYBR Green[1] (Thermo Scientific)	1×
Specific forward primer	0.2 µM
Specific reverse primer	0.2 µM
DNA (volume)	3 µl
Cycling parameters	
Initial denaturation	95 °C for 15 min
Number of cycles	60
Denaturation	95 °C for 30 s
Annealing	63 °C for 60 s
Elongation	72 °C for 30 s
Expected amplicons	

Size	325 bp for *Aphelenchoides besseyi* 470 bp for *A. fragariae* 347 bp for *A. ritzemabosi*	

† For a final reaction volume of 25 µl.

bp, base pairs; PCR, polymerase chain reaction.

4.5.3 PCR for *Aphelenchoides fragariae*

This internal transcribed spacer (ITS)1-PCR was described by McCuiston *et al.* (2007) as a diagnostic test for early detection and identification of *A. fragariae* directly in host plant material using the species-specific primers given below. These specific primers amplify DNA from *A. fragariae* and do not amplify DNA from other plant-parasitic nematode species (*Meloidogyne incognita*, *Heterodera schachtii*, *Pratylenchus penetrans*, *Caenorhabditis elegans*, *D. dipsaci*, *A. besseyi* and *A. ritzemabosi*). The PCR is sensitive, detecting a single nematode in a background of plant material extract. The test has accurately detected *A. fragariae* in more than 100 naturally infected samples, including 50 ornamental plant species. Total DNA was extracted from infected plant material using the DNeasy Plant Mini Kit[1] (Qiagen).

The primers (within the rDNA-ITS1 region) used in this PCR are:

AFragF1 (forward): 5'-GCA AGT GCT ATG CGA TCT TCT-3'

AFragR1 (reverse): 5'-GCC ACA TCG GGT CAT TAT TT-3'

Each real-time PCR is performed according to the conditions described in Table 8.

Table 8. ITS1 rDNA conventional PCR master mix composition, cycling parameters and amplicons (McCuiston *et al.*, 2007)

Reagent	**Final concentration**
PCR-grade water	–†
PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl)	1×
$MgCl_2$	1 mM
dNTPs	0.2 mM
Primer AFragF1 (forward)	0.4 µM
Primer AFragR1 (reverse)	0.4 µM
DNA polymerase (GoTaq Flexi DNA Polymerase[1] (Promega))	1.25 U
DNA (volume)	2 µl
Cycling parameters	
Initial denaturation	94 °C for 2 min
Number of cycles	40
Denaturation	94 °C for 1 min
Annealing	53 °C for 40 s
Elongation	72 °C for 1 min
Final elongation	72 °C for 10 min
Expected amplicons	
Size	169 bp

† For a final reaction volume of 25 µl.

bp, base pairs; ITS, internal transcribed spacer; PCR, polymerase chain reaction; rDNA, ribosomal DNA.

4.5.4 Controls for molecular tests

For the test result obtained to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid isolation and amplification of the target pest or target nucleic acid. For PCR a positive nucleic acid control and a negative amplification control (no template control) are the minimum controls that should be used.

Positive nucleic acid control. This control is used to monitor the efficiency of the test method (apart from the extraction), and specifically the amplification. Pre-prepared (stored) nematode nucleic acid may be used.

Negative amplification control (no template control). This control is necessary for conventional and real-time PCR to rule out false positives due to contamination during preparation of the reaction mixture. PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage instead of DNA solution.

4.5.5 Interpretation of results from PCR

Conventional PCR

The pathogen-specific PCR will be considered valid only if the positive control produces an amplification product of the expected size for the target nematode and the negative control produces no amplification product of the expected size for the target nematode.

Real-time PCR

The real-time PCR will be considered valid only if the positive control produces an amplification curve with the pathogen-specific primers and the negative control produces no amplification curve.

If internal control primers are also used, the positive control and each of the test samples should produce an amplification curve.

5. Records

Records and evidence should be retained as described in ISPM 27 (*Diagnostic protocols for regulated pests*).

In cases where other contracting parties may be affected by the results of the diagnosis, in particular in cases of non-compliance (ISPM 13 (*Guidelines for the notification of non-compliance and emergency action*)) and where the nematode is found in an area for the first time, the following records and evidence and additional material should be kept for at least one year in a manner that ensures traceability:

- the original sample of the infested material
- description and photographs of the symptoms and damage
- measurements and drawings or photographs of the nematode
- permanent slides or culture of the nematode
- if relevant, DNA extracts and PCR amplification products, stored at −80 °C and −20 °C, respectively.

6. Contact Points for Further Information

Further information on this protocol can be obtained from:

United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Plant Protection and Quarantine (PPQ), 389 Oyster Point Blvd, Suite 2, South San Francisco, CA 94080, United States (Fengru Zhang; e-mail: fzhang@aphis.usda.gov; tel.: +1 650 876 9098; fax: +1 650 876 0915).

Laboratory of Plant Nematology and Research Center of Nematodes of Plant Quarantine, College of Natural Resources and Environment, South China Agricultural University, Wushan Street, Guangzhou City, Guangdong Province 510642, China (Hui Xie; e-mail: xiehui@scau.edu.cn; tel.: +86 020 3829 7432; fax: +86 020 3829 7286).

Directorate Inspection Services, Department of Agriculture, Forestry and Fisheries, Private Bag X5015, Stellenbosch 7599, South Africa (Rinus Knoetze; e-mail: RinusK@daff.gov.za; tel.: +27 021 809 1621).

Nematology Unit, Fera Science Limited, Sand Hutton, York YO1 1LZ, United Kingdom (Sue Hockland; e-mail: sue.hockland@plantparasiticnematodes.com).

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will in turn forward it to the Technical Panel on Diagnostic Protocols (TPDP).

7. Acknowledgements

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The information for *A. besseyi* is adapted from the EPPO Diagnostic Protocol for *Aphelenchoides besseyi* (PM 7/39(1)) (see EPPO, 2004), which was produced by S. Hockland with the cooperation of the members of the EPPO Panel for Diagnostics in Nematology, the EPPO Secretariat, David Hunt and Mischa Aalten.

8. References

The present annex refers to ISPMs. ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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9. Figures

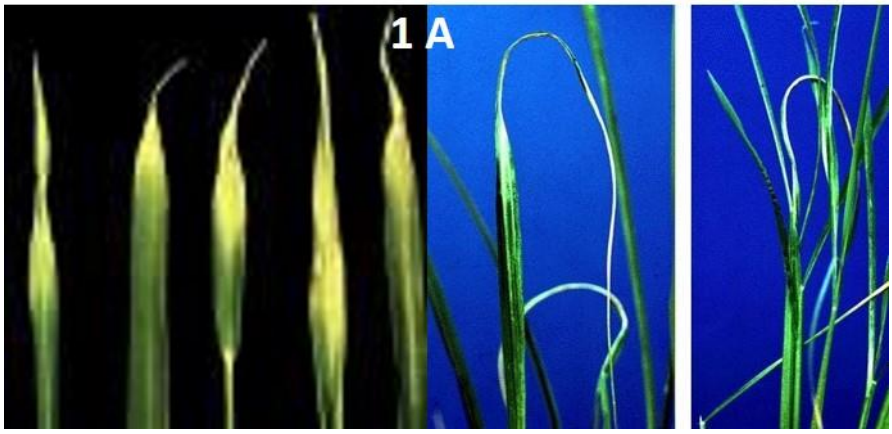


Figure 1(A). Symptoms caused by *Aphelenchoides besseyi* on *Oryza sativa* leaves: white tip (left and middle) and necrotic patches and crinkled leaves (right).
Photo courtesy Society of Nematologists (1980) (left) and CABI (2006) (middle and right).

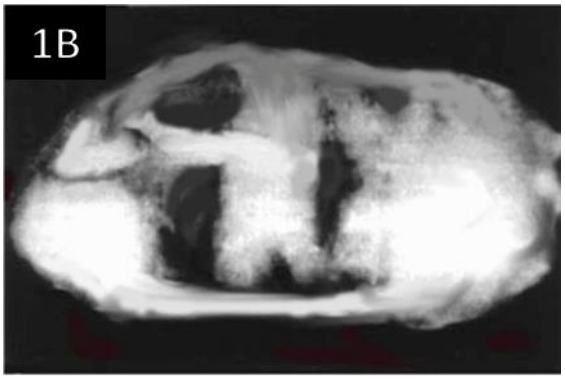


Figure 1(B). Necrotic lesions caused by *Aphelenchoides besseyi* in the endosperm of a rice kernel.
Photo courtesy Bridge et al. *(1990)*.

Figure 1(C). Symptoms caused by *Aphelenchoides besseyi* on strawberry.
Photo courtesy Jeffrey Lotz, Florida Department of Agriculture and Consumer Services, Gainesville, FL, United States.

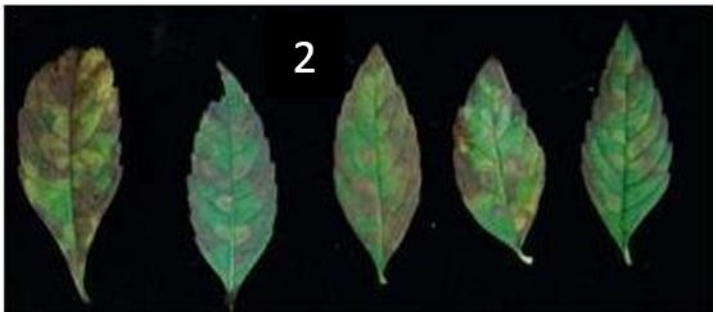


Figure 2. Symptoms caused by *Aphelenchoides fragariae* on *Stachys riederi* var. *japonica.*
Photo courtesy Khan et al. *(2008)*.

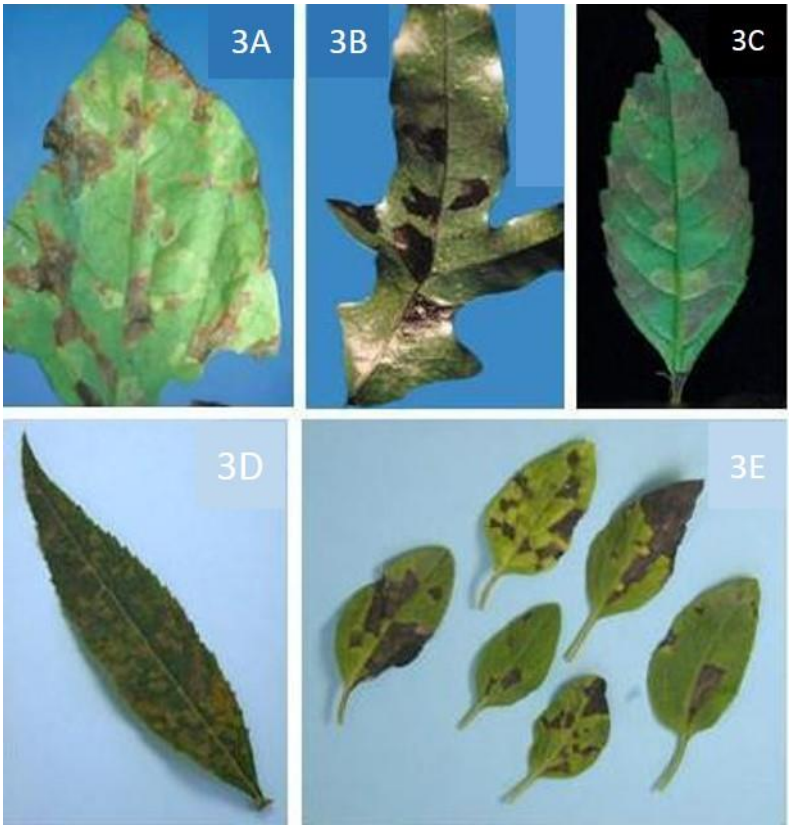


Figure 3. Symptoms of *Aphelenchoides fragariae* attack in: (A) *Convolvulus arvensis*; (B) *Phymatodes diversifolium*; (C) *Stachys riederi*; (D) *Buddleja* sp.; and (E) *Salvia* sp.
Photo courtesy (A, B) Knight et al. *(2002); (C) Khan* et al. *(2008); and (D, E) Kohl (2011)*.

Figure 4. Different degrees of symptom severity on *Hosta* leaves caused by *Aphelenchoides fragariae.*
Photo courtesy Zhen et al. *(2012)*.

Figure 5. Leaf blotch symptoms on plants infested with *Aphelenchoides fragariae*: (A) ferns; (B) *Pteris cretica*; and (C) *Stenochlaena tenuiflolia.*
Photo courtesy (A) Cobon and O'Neill (2011) and (B, C) Chizov et al. *(2006)*.

Figure 6. Leaf blotch symptoms on plants infested by *Aphelenchoides fragariae:* (A) *Begonia* sp. and (B) *Andrographis paniculata.*
Photo courtesy (A) Department of Crop Sciences, University of Illinois at Urbana–Champaign, Champaign, IL, United States, and (B) dan Supriadi (2008).

Figure 7. *Fragaria* spp. plants infested with *Aphelenchoides fragariae:* (A) tight aggregation of crown with malformed leaves; (B) abnormal plant growth with stunting and deformation; (C) an uninfested plant; and (D) malformed leaves.
Photo courtesy (A–C) Cobon and O'Neill (2011) and (D) Adam Szczygieł, formerly Institute of Pomology and Floriculture, Experimental Research Station at Brzezna, Poland.

Figure 8. Symptoms (interveinal necrosis) caused by *Aphelenchoides ritzemabosi* on *Chrysanthemum* leaves.
Photo courtesy J. Bridge, CABI BioScience.

Figure 9. *Aphelenchoides* spp.: (A) female; (B) male; (C) female anterior end; (D) lateral field; (E) female tail; (F) male tail; (G) female tail terminal mucro; and (H) male tail terminal mucro.
Photo courtesy (A, B, E) Wang et al. *(2013); (D, G) Deimi* et al. *(2006); (H) Yu and Tsay (2003); and (C, F) Z. F. Yang and H. Xie, South China Agricultural University, Guangzhou, China)*.

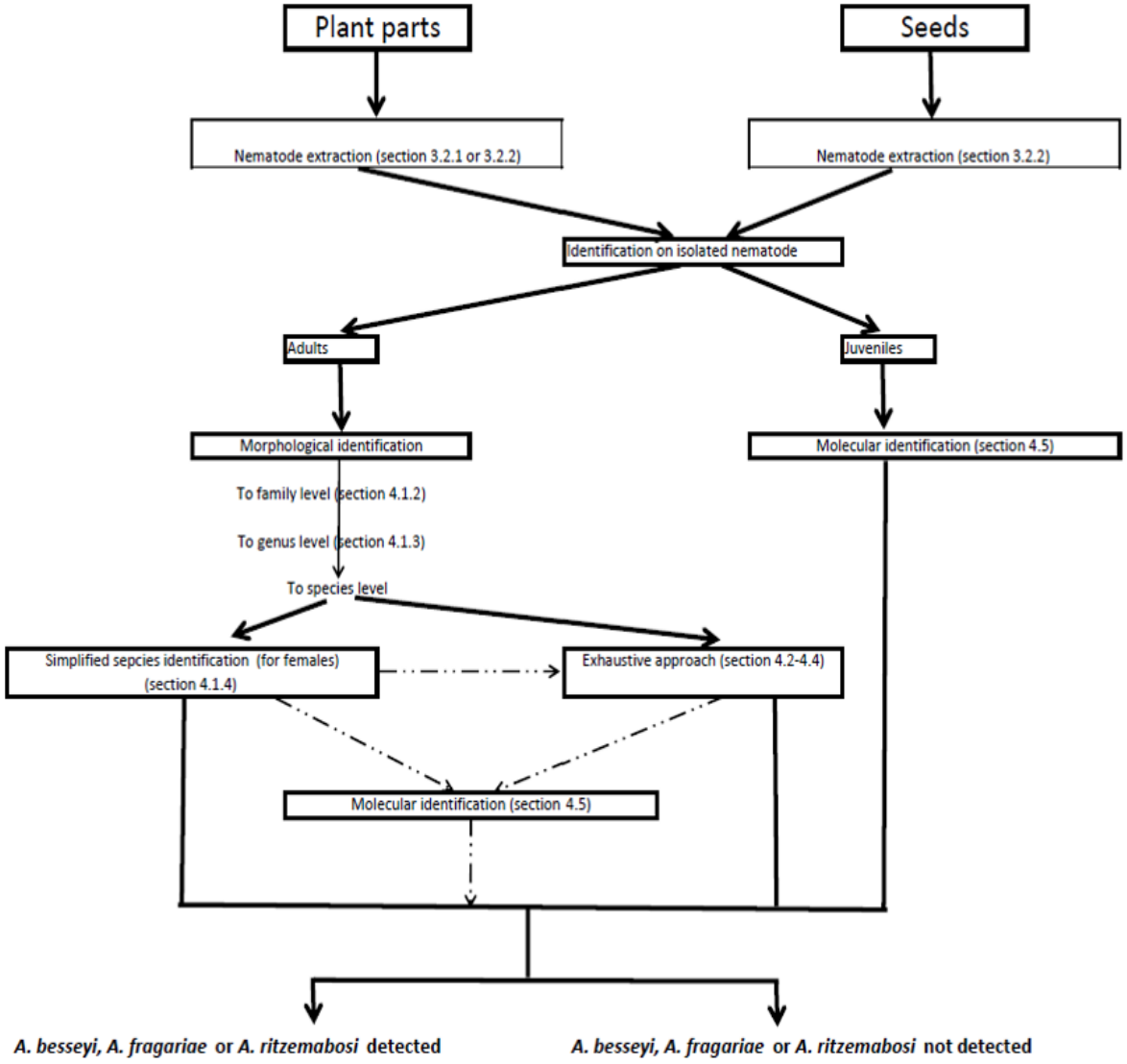


Figure 10. Flow diagram of the process to identify *Aphelenchoides* species.

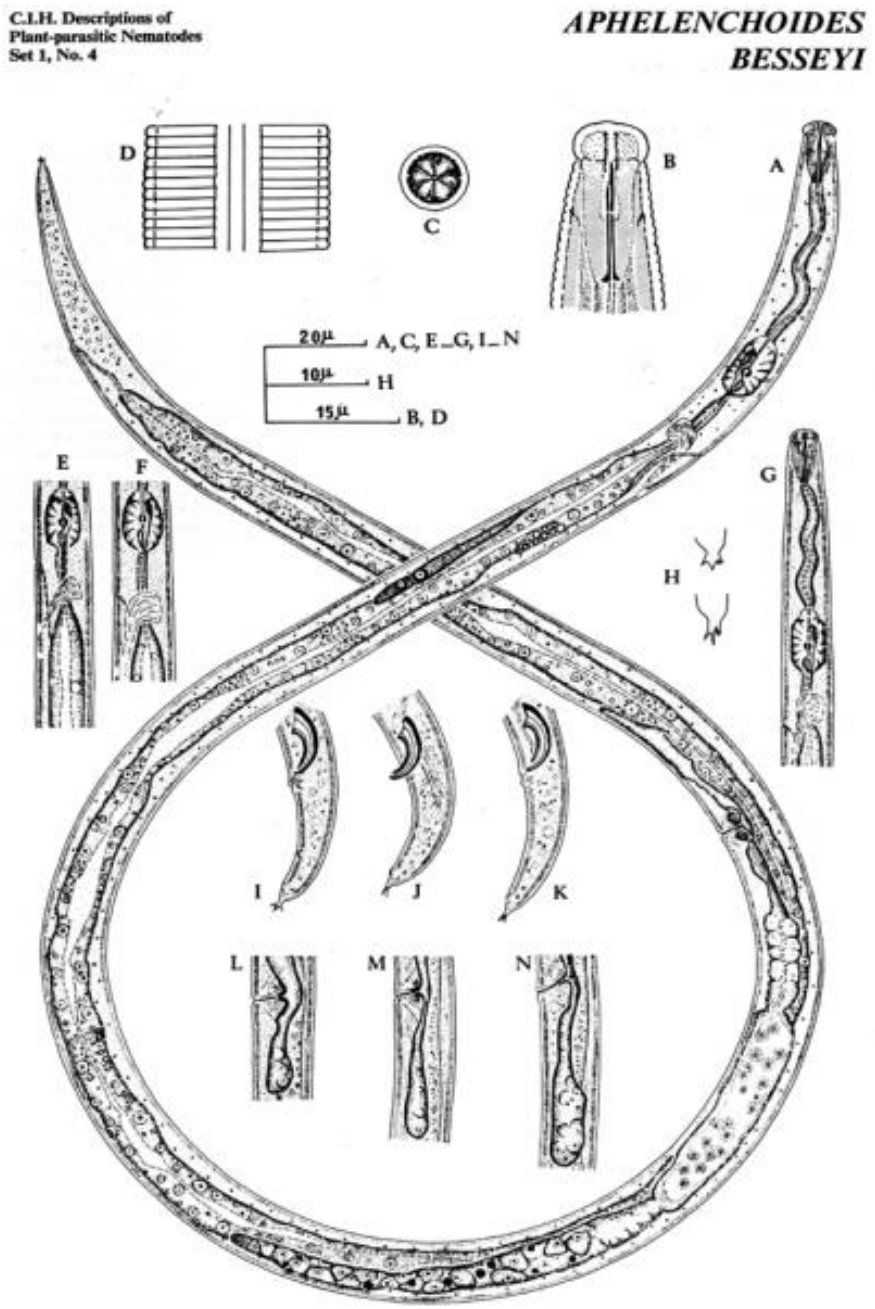
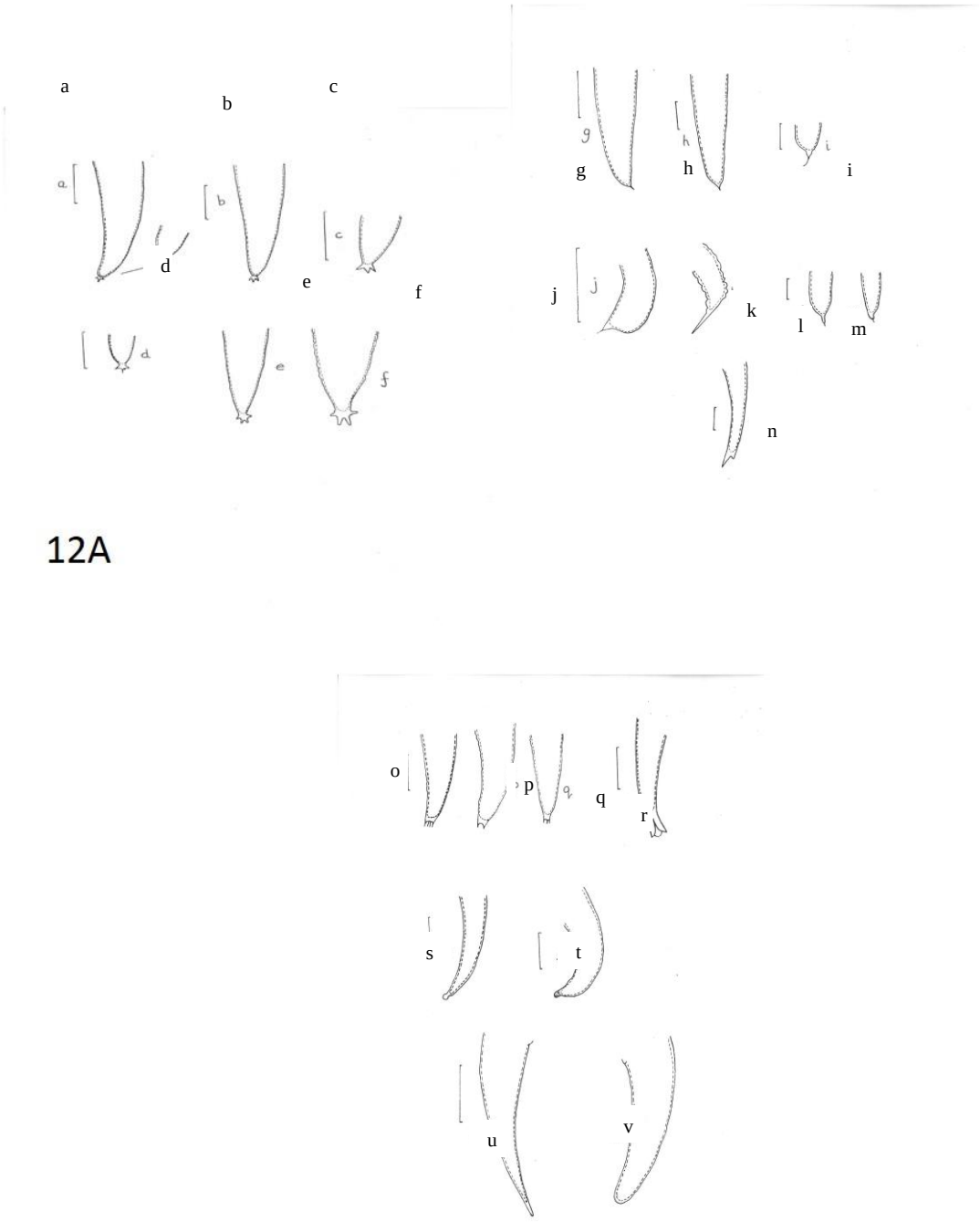


Figure 11. *Aphelenchoides besseyi*: **(**A) female; (B) female head end; (C) female *en face* view; (D) lateral field; (E, F) variation in female metacorpus and pharynx region and position of excretory pore with respect to nerve ring; (G) male anterior end; (H) female tail termini showing variation in shape of mucro; (I–K) male tail ends; and (L–N) variation in post-vulval sac.
Source: Fortuner (1970), except (D) Franklin and Siddiqi (1972).



12A

Figure 12(A). Tail terminus types of *Aphelenchoides* species (code numbers according to the polytomous key in section 4.2.2.2): (a–f) star shape: (a) *A. aligarhiensis*, (b) *A. asterocaudatus*, (c) *A. besseyi* and (d) *A. goodeyi* (all scale bars = 10 µm); (e, f) *A. nonveilleri* (×1 100 and ×2 200, respectively); (g–m) single terminal mucro: (g) *A. richardsoni*, (h) *A. nechaleos*, (i) *A. vaughani,* (j) *A. sp.* (k) *A. tsalolikhini* and (l, m) *A. submersus*; (n) bifurcate: *A. bicaudatus* (all scale bars = 10 µm); (o–t) other: (o–q) *A. ritzemabosi*, (r) *A. sphaerocephalus*, (s) *A. gynotylurus* and (t) *A. helicosoma* (all scale bars = 10 µm); and (u, v) no mucro: (u) *A. microstylus* (scale bar = 10 µm) and (v) *A. obtusus* (×1 250).
Drawing Sue Hockland, Fera Science Limited, York, United Kingdom.

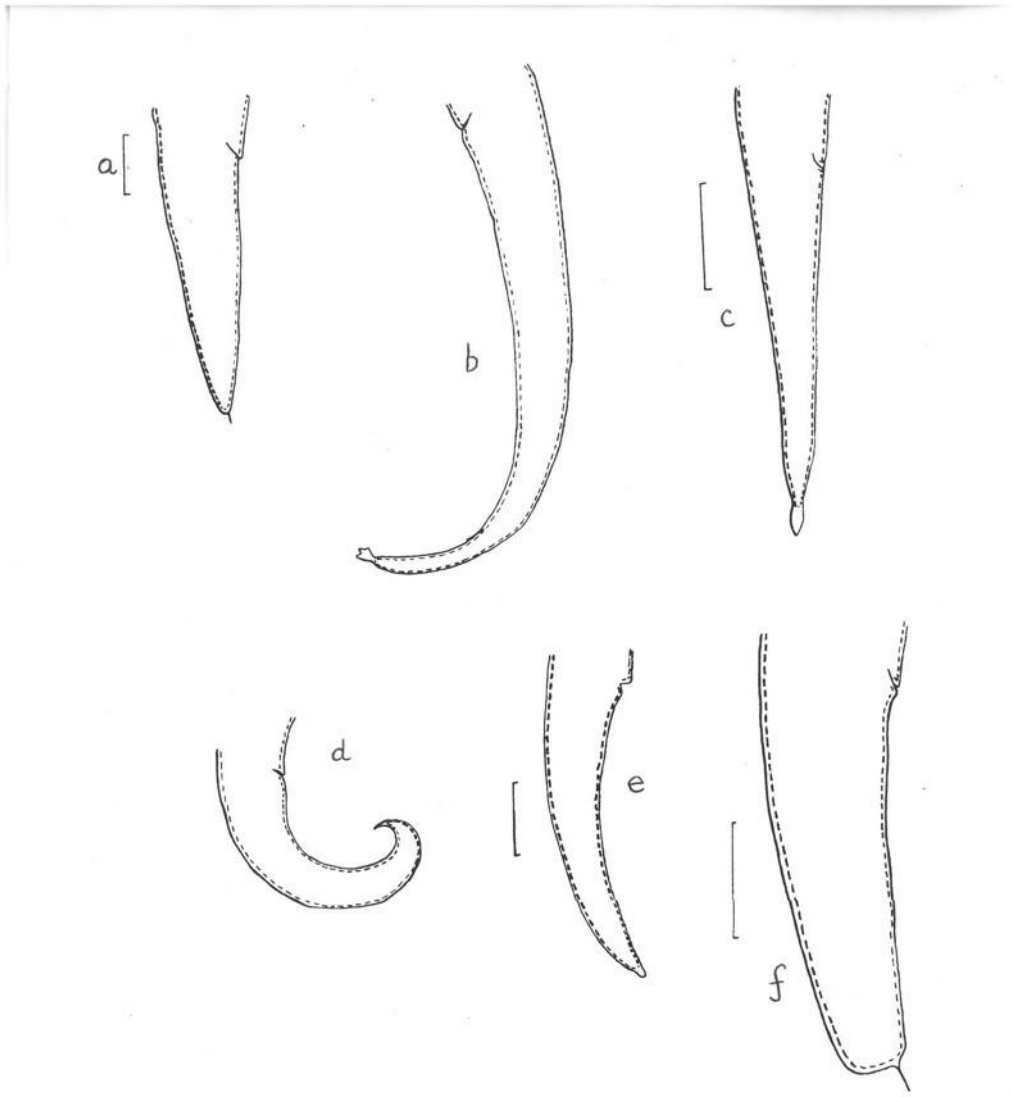
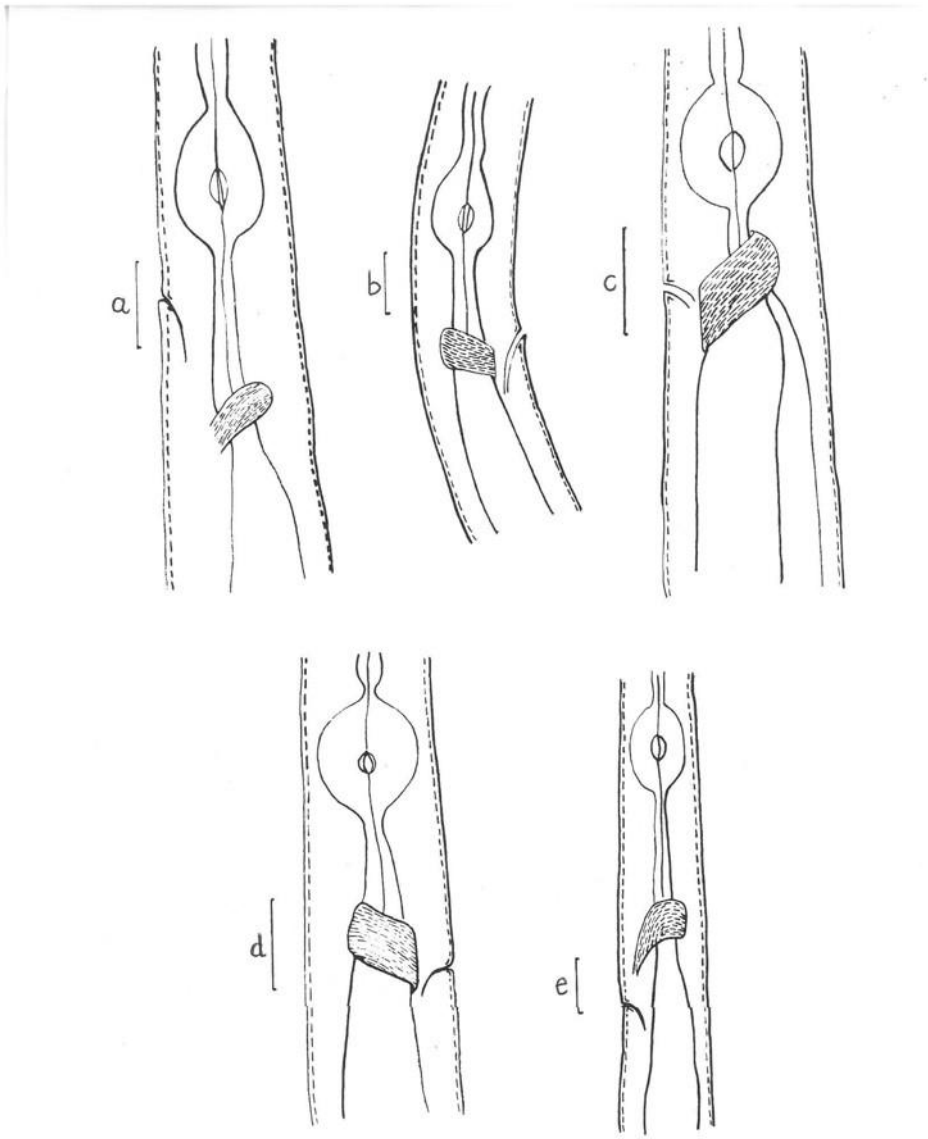


Figure 12(B). Tail shapes in *Aphelenchoides* species (scale bars = 10µm): conoid: (a) *A. blastophthorus*; elongate conoid: (b) *A. andrassyi* (no scale bar) and (c) *A. chalonus*; dorsally convex conoid: (d) *A. fluviatilis* (×1 100) and (e) *A. franklini*; and subcylindroid: (f) *A. subtenuis.*
Drawing Sue Hockland, Fera Science Limited, York, United Kingdom.



12C

Figure 12(C). Positions of the excretory pore relative to the nerve ring in *Aphelenchoides* species: excretory pore anterior to or level with the anterior edge of the nerve ring: (a) *A. longiurus* and (b) *A. blastophthorus*; excretory pore level with the nerve ring (from behind the anterior point to in front of the posterior point): (c) *A. cibolensis*; and excretory pore level with the posterior edge of the nerve ring: (d) *A. arcticus* or posterior to it: (e) *A. ritzemabosi* (all scale bars = 10 µm).
Drawing Sue Hockland, Fera Science Limited, York, United Kingdom.

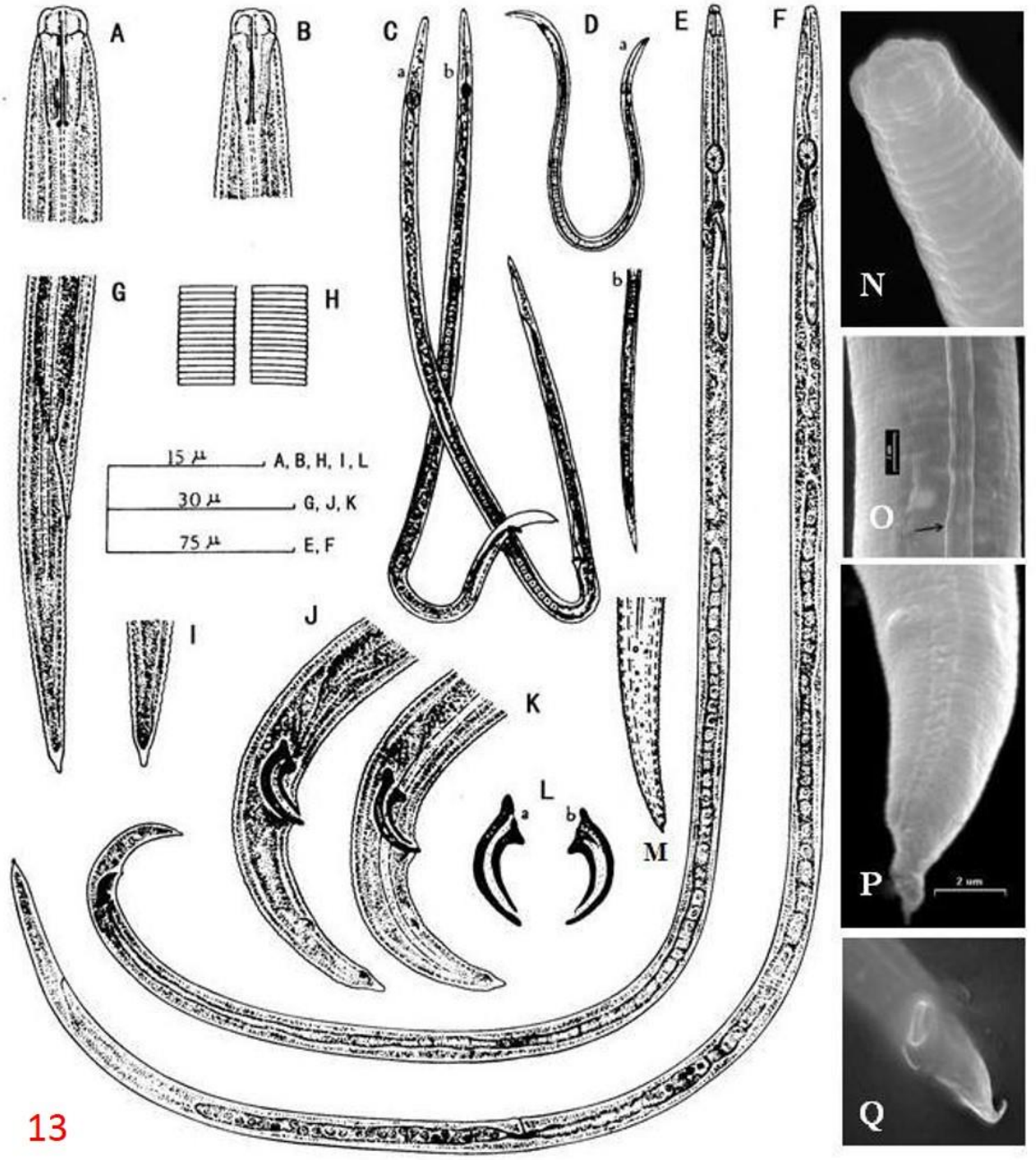


Figure 13. *Aphelenchoides fragariae*: **(**A, N) female anterior or lip region; (B) male anterior or lip region; (C) (a) female and (b) male of *A. olesistus* Ritzema Bos, 1893 (= *A. fragariae*); (D) (a) male and (b) posterior portion of female of *Aphelenchus fragariae* Ritzema Bos, 1891; (E) male; (F) female; (G) female tail; (H, O) lateral field; (I, M, P) female tail tip; (J, K, Q) male tails; and (L) spicules.
Photo courtesy (A, B, E–L) Siddiqi (1975); (C) Ritzema Bos (1893); (D) Ritzema Bos (1891); (M) Allen (1952); (N, Q) Kohl (2011); and (O, P) Khan et al. *(2008)*.

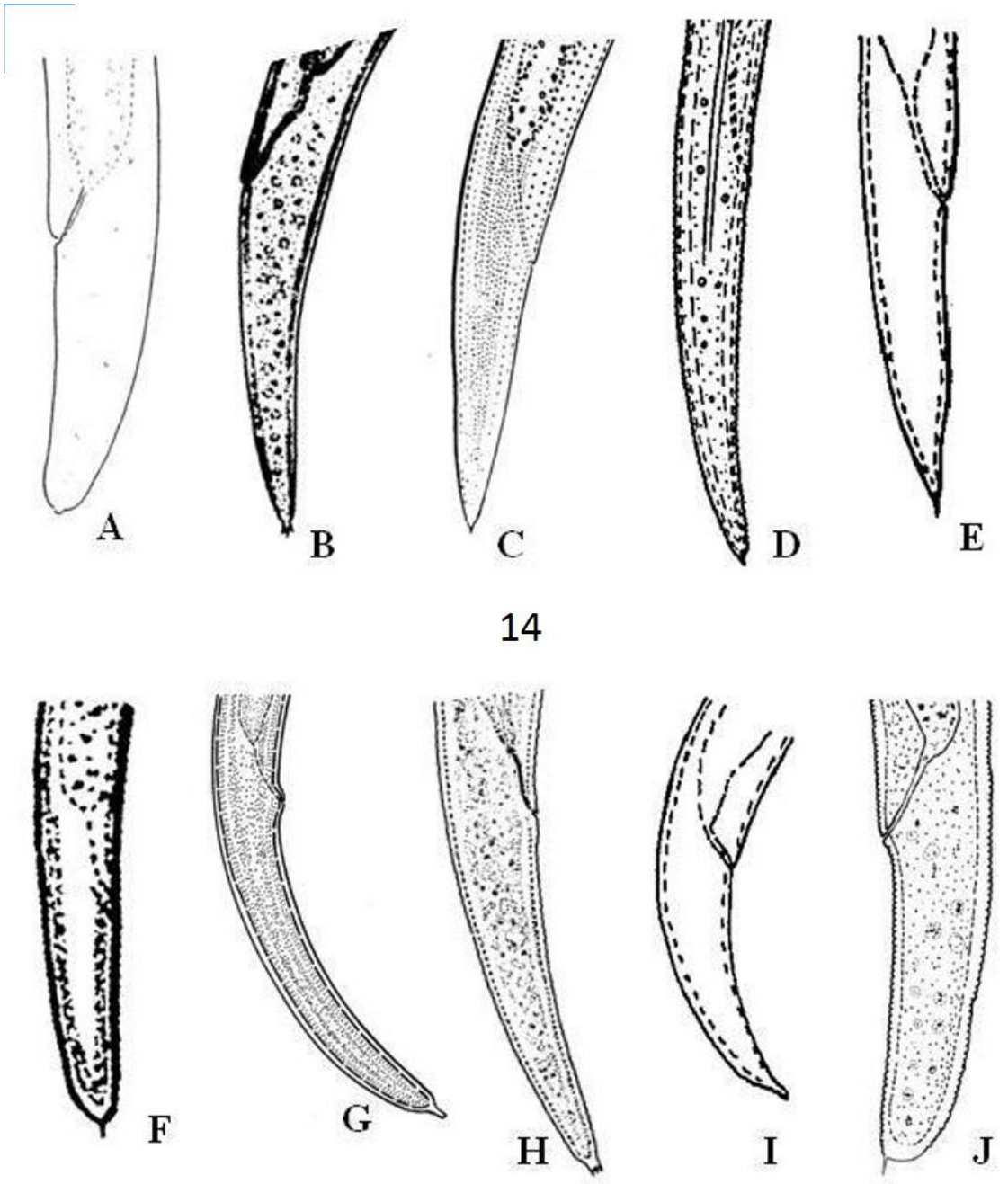


Figure 14. Tails of Aphelenchoides fragariae and related species of Aphelenchoides: (A) A. arachidis; (B) A. besseyi; (C) A. blastophthorus; (D) A. fragariae; (E) A. helophilus; (F) A. resinosi; (G) A. rhytium; (H) A. ritzemabosi; (I) A. saprophilus; and (J) A. subtenuis.
Photo courtesy (A) Bridge and Hunt (1985); (B) Franklin and Siddiqi (1972); (C) Hooper (1975); (D) Allen (1952); (E) Shahina (1996); (F) Kaisa et al. *(1995); (G) Massey (1974); (H) Siddiqi (1974); (I) Shahina (1996); and (J) Deimi* et al. *(2006).*

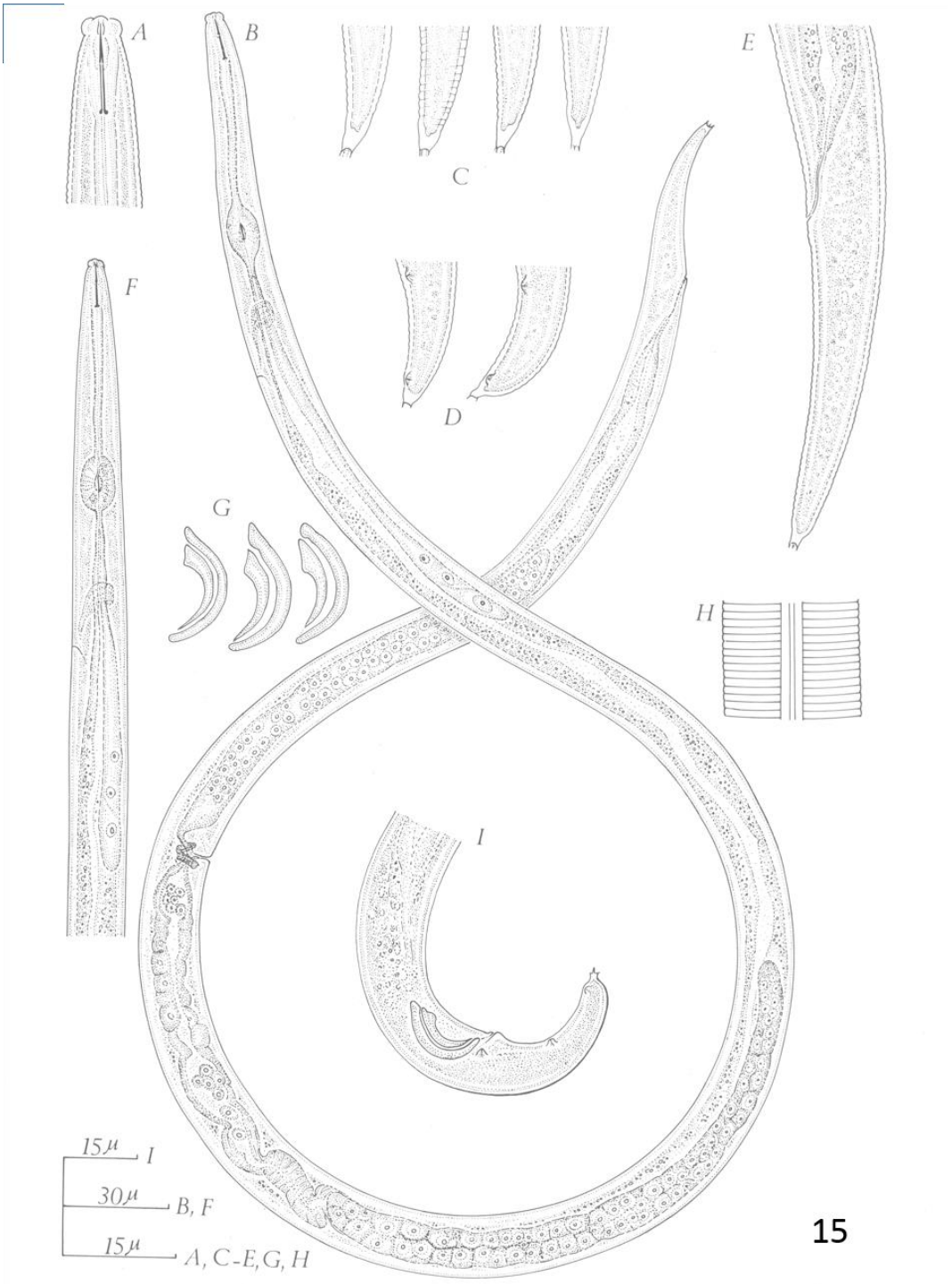


Figure 15. *Aphelenchoides ritzemabosi:* (A) female head end; (B) female; (C) female tail ends; (D) male tail ends; (E) female tail; (F) female pharyngeal region; (G) spicules; (H) lateral field; and (I) male tail region.
Photo courtesy Siddiqi (1974).

Publication history

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ISPM 27. Annex 17. *Aphelenchoides besseyi, A. fragariae* and *A. ritzemabosi* (2016). Rome, IPPC, FAO.

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ISPM 27
Diagnostic protocols for regulated pests

DP 18: *Anguina* spp.

Adopted 2017; published 2017

CONTENTS

1. Pest Information

The Anguinidae family of nematodes contains both mycophagous species and species that parasitize bulbs, tubers and aerial parts of plants. The gall-forming nematodes of the subfamily Anguininae are obligate parasites of plants. More than 40 nominal species of gall-forming Anguinidae have been described. The seed gall nematodes, *Anguina* spp., inhabit the aerial parts of cereals and forage grasses.

Species of *Anguina* invade ovules where they induce galls, lay eggs and reside as second-stage juveniles. This juvenile stage can remain as anhydrobiotes within dried seed galls for many years. A single wheat gall formed by *Anguina tritici* (Steinbuch, 1799) Filipjev, 1936 usually contains 11 000–18 000 nematodes, although galls with as many as 90 000 have been recorded (Decraemer and Hunt, 2013). The nematodes can be retrieved from galls for up to 30 years after forming if kept dry.

Three *Anguina* species, *A. tritici* (Steinbuch, 1799) Filipjev, 1936, *A. agrostis* **(**Steinbuch, 1799) Filipjev, 1936 and *A. funesta* Price, Fisher & Kerr, 1979 are considered of economic importance as agricultural and quarantine pests in various countries (Chizhov and Subbotin, 1990; Krall, 1991) This diagnostic protocol covers morphological and molecular identification of the genus and these species of major economic importance. Other species with importance in a limited geographical range include:

A. agropyri Kirjanova, 1955

A. agropyronifloris Norton, 1965

A. amsinckiae (Steiner & Scott, 1935) Thorne, 1961

A. australis Steiner, 1940

A. balsamophila (Thorne, 1926) Filipjev, 1936

A. caricis Solovyeva & Krall, 1982

A. cecidoplastes (Goodey, 1934) Filipjev, 1936

A. graminis (Hardy, 1850) Filipjev, 1936

A. microlaenae (Fawcett, 1938) Steiner, 1940

A. pacificae Cid del Prado Vera & Maggenti, 1984 (Ferris, 2013).

A. tritici (wheat gall nematode, bunted wheat) has been recorded in major wheat growing areas on all continents (Southey, 1972) and was historically widely distributed (EPPO, 2015). This species can cause severe crop losses to *Secale cereale* L. (rye) (35–65%) and *Triticum aestivum* L. (wheat) (20–50%) (Leukel, 1929, 1957; Anwar *et al.*, 2001). However, the use of modern seed cleaning methods that separate galls from healthy grains has almost eliminated this species from commercial wheat production in developed countries. For example, recent surveys for *A. tritici* in stored grain harvested from states of the United States of America with records of this nematode did not provide any evidence that it was still present in the country (CABI, 2001, 2014b). Recorded hosts are *T. aestivum*, *Triticum dicoccum* Schrank, *Triticum durum* Desf., *Triticum monococcum* L. (emmer), *Triticum spelta* L. (spelt), *Triticum ventricosum* Ces and *S. cereale*. *Hordeum vulgare* L. (barley) is a very poor host (Southey, 1972). There is little evidence that this nematode reproduces on *Avena sativa* L. (oat) and other grasses; although there are some reports of damage to oat at seedling stage by second-stage juveniles, no galls have been observed on this host.

Clavibacter tritici, the bacterium that is the causal agent of yellow ear rot or "tundu" of *T. aestivum*, is associated with the presence of *A. tritici*. Freshly harvested infected wheat cockles containing the bacterium are toxic to cattle and sheep (Anwar *et al.*, 2001). *A. tritici* has been shown to be vector of *Rathayibacter toxicus* (toxic yellow slime bacterium) under experimental conditions (Riley and McKay, 1990).

A. agrostis (bentgrass nematode) has been reported from Asia, Australia, Europe, New Zealand, North America and Republic of South Africa (CABI, 2014a). It is considered to be a species complex with pathotypes differing in host range (Krall, 1991; Brzeski, 1998). Subbotin *et al.* (2004) supported the concept of narrow specialization of seed gall nematodes, concluding that *A. agrostis* occurs in only

one host, *Agrostis capillaris*, and that other *Agrostis* species are hosts for two further undescribed species of *Anguina*. The type host of *A. agrostis* is *Agrostis tenuis* Sibth. In addition to various bentgrass species, *A. agrostis sensu lato* has been reported from other grass genera, including *Apera*, *Arctagrostis*, *Calamagrostis*, *Dactylis*, *Eragrostis*, *Festuca*, *Hordeum*, *Koeleria*, *Lolium*, *Phalaris*, *Phleum*, *Poa*, *Puccinellia*, *Sporobolus* and *Trisetum* (although certain records may relate to *A. funesta* (CABI & EPPO, 2004)).

A. agrostis has been shown to vector *R. toxicus* under experimental conditions (Riley and McKay, 1990). Several older references (e.g. Goodey, 1960) to this species being the causal agent of disease in livestock relate to galls on *Festuca* spp. *A. agrostis* may actually refer to the species *A. funesta* (Southey, 1973).

A. funesta has been described from Australia, and recently has been reported from Oregon in the United States of America (Meng *et. al.*, 2012). The principal host of *A. funesta* is *Lolium rigidum* (annual ryegrass).

A. funesta is recorded as a vector of *R. toxicus*, which causes the disease annual ryegrass toxicity when consumed by livestock. Annual ryegrass toxicity is responsible for severe losses in the livestock industry in Australia (Price *et al.*, 1979). Rangeland infested by the nematode and bacterium is unusable for grazing (Figures 1 to 3).

2. Taxonomic Information

Name: *Anguina* Scopoli, 1777

Synonyms: *Angvina* (= original spelling, amended to *Anguina* by later workers); *Anguillulina* Gervais & Van Beneden, 1859; *Anguillulina* (*Anguina* Scopoli) (Schneider, 1939); *Paranguina* Kirjanova, 1954; *Paranguina* Kirjanova, 1955

Taxonomic position: Nematoda, Chromadorea, Chromadoria, Rhabditida, Tylenchina, Tylenchomorpha, Sphaerularioidea, Anguinidae, Anguininae (after Decraemer and Hunt, 2013)

Common names: Seed and leaf gall nematode, seed-gall nematode. Other common names in various languages are listed in CABI (2013).

Name: *Anguina tritici* (Steinbuch, 1799) Chitwood, 1935

Synonyms: *Vibrio tritici* Steinbuch, 1799; *Rhabditis tritici* (Steinbuch) Dujardin, 1845; *Anguillula tritici* (Steinbuch) Grube, 1849; *Anguillulina tritici* (Steinbuch) Gervais & Van Beneden, 1859; *Tylenchus tritici* (Steinbuch) Bastian, 1865; *Anguillula scandens* Schneider, 1866; *Tylenchus scandens* (Schneider) Cobb, 1890; *Anguillulina scandens* (Schneider) Goodey, 1932

Common names: Wheat seed gall nematode, wheat cockle nematode

Name: *Anguina agrostis* (Steinbuch, 1799) Filipjev, 1936

Synonyms: *Vibrio agrostis* Steinbuch, 1799; *Anguillula agrostis* (Steinbuch) Ehrenberg, 1838; *Tylenchus agrostis* (Steinbuch) Goodey, 1930; *Anguillulina agrostis* (Steinbuch) Goodey, 1932; *Vibrio phalaridis* Steinbuch, 1799; *Anguilllula phalaridis* (Steinbuch) Ehrenberg, 1838; *Tylenchus phalaridis* (Steinbuch) Örley, 1880; *Anguillulina phalaridis* (Steinbuch) Goodey, 1932; *Anguina phalaridis* (Steinbuch) Chizhov, 1980; *Tylenchus agrostidis* Bastian, 1865; *Anguillula agrostidis* (Bastian) Warming, 1877; *Tylenchus phlei* Horn, 1888; *Anguina poophila* Kirjanova, 1952; *Anguina lolii* Price, 1973

Common names: Bentgrass nematode, grass seed nematode

Name: *Anguina funesta* Price, Fisher & Kerr, 1979

Synonyms: *A. lolii* Bird & Stynes, 1977 (in part)

Common name: Seed gall nematode

3. Detection

3.1 Symptoms specific to *Anguina* species

3.1.1 *Anguina tritici* (after Southey, 1972; Krall, 1991)

A. tritici incites seed galls (ear cockles) in cereals (Figure 4(A)). Invasive juveniles emerge from the seed galls in the soil and attack newly germinated seedlings. They establish infection on the tissues of young leaves near the growing point where they feed as an ectoparasite causing leaf distortion and crinkling (Figure 4(B)). Infected hosts become stunted and exhibit shorter and deformed stems and leaves (Figures 4(B) and 4(C)). Severely infected plants do not form ears or form only stunted ears on stunted stems. A diseased ear is much wider and shorter than a normal ear and has short deformed awns (Figure 4(C)). Slight elevations occur on the upper leaf surface with indentations on the lower side. Other leaf symptoms include wrinkling, twisting, curling of the margins towards the midrib, distortion, buckling, swelling and bulging. A tight spiral coil evolves, and dwarfing, loss of colour or development of a mottled yellowed appearance, and stem bending may also occur. In severe infection, the entire above-ground plant is distorted to some degree and therefore the disease is usually obvious (CABI, 2015). The second-stage juveniles stimulate the formation of galls in floral tissues in place of seed development. Galls vary from light and dark brown to almost black (Figure 5(A)). They are smaller than healthy grains (Figure 5(B)). The nematodes can survive in a quiescent stage in seed galls (Figure 6).

3.1.2 *Anguina agrostis* (after Southey, 1973; Krall, 1991)

A. agrostis is considered to be an economically important nematode pest of bentgrass. In grasses, seed galls are difficult to detect as they are covered by lemmas and paleae. A small scarifier can be used to remove lemmas and paleae without damage to seeds or galls. This allows visual identification of galls (Alderman *et al.*, 2003). Galled flowers have glumes of two or three times the normal length, lemmas five to eight times the normal length, projecting beyond the glumes as a sharp point, and paleae developing to about four times the normal length. Galls are at first greenish, and later become dark purple–brown. They reach 4–5 mm long (Figures 7 and 8). Lodicules, stamens and sometimes other flower parts are suppressed in parasitized flowers. Symptoms of the inflorescence also include elongated flower galls, which are modified ovaries that look greenish or purple and may be 4–5 mm long. Seed galls containing the nematodes are dark brown. They may look similar to normal seeds but are less heavy and hence can be separated mechanically from them.

3.1.3 *Anguina funesta*

The life cycle of *A. funesta* is similar to that of *A. agrostis*. During dry summers, *A. funesta* survives within seed galls as anhydrobiotic second-stage juveniles. During winter, the nematodes are released from decaying galls and via water droplets in moist soil they invade new host seedlings, where they feed upon the young leaves. The nematodes congregate near the apical meristems until ovary initiation then stimulate ovary primordia to develop into galls. Occasionally, galls are produced in stamen primordia or, in very heavily infested plants, on glumes or rachis (McCay and Ophel, 1993). Information on the biology of this species can be found in Price *et al.* (1979). Symptoms of infestation are shown in Figures 1 to 3.

3.2 Nematode extraction

3.2.1 Direct examination

Symptomatic foliage and seed suspected to be infested with Anguininae can be processed by dissecting foliage tissue and galls immersed in tap water in a Petri dish. Specimens of motile and immotile stages may be observed under a stereomicroscope, usually within 30 min if the host plant is heavily infested.

Seed cleaning can be achieved most effectively by modern equipment used for this purpose. Galls may be removed by a salt brine method in which the seeds are stirred into a 20% salt solution. Galls and debris float to the surface from where they are skimmed then steamed, boiled or chemically treated to kill the nematodes. The salt solution containing healthy seeds is drained and the seeds are rinsed several times in freshwater to remove the salt, then spread in thin layers on a clean surface to dry.

3.2.2 Extraction from soil and plant material

Detailed descriptions of extraction equipment and procedures can be found in the European and Mediterranean Plant Protection Organization (EPPO) standard on nematode extraction (EPPO, 2013a).

All stages of anguinid nematodes can be extracted from plant tissue, and infective juveniles can also be isolated from soil or growing medium, using the Baermann funnel technique, the modified Baermann tray method (Hooper and Evans, 1993), an adapted sugar flotation method (Coolen and D'Herde, 1972) or the mistifier technique (Hooper *et al.*, 2005). These extraction methods should be conducted for 48 h at room temperature to detect low levels of infestation. Any plant material to be tested should be cut into pieces or sliced before extracting, for increased efficacy of extraction. The number of infective juveniles that may be recovered from soil depends on soil type, sampling depth, host plant and seasonal factors (Hooper, 1986). A large amount of fresh organic matter in the soil sample (e.g. plant residue after harvest) can influence nematode numbers because of its decomposition process, which might be toxic to nematodes or increase the number of saprophytic nematodes, or because the organic matter hampers extraction by clogging sieves or contaminating the supernatant obtained in density-based methods.

The Baermann funnel technique (and modifications of it, such as the tray method, or Seinhorst mistifier, described by Hooper (1986)) is a reference technique for extraction of nematodes from soil and plant material. A piece of rubber tubing is attached to the stem of a glass funnel (with a recommended slope of approximately 30 degrees) and is closed by a spring or screw clip. The funnel is placed in a support and filled almost to the top with tap water. A plastic sieve or wire basket with a large enough aperture size to allow nematodes to actively pass through is placed just inside the rim of the funnel. Plant tissue cut into small pieces or soil is placed either directly onto the mesh or onto a single-ply tissue supported by the mesh, and the water level is adjusted so the substrate is only just submerged. Active nematodes pass through the mesh and sink to the bottom of the funnel stem. Alternatively, funnels made of plastic or stainless steel, or tubing made of silicone, can be used. However, regarding the latter, diffusion of oxygen into water is lower than for polyethylene (Stoller, 1957), which can lead to slow asphyxiation of the nematodes. Depending on the plant tissue, most (50–80%) of the motile nematodes present will be recovered within 24 h; however, samples can be left on the funnel for up to 72 h to increase the recovery rate. For longer extraction periods, regular tapping of the funnel and addition of freshwater increases nematode motility and compensates for evaporation and lack of oxygen, thereby improving the recovery rate. The efficacy of extraction can also be improved by adding 1–3% hydrogen peroxide (H_2O_2) for oxygen supply (Tarjan, 1967, 1972). Following the extraction period, a small quantity of water containing the nematodes is run off and observed under a stereomicroscope (Flegg and Hooper, 1970).

Motile and immotile nematodes can be extracted from plant material by the sugar flotation method (Coolen and D'Herde, 1972). The plant material is washed, cut into pieces of about 0.5 cm, and 5 g portions are macerated in 50 ml tap water in a domestic blender at the lowest mixing speed for 2 min. The suspension of nematodes and tissue fragments is washed through a 750 µm sieve placed on top of

a 45 µm sieve. The residue on the 45 µm sieve is collected and poured into two 50 ml centrifuge tubes. About 1 ml kaolin is added to each tube, the mixture is thoroughly stirred and then it is centrifuged at 1500 *g* for 5 min. The supernatant is decanted and sucrose solution (density 1.13 g/cm^3) is added to the tubes. The mixture is thoroughly stirred and centrifuged at 1800 *g* for 4 min. The supernatant is washed through a 45 µm sieve, the residue is collected and the nematodes are studied under a stereomicroscope. Instead of sucrose, zinc sulphate ($ZnSO_4$), magnesium sulphate ($MgSO_4$) or colloidal silica can be used.

3.2.3 Extraction from seed

Infective juveniles can be extracted from infested seed using a number of methods, including the Baermann funnel technique, which is summarized in section 3.2.2. A comparative study of the efficacy of various methods of extraction from seed, including water-agar blend, sieve blend, misting and blender-funnel-host stimulant, are presented by Griesbach *et al.* (1999).

In the blender-funnel-host stimulant method, which is described in Griesbach *et al.* (1999) as the most effective method for the recovery of *A. agrostis* from *Dactylis glomerata* and *Agrostis* spp. seed, 50 g seed is placed in a blender with 300 ml tap water and blended for 15 s, shaken, and blended again for 15 s. The mixture is placed on a single tissue draped on an 850 µm pore size sieve supported over a large funnel containing tap water. Approximately 0.1 g orchardgrass leaves are added to the funnel as a stimulant, and the water column is aerated. Check valves at the funnel base enable the suspension to be drawn off after 24–48 h. The suspension is finally passed through a 25 µm pore size sieve before examination.

4. Identification

The scope of this diagnostic protocol is to facilitate identification of *Anguina* to the genus level. Both morphological (section 4.1) and molecular (section 4.2) approaches are presented.

4.1 Morphological identification

Information for morphological identification of valid genera within the Anguinidae is provided in section 4.1.2.1. Species of Anguinidae are probably one of the most variable groups among the Tylenchina regarding morphological characters (Brzeski, 1998). Identification to species can be unreliable if morphological characters are used in isolation; information regarding biology, host plant and symptoms of infection should also be taken into consideration. Often, only juveniles are found in seed galls, which can further complicate identification as important morphological features in adult specimens cannot be observed. Morphological information for the three species of economic importance is provided in section 4.1.2.2; however, this information should be used in combination with other sources to confirm diagnosis. Keys to species have been provided by Krall (1991) (ten species) and Brzeski (1998) (four species recorded from temperate Europe).

4.1.1 Preparation of nematode specimens

As with other species of plant-parasitic nematodes, morphological observation should be carried out on as many adult specimens as possible. There are numerous published methods for fixing and processing nematode specimens for study, most recently summarized in Manzanilla-López and Marbán-Mendoza (2012). Nematodes processed with anhydrous glycerol are recommended for examination as important taxonomic features can be obscured if specimens are not cleared sufficiently.

If possible, permanent slides should be prepared for future reference and deposited in nematode reference collections. Methods of preparing permanent slide mounts of nematodes have been described in detail elsewhere (Seinhorst, 1962; Hooper, 1986). The slow evaporation method as described by Hooper (1986), which preserves the structures and characteristics of the nematodes, is outlined in section 4.1.1.2.

Temporary microscope slide preparations can be made quickly for instant examination but such slides may remain usable for only a few weeks.

4.1.1.1 Temporary preparations

A small drop of water is placed on a glass cavity slide, enough to sufficiently fill the well. The nematode specimens are transferred to the water and heated to 65 °C. It is critical that the heat should be applied just long enough to kill the nematodes, as prolonged heating will result in their distortion and deterioration. In practice, 10–15 s on a hotplate will be sufficient time for most species, but it is recommended that the slide be checked at intervals to monitor progress and removed from the heat only when movement of all the nematodes has ceased.

A glass slide, free of dust, is placed on the side of the microscope stage. A small drop of single strength triethanolamine and formalin (TAF) fixative (10 ml formalin[1] (35% formaldehyde in water) mixed with 1 ml triethanolamine and 89 ml distilled water) or another appropriate fixative is put in the centre of the slide and an appropriate amount of paraffin wax shavings is positioned around the drop (the wax will help support the coverslip and seal it to the slide).

The nematodes are transferred from the cavity slide to the fixative so that they are positioned beneath the meniscus in the centre of the drop and not overlapping one another. The number of specimens able to fit on a slide will vary according to the size of the nematodes.

An appropriately sized coverslip is carefully cleaned with lens tissue. It is gently lowered onto the wax shavings so that contact is made with the drop of fixative. The slide is placed on a hotplate and monitored until the wax has just melted; the air that may be lodged under the coverslip is removed by gently tapping the slide. The slide is then removed from the heat and examined.

There should be a clear area of fixative containing the nematodes in the centre and a complete ring of wax to seal the slide.

Should the seal be broken or the nematodes become embedded in the wax, the slide is heated again, the coverslip carefully removed, and the nematodes recovered and remounted on a new slide. If the wax has spread beyond the coverslip, it is cleared away with a fine blade.

The coverslip is sealed with a ring of clear nail varnish. When the varnish has dried, the specimens are ready for study.

4.1.1.2 Permanent preparations

A small drop of water is placed on a glass cavity slide, enough to sufficiently fill the well. The nematode specimens are transferred to the water and heated to 65 °C. It is critical that the heat should be applied just long enough to kill the nematodes, as prolonged heating will result in their distortion and deterioration. In practice, 10–15 s on a hotplate will be sufficient time for most species, but it is recommended that the slide be checked at intervals to monitor progress and removed from the heat only when movement of all the nematodes has ceased.

The nematodes are transferred to an embryo dish or suitable watch glass half full of single strength TAF fixative (7 ml formalin[1] (40% formaldehyde in water) mixed with 2 ml triethanolamine and 91 ml distilled water). The dish is covered and left to fix for a minimum of one week.

The specimens are transferred to a watch glass containing a 3% glycerol solution with a trace amount of TAF fixative. The nematodes should be submerged. A coverslip is placed over the watch glass and left on it overnight.

The coverslip is slightly moved so that a small gap is produced to allow evaporation, and the watch glass is left in an incubator (approximately 40 °C) until all the water has evaporated (this may take one week or longer). At the same time, a small beaker of glycerol is placed in the incubator to ensure it becomes anhydrous.

[1] Formalin comprises 35–40% formaldehyde in water.

A small drop of the anhydrous glycerol is dispensed using a syringe or dropper onto the centre of a glass slide and the nematodes are transferred to this, arranged centrally.

Three coverslip supports, such as glass beads, of similar diameter to that of the nematodes are placed at intervals in the margin of the glycerol drop so that they form an even support.

Small amounts of paraffin wax shavings are placed at regular intervals around the circumference of the glycerol drop.

A coverslip is heated on a hotplate for a few seconds. The coverslip is cleaned with lens tissue and gently lowered on to the wax, so that contact is just made between coverslip and glycerol.

The slide is placed on the hotplate and as soon as the wax has melted and any air bubbles have been expelled by the settling coverslip, the slide is removed from the heat and the wax is allowed to reset.

When the wax is completely hard, any excess wax is removed from around the coverslip with a scalpel.

The coverslip is sealed with a ring of sealant such as Glyceel or clear nail varnish. The slide is labelled with an indelible marker, or affixed with a slide label. Information includes classification, date of slide preparation, host, locality, sample number (if applicable) and method of preservation.

4.1.2 Morphological identification

4.1.2.1 Morphological identification at the genus level

Comparative morphology of genera assigned to the Anguininae is presented in Table 1. Definitions of terminology used in the following sections can be found in EPPO's *Diagnostic protocols for regulated pests: Pictorial glossary of morphological terms in nematology* (EPPO, 2013b).

Diagnosis of the Anguininae and the genus *Anguina* has been described by Siddiqi (2000), as follows and as described in Table 1, with key characters for identification shown in **bold**. Medium to large in size (1.0–2.7 mm), obese; **mature female curved generally in one to one-and-a-half spirals. Metacorpus muscular.** Basal bulb in adults enlarged, continuous or offset from isthmus by a constriction, base usually extending over anterior end of intestine. Ovary with one or two flexures anteriorly due to excessive growth; **oocytes in multiple rows, arranged about a rachis. Crustaformeria a long tube formed by a large number of cells in multiple irregular rows.** Spermatogonia in multiple rows. **Bursa subterminal.** Second-stage juveniles generally resistant, and the infective stage. Obligate plant parasites incite galls in seeds of cereals and grasses as well as stems, leaves and inflorescence of various monocotyledonous plants; type species causes wheat seed galls (ear cockles); only *A. amsinckiae* and *A. balsamophila* are known to parasitize dicotyledonous plants.

There has been little recent morphological work on the genus and no reliable and up-to-date morphological keys to species are available. Therefore, identification at the genus level is described with summary information only for the three economically important pest species *A. tritici*, *A. Agrostis* and *A. funesta*.

Genus	Galls	Female body shape	Crustaformeria Quadri-columella	Rachis	Vulval flaps	Post-uterine sac	Median pharyngeal bulb	Terminal bulb	Incisures in lateral fields	Excretory pore	Guber-naculum	Bursa	Tail shape
Litylenchus Zhao *et al.*, 2011	–	Slender and semi-obese	+	–	–	+	+, non-muscular	Abuts	4	Posterior to nerve ring	+	Extends to near tail tip	Conoid
Anguina Scopoli, 1977	+	Obese, spiral	–	+	–	+	+, non-muscular or muscular	Base extends over intestine	4, unclear	Posterior to nerve ring	+	Small, subterminal	Conoid
Diptenchus Khan, Chawla & Seshadri, 1969	? (roots)	Slender	+	–	–	–	+, muscular	Abuts	5	At base of pharynx	+	To two-thirds tail length	Conical
Ditylenchus Filipjev, 1936	– (+ from India)	Slender	+	–	–	+	+, non-muscular or muscular	Abuts or overlaps a little	4 or 6	Posterior to nerve ring	+	Adanal to subterminal	Elongate conoid to filiform
Indotylenchus Sinha, Choudhury & Baqri, 1985	? (mangroves)	Slender	Not available	–	–	+	+, muscular	Offset	4	Anterior to median bulb	+	To two-fifths tail length	Elongate conoid
Nothanguina Whitehead, 1959	+	Obese, spiral	–	+	–	+	–	Offset or small dorsal overlap	Not available	Posterior to nerve ring	-	To half tail length	Conoid
Nothotylenchus Thorne, 1941	–	Slender	+	–	–	+	+, non-muscular	Offset from intestine	4 or 6	Posterior to nerve ring	+	To half tail length	Elongate conoid
Orrina Brzeski, 1981	+	Slender	+	–	–	+	+, non-muscular	Overlaps a little	4	Posterior to nerve ring	+	To two-thirds tail length	Conoid
PseudhalenchusTarjan, 1958	Not available	Slender	+	–	–	+	+, muscular	Overlaps	4	Posterior to nerve ring	+	To one-third tail length	Elongate conoid
Pterotylenchus Siddiqi & Lenné, 1984	+	Slender	–	–	+	+	–	Overlaps dorsally	4	Posterior to nerve ring	Not available	Not available	Elongate conoid
Safianema Siddiqi, 1980	– (fungal feeder)	Slender	+	–	–	+	+, muscular	Overlaps laterally	6	Posterior to nerve ring	+	Adanal to subterminal	Elongate conoid to filiform
Subanguina Paramonov, 1967	+	Slender or semi-obese	–	–	–	+	+, muscular	Abuts or overlaps	Not available	Posterior to nerve ring	+	Subterminal	Conoid
Zeatylenchus Zhao *et al.*, 2013	–	Slender to semi-obese	+	–	–	+	+, non-muscular, fusiform	Subventral glands overlap intestine	Unable to discern in ♀, 3 at mid-body in ♂	In region of retracted stylet	+	Leptoderan, to*ca* 30% distance to tail tip	Conoid, terminus with a ventral spike

Table 1. Comparative morphology and feeding habits of Anguininae

Source: After Zhao *et al.* (2011, 2013).

4.1.2.2 Morphological identification of selected **Anguina** *species*

Anguina tritici

Description after Southey (1972) and Krall (1991). Refer to Table 2, and Figure 9 and Figure 10.

Mature females. Body obese, spirally coiled ventrally. Lip region low and flattened, slightly offset, cephalic framework weak. Cuticle very finely annulated. Procorpus often swollen by gland secretions but constricted at junction of metacorpus. Isthmus sometimes posteriorly swollen, offset from pharyngeal glands by a deep constriction. Pharyngeal glands not overlapping, or with very slight overlap of intestine. Excretory pore near junction of pharynx and intestine or slightly more posterior. Vulval lips protruding, orifice of small glands visible on vulval lips anterior and posterior to vulva. Ovary with two or more flexures, often reaching to pharyngeal region with oogonia in multiple rows arranged around a rachis. Spermatheca pyriform, separated from oviduct by a sphincter. Postvulval uterine sac present. Tail conoid, tapering to an obtuse or rounded tip, not mucronate.

Males. More slender than females. Habitus upon heat relaxation curved either ventrally or dorsally. Testis with one or two flexures. Spicules stout, arcuate, with two ventral ridges running from tip to widest part. Capitulum with distinct ventral folding at anterior. Gubernaculum simple, trough-like. Bursa leptoderan. Tail conoid, tip rounded or obtuse.

Second-stage juveniles. Body slender, not spirally coiled. Tail conoid, pointed (Figure 10). For measurements, see Table 2.

Anguina agrostis

Description after Southey (1973) and Krall (1991). Refer to Tables 1 and 2, and Figures 11 and 12.

Mature females. Based on specimens from the type host. Body obese, C-shaped to spirally coiled ventrally. Lip region low and flattened, offset by a fine constriction. Cuticle marked by fine annulations. Lateral fields not discernible on fully developed adult females, six incisures are visible on immature specimens. Neither procorpus nor isthmus exhibiting marked swellings, the former slightly contracted at its junction with the metacorpus. Isthmus occasionally folded in mature specimens by forward pressure of the gonad. Pharyngeal glands not overlapping, or with very slight overlap of intestine. Vulval lips prominent. Ovary usually with two flexures, often reaching to pharyngeal region with oogonia in multiple rows arranged around a rachis. Spermatheca pyriform, separated from oviduct by a constriction. Postvulval uterine sac present, 36–63% of vulva–anus distance. Tail conoid with an acute terminus.

Males. Smaller and more slender than females. Habitus upon heat relaxation curved ventral to almost straight. Lateral field difficult to discern on mature specimens, reported to have six incisures. Testis usually reflexed once, spermatocytes arranged about a rachis. Spicules more slender in build than those of *A. tritici*, the capitulum showing little or no ventral folding at anterior. Two ventral ridges running from tip of each spicule to widest part, before converging and joining the capitulum. Gubernaculum simple, bursa leptoderan, ending just short of acute or finely mucronate tail tip.

Second-stage juveniles. Body slender, not spirally coiled; tail conoid, pointed (Figure 12(A)). For measurements, see Tables 2 and 3.

Anguina funesta

Description after Price *et al.* (1979). Refer to Table 1 and Table 2, and Figure 13.

Mature females. Length of postvulval uterine sac 62–112 µm; stylet length 7–10 µm. Young females are fully motile, but older females, in which gross expansion of the ovary has occurred, are strongly ventrally curved and capable of only weak movements of head and tail. Habitus following heat relaxation ventrally curved forming a complete circle, with head and tail overlapping. Lips slightly offset and rounded in front, cephalic framework lightly cuticularized. Stylet with conus and shaft of

roughly equal length and with well-developed knobs. Pharynx 64–178 µm long with wide procorpus opening to a muscular metacorpus, ovate to spheroid, 17–25 µm long. Pharyngeal–intestinal junction obscured by the large dorsal gland, 45–72 µm in length, ovate to spatulate with a prominent nucleus. Pharyngeal glands slightly overlapping the intestine. Hemizonid between base of metacorpus and anterior end of dorsal pharyngeal gland, at 80–100 µm from anterior. Excretory pore located more posteriorly, 105–155 µm from anterior. Lateral field difficult to discern. Vulva with prominent lips. Anterior ovary with one or two flexures. Rarely, the gonad not reflexed but rather extended anteriorly to the base of the procorpus. Oocytes arranged in three or four rows about a rachis, except near the base of the ovary where this increases to five or six rows. Spermatheca ovate or elongated, long and 25–40 µm wide. Crustaformeria long and slender, made up of more than four columns of cells and up to 350 µm long, separated from spermatheca and uterus by short constrictions, 10–15 µm long. Crustaformeria often containing sperm cells and up to eight eggs. Uterus thick-walled, 70–100 µm long. Postvulval uterine sac approximately the same length as the uterus, 62–112 µm. Tail 48–112 µm long, vulva–anus distance 86–73 µm. Body width at anus approximately half that at vulva. Tail occasionally bluntly rounded, more usually conically pointed, sometimes with a mucronate tip.

Males. Males shorter and thinner than females. Habitus upon heat relaxation straight or slightly curved. Lip and pharyngeal regions of the male similar to that of the female, although dorsal pharyngeal gland larger, almost rectangular, 50–78 µm long. Hemizonid and excretory pore 71–90 µm and 102–147 µm from anterior, respectively. Lateral field with five or six incisures, broken at intervals and occupying one-quarter to one-third of body width. Testis nearly always reflexed once. Testis with spermatocytes in multiple rows about a rachis. Spicules paired, non-fused and arcuate, each 16–28 µm long with characteristic bulges on the manubrium and where the manubrium and shaft join. Gubernaculum slim and trough-like. Bursa leptoderan, extending almost to tail tip, 44–114 µm in length. Tail 43–72 µm long with terminus conically pointed. Body width at cloaca 17–43 µm. For measurements, see Tables 2 and 3.

Table 2. Morphometric data for *Anguina tritici*, *Anguina agrostis* and *Anguina funesta*

Morphometric characters	**Range, numerous populations**		
	A. tritici	***A. agrostis* (restricted to *Agrostis* spp.)**	***A. funesta***
Mature females			
L (mm)	3.0–5.2	1.3–2.7	1.65–2.44
Stylet (µm)	8–11	8–12	7–10
a (nematode body length/greatest width (usually at mid-body))	13–30	13.8–25.4	16.8–20.1
b (nematode body length/pharynx length from lips to pharyngo-intestinal valve)	9.8–25.0	8.0–28.7	9.3–34.0
c (body length/tail length)	24–63	25.2–44.0	18.1–41.2
V (%)	70–95	87–92	86.9–94.0
Males			
L (mm)	1.9–2.5	1.05–1.68	0.78–1.52
Stylet (µm)	8–11	10–12	7–10
a (nematode body length/greatest width (usually at mid-body))	21–30	23–38	20.3–30.9
b (nematode body length/pharynx length from lips to pharyngo-intestinal valve)	6.3–13.0	6–9	6.3–9.5
c (body length/tail length)	17–28	20.0–28.4	16.1–24.9
Spicules (µm)	35–40	25–40	16–28
Gubernaculum (µm)	Approximately 10	10–14	9–14
Second-stage juveniles			
L (mm)	0.75–0.95	0.55–1.25	0.81–0.87
Stylet (µm)	Approximately 10	Approximately 10	7–10
a (nematode body length/greatest width (usually at mid-body))	47–59	44–71	48–53
b (nematode body length/pharynx length from lips to pharyngo-intestinal valve)	4.0–6.3	3.2–6.1	4.2–4.6
c (body length/tail length)	23–28	11.7–20	12.3–15.1

Source: After Southey (1972, 1973), Price *et al.* (1979), Chizhov (1980), Krall (1991), Brzeski (1998) and Meng *et al.* (2012).
L, length; V, distance from the anterior end to the vulva divided by nematode body length (%).

Additional data for populations of *A. agrostis* and *A. funesta* (infective juveniles) are presented in Table 3.

Table 3. Morphometric data of juveniles of an *Anguina funesta* population from annual ryegrass, an *Anguina agrostis* population from bentgrass and an *Anguina agrostis* population from orchardgrass from commercial seed production fields in Willamette Valley, Oregon, the United States of America

Characters (*n* = 20)	**Mean ± standard deviation (range) (µm)**		
	***A. funesta* (annual ryegrass)**	***A. agrostis* (bentgrass)**	***A. agrostis* (orchardgrass)**
Body length	836.2 ± 14.6 (815.9–865.7)	795.0 ± 33.8 (726.4–875.6)	739.8 ± 20.0 (726.4–796.0)
Genital primordium to posterior	396.7 ± 31.1 (351.5–480.2)	354.9 ± 25.0 (311.9–415.8)	350.0 ± 21.6 (297.0–381.2)
Tail length	63.3 ± 3.3 (55.9–68.0)	57.7 ± 2.1 (53.5–60.8)	61.5 ± 4.2 (55.9–70.5)
Anterior to excretory pore	122.5 ± 2.5 (119.1–128.8)	122.2 ± 2.1 (116.6–126.4)	122.5 ± 2.5 (119.1–128.8)
Pharyngeal length	183.0 ± 7.1 (172.5–194.4)	186.9 ± 10.5 (158.0–199.3)	183.7 ± 8.8 (167.7–194.4)
Genital primordium length	20.4 ± 1.5 (18.0–23.0)	16.5 ± 1.5 (13.5–19.0)	18.1 ± 1.8 (15.0–23.0)
Genital primordium width	8.8 ± 1.2 (6.0–11.0)	7.5 ± 0.9 (6.0–10.0)	8.1 ± 0.7 (7.0–10.0)
Body width	16.6 ± 0.7 (15.0–18.0)	14.0 ± 0.5 (13.0–15.0)	14.6 ± 0.6 (14.0–15.0)
Metacorpus length	17.0 ± 1.1 (15.0–19.0)	16.2 ± 1.6 (12.0–19.0)	15.9 ± 1.0 (15.0–17.0)
Metacorpus width	8.5 ± 0.5 (8.0–9.0)	8.7 ± 0.8 (7.5–10.0)	7.9 ± 0.7 (7.0–9.0)
Stylet length	8.0 ± 1.0 (7.0–10.0)	8.0 ± 1.0 (7.0–10.0)	8.0 ± 0.7 (7.0–10.0)
Anterior to base of stylet	10.1 ± 0.3 (10.0–11.0)	10.1 ± 0.5 (8.0–11.0)	10.5 ± 0.4 (10.0–11.0)
Pharyngeal length	0.9 ± 0.4 (0.5–1.5)	1.2 ± 0.4 (0.5–1.5)	1.0 ± 0.3 (0.5–1.5)
a (nematode body length/greatest width (usually at mid-body))	50.4 ± 1.8 (48.0–52.9)	56.9 ± 3.7 (49.1–62.8)	50.6 ± 2.1 (45.4–54.7)
b (nematode body length/pharynx length from lips to pharyngo-intestinal valve)	4.6 ± 0.2 (4.2–4.6)	4.3 ± 0.2 (4.0–4.7)	4.0 ± 0.2 (3.8–4.4)
c (body length/tail length)	13.2 ± 0.8 (12.3–15.1)	13.8 ± 0.6 (12.8–15.0)	12.1 ± 1.0 (11.0–15.4)
Anterior to excretory pore as % of length	14.6 ± 0.3 (14.4–16.0)	15.4 ± 0.6 (14.2–16.7)	16.6 ± 0.5 (15.3–17.1)
Genital primordium to tail as % of length	47.4 ± 3.6 (43.2–56.8)	44.7 ± 2.7 (36.2–49.7)	47.3 ± 3.0 (39.8–51.8)

Source: Reproduced from Meng *et al.* (2012), courtesy Plant Management Network, *Plant Health Progress*.

4.2 Molecular identification

This section provides information on molecular tests that allow the identification of isolated nematodes of the major *Anguina* species. The tests are generally performed following a morphological examination in order to confirm the results obtained.

Molecular diagnosis of *Anguina* spp. is based on polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) (Powers *et al.*, 2001), real-time PCR (Ma *et al.*, 2011; Li *et al.*, 2015) or sequencing of the internal transcribed spacer (ITS) region of ribosomal (r)RNA (Subbotin *et al.*, 2004). The choice of test depends on whether identification requires confirmation of both the presence and the absence of particular species, and on the availability of species standards for controls. The method described by Ma *et al.* (2011) is limited to positive identification of *A. agrostis*, while the other methods are able to simultaneously distinguish multiple species within the same test. PCR combined with analysis of RFLP is the most common way in which to simultaneously distinguish a range of *Anguina* species from each other (Powers *et al.*, 2001).

Powers *et al.* (2001) first sequenced the ITS1 region for *Anguina* spp. Subbotin *et al.* (2004) subsequently sequenced 58 populations of *Anguina*, *Ditylenchus*, *Heteroanguina* and *Mesoanguina* for phylogenetic analysis. There are 71 sequence accessions of rRNA fragments obtained from *Anguina* spp. collected from different localities and host plants presently available in the United States National Center for Biotechnology Information (NCBI) public database.

ITS DNA PCR fragments may also be used for DNA sequence analysis, as described in section 4.2.5.

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

4.2.1 DNA extraction

A single juvenile (or adult, if available) is processed for PCR by placing it in a 15 µl drop of double distilled sterile water on a glass slide and manually disrupting it by cutting it into several pieces with a knife under a stereomicroscope (Powers and Harris, 1993).

The nematode pieces in 8 µl double distilled sterile water are transferred to a microcentrifuge tube containing, for example and depending on size and number of nematodes, 10 µl nematode extraction buffer (10 mM Tris, pH 8.2; 2.5 mM $MgCl_2$; 50 mM KCl; 0.45% Tween 20; 0.05% gelatin; 60 µg/ml proteinase-K) (Thomas *et al.*, 1997) and frozen at −70 °C for 15 min or until needed. The extract is thawed and incubated at 60 °C for 60 min then the proteinase-K is denatured by heating at 95 °C for 15 min. In the protocol by Ma *et al.* (2011) the nematode is cut in 8 µl double distilled water and this suspension is transferred to a tube containing 1 µl PCR buffer with 1 µl proteinase-K (1 µg/ml), with freezing as described above and incubation at 65 °C for 60 min followed by 95 °C for 10 min.

There are no published protocols designed specifically for bulk DNA extraction from *Anguina* spp.; however, methods described for other nematodes can be adapted as required. For example, the commercially available QIAamp DNA Micro Kit (Qiagen[2]) was used for DNA extraction from reniform nematodes following Baermann extraction and sugar centrifugal flotation to isolate nematodes from soil (Sayler *et al.*, 2012). Quantification of extracted DNA is measured with the NanoDrop ND-1000 spectrophotometer (Thermo Scientific[2]).

[2] In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

4.2.2 ITS1 rRNA PCR-RFLP for identification of *Anguina* spp. (Powers *et al.*, 2001)

The ITS rRNA universal primers described in this test are:

rDNA2 (forward): 5′-TTGATTACGTCCCTGCCCTTT-3′ (Vrain *et al.*, 1992)

rDNA1.58S (reverse): 5′-ACGAGCCGAGTGATCCACCG-3′ (Cherry *et al.*, 1997)

The PCR and the cycling parameters as described by Szalanski *et al.* (1997) are presented in Table 4. Alternatively, the amplification can be conducted according to Meng *et al.* (2012) (Table 5). After PCR, 5 µl of the product is analysed electrophoretically on a 1.5% agarose gel in Tris-acetate-ethylenediaminetetraacetic acid (EDTA) (TAE) buffer. The gel can be stained with ethidium bromide and photographed using a gel imaging system with an ultraviolet light filter.

The PCR products are purified with the Geneclean II Kit (MP Biomedicals[2]) or a similar PCR purification kit. The restriction enzymes *Alu*I, *Bsr*I, *Eco*RI, *Hae*III, *Hha*I, *Hinf*I and *Taq*I are required for identifying *Anguina* spp. The reactions are conducted separately (one tube for each enzyme) and according to the individual enzyme manufacturer's recommendations (Table 6). A positive restriction control should be included in the RFLP step to confirm the success of the enzymatic digestion.

The lengths of the restriction fragments generated by these diagnostic enzymes and the restriction pattern of each species are given in Table 7.

Table 4. ITS1 rRNA conventional PCR master mix composition, cycling parameters and amplicons (after Szalanski *et al.*, 1997)

Reagent	**Final concentration**
PCR-grade water	–[†]
PCR buffer (including $MgCl_2$)	1×
dNTPs	0.8 mM
Primer rDNA2 (forward)	0.4 mM
Primer rDNA1.58S (reverse)	0.4 mM
DNA polymerase	2.5 U
DNA (volume)	1 µl
Cycling parameters[‡]	
Initial denaturation	94 °C for 3 min
Number of cycles	40
Denaturation	94 °C for 45 s
Annealing	55 °C for 1 min
Elongation	72 °C for 2 min
Final elongation	72 °C for 10 min
Expected amplicons	
Size	From 547 to 553 bp for *Anguina* spp. (except for *Astrebla* genus: 575 bp)

[†] For a final reaction volume of 50 µl.

[‡] According to the DNA polymerase manufacturer's instruction.

bp, base pairs; ITS, internal transcribed spacer; PCR, polymerase chain reaction; rRNA, ribosomal RNA.

Table 5. ITS1 rRNA conventional PCR master mix composition, cycling parameters and amplicons (after Meng *et al.*, 2012)

Reagent	**Final concentration**
PCR-grade water	–†
PCR buffer	1×
$MgCl_2$	1 mM
dNTPs	0.4 mM
Primer rDNA2 (forward)	0.2 mM
Primer rDNA1.58S (reverse)	0.2 mM
DNA polymerase (GoTaq Flexi (Promega[2]))	2.0 U
DNA (volume)	15 µl
Cycling parameters‡	
Initial denaturation	94 °C for 3 min
Number of cycles	40
Denaturation	94 °C for 1 min
Annealing	55 °C for 1 min
Elongation	72 °C for 1 min
Final elongation	72 °C for 10 min
Expected amplicons	
Size	From 547 to 553 bp for *Anguina* spp. (except for *Astrebla* genus: 575 bp)

† For a final reaction volume of 50 µl.

‡ According to the DNA polymerase manufacturer's instruction.

bp, base pairs; ITS, internal transcribed spacer; PCR, polymerase chain reaction; rRNA/DNA, ribosomal RNA/DNA.

Table 6. Master mix composition, template, reaction conditions and amplicons for RFLP

Reagent	**Final concentration**
PCR-grade water	–†
Enzyme mix	1×
Restriction enzyme	10 U
PCR product (volume)	6 µl
Reaction conditions‡	37 °C or 65 °C for 8 h
Expected amplicons	
Size	See Table 7

† For a final reaction volume of 14 µl.

‡ 37 °C for all enzymes except *Taq*I, for which incubation should be performed at 65 °C.

PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

Table 7. Restriction fragment sizes for *Anguina* species and associated restriction fragment length polymorphism (RFLP) patterns (after Powers *et al.*, 2001)

Species	*Alu*I	*	*Bsr*I	*	*Eco*RI	*	*Hae*III	*	*Hha*I	*	*Hinf*I	*	*Taq*I	*
A. agrostis	548	A	295, 238, 15	B	299, 249	A	548	A	548	A	448, 100	B	355, 135, 58	A
A. agropyronifloris	547	A	547	C	547	B	317, 230	B	547	A	447, 100	B	489, 58	B
A. funesta	548	A	295, 238, 15	B	299, 249	A	548	A	548	A	448, 100	B	490, 58	B
A. graminis	548	A	310, 238	B	548	B	548	A	548	A	249, 199, 100	C	490, 58	B
A. microlaenae	550	A	550	C	301, 249	A	550	A	550	A	449, 52, 49	D	357, 135, 58	A
A. pacificae	549	A	239, 225, 85	D	549	B	319, 230	B	549	A	448, 101	B	491, 58	B
A. tritici	277, 274	B	550	C	550	B	550	A	462, 88	B	550	E	492, 58	B
A. sp. / *Dactylis*	550	A	297, 253	B	550	B	320, 230	B	550	A	252, 198, 100	C	357, 135, 58	A
A. sp. / *Agrostis*	553	A	301, 237, 15	B	553	B	333, 220	B	303, 250	C	454, 99	B	360, 135, 58	A
A. sp. / *Polypogon*	553	A	301, 237, 15	B	553	B	333, 220	B	553	A	454, 99	B	360, 135, 58	A
A. sp. / *Stipa*	548	A	467, 81	A	299, 249	A	548	A	548	A	375, 98, 46, 29	F	355, 135, 58	A
A. sp. / *Astrebla*	359, 216	C	575	C	575	B	575	A	575	A	401, 128, 46	A	517, 58	B
A. sp. / *Holcus*	550	A	310, 240	B	301, 249	A	550	A	550	A	450, 100	B	303, 135, 58, 54	C

* Code for the RFLP profile for each restriction enzyme.

4.2.3 TaqMan real-time PCR for identification of *Anguina agrostis* (Ma *et al.*, 2011)

This test developed by Ma *et al.* (2011) was designed as a species-specific real-time PCR to identify juveniles of *A. agrostis.* It was evaluated against the target species *A. agrostis* as well as non-target species *A. tritici*, *A. wevelli* and *Ditylenchus destructor*. While described for the "detection" of nematodes isolated from seed galls or plant material, the test was not specifically evaluated for its ability to quantify nematodes from quarantine samples.

The ITS rRNA species-specific primers described in this test are:

PF: 5′-GTTTGCCTACCGGTTGTTTACG-3′

PR: 5′-CCACATGCAGTCGGTGTGAA-3′

TaqMan probe Pb: 5′-FAM-TCATGTCTTGGCTATTGTAGACGTATCTGA-TAMRA-3′

The amplification is performed in a real-time PCR using the LightCycler (Roche[2]) according to the cycling parameters described in Table 8.

Table 8. Real-time PCR master mix composition, cycling parameters and amplicons (after Ma *et al.*, 2011)

Reagent	Final concentration
PCR-grade water	–†
PCR buffer	1×
$MgCl_2$	1.25 mM
dNTPs	0.2 mM
Primer PF (forward)	0.4 µM
Primer PR (reverse)	0.4 µM
Probe Pb	0.02 µM
DNA polymerase	0.5 U
DNA (volume)	1 µl
Cycling parameters	
Initial denaturation	94 °C for 3 min
Number of cycles	45
Denaturation	94 °C for 10 s
Annealing	60 °C for 30 s
Expected amplicons	
Size	88 bp

† For a final reaction volume of 10 µl.

bp, base pairs; PCR, polymerase chain reaction.

4.2.4 Real-time PCR for identification of *Anguina agrostis*, *A. funesta*, *A. pacificae* and *A. tritici* (Li et al., 2015)

Li *et al.* (2015) designed a TaqMan real-time PCR to identify *A. agrostis*, *A. funesta*, *A. pacificae* and *A. tritici*. This test includes forward and reverse genus-specific primers combined with a fluorescent probe (modified with TET dye and BHQ-2 Black Hole Quencher[2]). This primers and probe set was designed to serve as an internal control for confirming the presence of *Anguina* spp. as well as the integrity of the PCR components and user performance. The test also includes primers and probe sets specifically designed for the detection of each of the target species mentioned above and is intended for identification of single juveniles. Species-specific probes were modified with 6-FAM and BHQ-1 and were simultaneously detectable on a different fluorescent channel in duplex PCRs (i.e. with the species-specific and genus-specific primers and probe sets). The sensitivity of the test was demonstrated through construction of standard curves from reactions using serially diluted nematode DNA: the test was able to detect as little as 1.25 copies of the ITS rDNA. The specificity of each primers and probe set was demonstrated in singleplex and duplex reactions (i.e. with the species-specific and genus-specific primers and probe sets) tested against all of the target species as well as several non-target nematodes including *Anguina* spp., *Meloidogyne* spp., *Pratylenchus* spp. and *Ditylenchus* spp.

The ITS rRNA genus- and species-specific TaqMan primers and probes described in this test are:

***A. agrostis* (AAfpr primers-probe set)**

AAf (forward): 5′-CGGTTGTTTACGGCCGT-3′

AAr (reverse): 5′-ATGTAGTCGGTGTGAAAACAGCCAT-3′

AAp (probe): 5′-6-FAM/ATCATGTCTTGGCTATTGTAGACGTATCTG/BHQ-1-3′

***A. funesta* (AFfpr primers-probe set)**

AFf (forward): 5′-GGTTGCTTACGGCCC-3′
AFr (reverse): 5′-GTGTAATCGATGTGATACAGCCCC-3′
AFp (probe): 5′-6-FAM/ATCATGTCTTGGCTATTATAGACGTATCTG/BHQ-1-3′

***A. pacificae* (APfpr primers-probe set)**

APf (forward): 5′-ACCGGTTGAATATTGGCTGT-3′
APr (reverse): 5′-ATGTAATCGATGTGAAACAGCCGT-3′
APp (probe): 5′-6-FAM/ATCATGTCTTGGAAAGTTTAGACGTATCTG/BHQ-1-3′

***A. tritici* (ATfpr primers-probe set)**

ATf (forward): 5′-GTTGCCTACGGCCGT-3′
ATr (reverse): 5′-ATGTAATCGATGTGGTACAGCCAT-3′
ATp (probe): 5′-6-FAM/ATCATGTCTTGGCTAGTGTAGACGTATCTG/BHQ-1-3′

***Anguina* spp. (ASfpr primers-probe set)**

ASf (forward): 5′-GTCTTATCGGTGGATCACTCGG-3′
ASr (reverse): 5′-TGCAGTTCACACCATATATCGCAG-3′
ASp (probe): 5′-TET/TCATAGATCGATGAAGAACGCAGCCA/BHQ-2-3′

The amplification reaction is performed in a real-time PCR using the SmartCycler II real-time PCR system (Cepheid[2]) according to the cycling parameters described in Table 9.

Table 9. Real-time PCR master mix composition, cycling parameters and amplicons (after Li *et al.*, 2015)

Reagent	**Final concentration**
PCR-grade water	–[†]
PCR buffer (including $MgCl_2$)	1×
$MgCl_2$	6.0 mM
dNTPs	0.24 mM
Species-specific primer (forward)	240 nM
Species-specific primer (reverse)	240 nM
Species-specific probe	120 nM
ASf internal control primer (forward)	160 nM
ASr internal control primer (reverse)	160 nM
ASp internal control probe	120 nM
DNA polymerase (Platinum Taq (Invitrogen[2]))	1.0 U
DNA (volume)	1 µl
Cycling parameters	
Initial denaturation	95 °C for 20 s
Number of cycles	40
Denaturation	95 °C for 1 s Optics OFF
Annealing	60 °C for 40 s Optics ON
Ramp	5 °C per s

Expected amplicons	
Size	74–85 bp

† For a final reaction volume of 25 µl.

bp, base pairs; PCR, polymerase chain reaction.

4.2.5 DNA sequence analysis of ITS1 and ITS2 rRNA

Molecular phylogenies of *Anguina* spp. provide a foundation for species identification based on alignment of rDNA sequences, including ITS1-partial 5.8S as described by Powers *et al.* (2001) or ITS1-5.8S–ITS2 as described by Subbotin *et al.* (2004). Sequences obtained from new isolates or unknown species are thus placed within the context of known species boundaries and phylogenetic relationships.

For amplification of complete ITS1, 5.8S rDNA and ITS2, the primers used are:

TW81 (forward): 5′-GTTTCCGTAGGTGAACCTGC-3′ (Joyce *et al.*, 1994)

AB28 (reverse): 5′-ATATGCTTAAGTTCAGCGGGT-3′ (Howlett *et al.*, 1992)

Alternatively, the following primer pair can be used:

rDNA2 (also known as 18S) (forward): 5′-TTGATTACGTCCCTGCCCTTT-3′ (Vrain *et al.*, 1992)

rDNA1 (also known as 26S) (reverse): 5′-TTTCACTCGCCGTTACTAAGG-3′ (Vrain *et al.*, 1992)

The PCR is run with the composition and cycling parameters shown in Table 10. The PCR products are analysed by agarose gel electrophoresis. DNA fragments are extracted from the gel using commercially available reagents (e.g. Qiaquick Gel Extraction Kit (Qiagen[2])) and sequenced with the same primers as for PCR. Alternatively, PCR products are cloned into a plasmid vector (e.g. TOPO TA cloning vector (Thermo Fisher Scientific[2]) or StrataClone vector (Agilent Technologies[2])) and transformed into competent *Escherichia coli*. Plasmid clones are isolated from transformed bacteria using blue-white colony selection and sequenced using universal vector primers (Zheng *et al.*, 2000).

The size of the complete ITS1-5.8S–ITS2 region is approximately 675 base pairs for *Anguina* spp.

DNA sequence alignment methods are numerous and rapidly evolving. DNA alignments with sequences obtained from GenBank are constructed with ClustalW, Clustal Omega or MAFFT from the European Bioinformatics Institute (available from http://www.ebi.ac.uk/Tools/msa) or by alignment plug-in modules within the commercial software packages Geneious (Biomatters[2]) and Chromas (Technelysium[2]). Pairwise genetic distances are calculated for all sequence combinations and expressed as percentage similarity or absolute number of nucleotide differences per aligned pair. Interspecific variation that exceeds the intraspecific variation generally indicates separation of species. A high degree of sequence similarity to named species should confirm results from PCR-RFLP and yield a definitive species diagnosis. A match to a previously identified unnamed population may yield the best possible conclusion for the circumstances.

Table 10. DNA sequence analysis of ITS1 and ITS2 rRNA: PCR master mix composition, cycling parameters and amplicons

Reagent	Final concentration
PCR-grade water	–[†]
Taq incubation buffer (Taq PCR Core Kit (Qiagen[2]))	1×
5× Q-solution (Taq PCR Core Kit (Qiagen[2]))	1×
dNTPs	0.2 mM
TW81 (forward)	1.5 µM
AB28 (reverse)	1.5 µM
DNA polymerase (Taq PCR Core Kit (Qiagen[2]))	0.8 U
DNA (volume)	10 µl
Cycling parameters	
Initial denaturation	94 °C for 4 min
Number of cycles	35
Denaturation	94 °C for 1 min
Annealing	55 °C for 1 min 30 s
Elongation	72 °C for 2 min
Final elongation	72 °C for 10 min
Expected amplicons	
Size	675 bp

[†] For a final reaction volume of 100 µl.

bp, base pairs; ITS, internal transcribed spacer; PCR, polymerase chain reaction; rRNA, ribosomal RNA.

4.2.6 Controls for molecular tests

For the test result to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid isolation and amplification of the target pest or target nucleic acid. For PCR a positive nucleic acid control, a negative amplification control (no template control) and a negative extraction control are the minimum controls that should be used.

Positive nucleic acid control. This control is used to monitor the efficiency of the test method (apart from the extraction), and specifically the amplification. Pre-prepared (stored) nucleic acid of the target nematode may be used.

Negative amplification control (no template control). This control is necessary for conventional PCR to rule out false positives due to contamination during preparation of the reaction mixture. PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage.

Negative extraction control. This control is used to monitor contamination during nucleic acid extraction. The control comprises nucleic acid extraction and subsequent amplification of extraction buffer only. It is recommended that multiple controls be included when large numbers of positive samples are expected.

Positive digestion control (for RFLP only). This control is used to monitor the efficiency of the enzymatic digestion. Amplicon obtained from pre-prepared (stored) nucleic acid of target nematode may be used, as long as the nematode has been accurately identified as belonging to one of the species described in the RFLP patterns.

4.2.7 Interpretation of results from PCR

4.2.7.1 Conventional PCR and PCR-RFLP

The pathogen-specific PCR will be considered valid only if these criteria are met:

- the positive control produces the correct size amplicon for the target nematode species
- the negative extraction control and the negative amplification control produce no amplicons of the correct size for the target nematode species
- the restriction enzyme patterns reveal only the bands expected for the species, with no additional bands and no missing bands.

4.2.7.2 Real-time PCR

The real-time PCR will be considered valid only if these criteria are met:

- the positive control produces an amplification curve with the species-specific primers and probe
- the negative extraction control and the negative amplification control produce no amplification curve or no exponential curve
- in the case of the Li *et al.* (2015) test, the genus-specific primers and probe produce an amplification curve in the presence of test sample DNA, indicating the presence of intact nematode DNA and integrity of the PCR components.

A sample will be considered positive if it produces an exponential amplification curve. If a cycle cut-off value is needed, its value has to be verified in each laboratory when implementing the test for the first time.

5. Records

Records and evidence should be retained as described in section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*).

In cases where other contracting parties may be affected by the results of the diagnosis, the following records and evidence and additional material should be kept for at least one year in a manner that ensures traceability: preserved or slide-mounted specimens, photographs of distinctive taxonomic structure, DNA extracts and photographs of gels.

For morphological evidence, critical features as outlined in the morphological section should be drawn or photographed while fresh material is available, and relevant measurements should be included.

Good photomicrographs (or scanning videos) of key morphological features are likely to be important for record keeping.

6. Contacts Points for Further Information

Further information on this protocol can be obtained from:

Nematology Laboratory, United States Department of Agriculture (USDA), Agricultural Research Service (ARS), 10300 Baltimore Ave., Bldg 010A BARC West, Rm 113, Beltsville, MD 20705, United States of America (Andrea Skantar; e-mail: Andrea.Skantar@ars.usda.gov; tel.: +1 301 504 5917).

Agri-Food and Biosciences Institute, Newforge Lane, Belfast BT9 5PX, United Kingdom (Colin Fleming; e-mail: Colin.Fleming@afbini.gov.uk).

Nematology Unit, Fera Science Limited, National Agri-Food Innovation Campus, Sand Hutton, York, YO41 1LZ, United Kingdom (Thomas Prior; e-mail: Colin.Fleming@afbini.gov.uk; tel.: +44 1904 462206).

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on

Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will in turn forward it to the Technical Panel on Diagnostic Protocols (TPDP).

7. Acknowledgements

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8. References

The present annex may refer to International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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9. Figures

Figure 1. Healthy *Lolium rigidum* seed (left), *Anguina funesta* gall (centre) and nematode gall colonized by *Rathayibacter toxicus* (right).
Photo courtesy I. Riley, South Australian Research and Development Institute (SARDI), Adelaide, Australia.

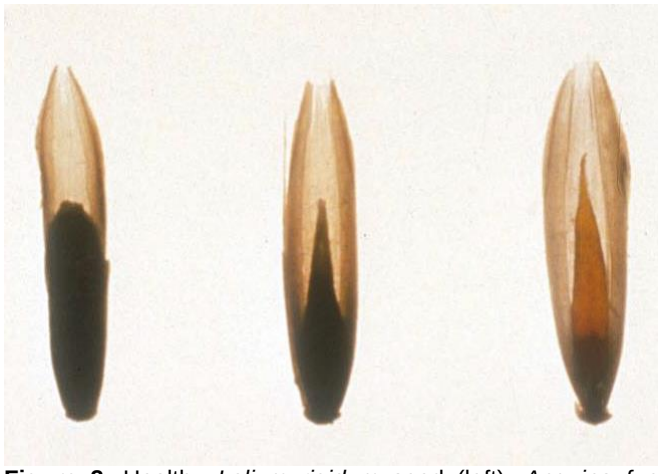

Figure 2. Healthy *Lolium rigidum* seed (left), *Anguina funesta*-infested nematode gall (centre) and *Anguina funesta*-infested bacterial gall (right).
Photo courtesy J. Allen, Western Australia Department of Agriculture and Food, Perth, Australia.

Figure 3. Gumming disease of *Lolium* due to *Rathayibacter toxicus*.
Photo courtesy J. Allen, Western Australia Department of Agriculture and Food, Perth, Australia.

A  B 

C 

Figure 4. (A) Healthy *Triticum aestivum* ears (left) and ears infested with *Anguina tritici* (right). (B) and (C) Symptoms of infestation of *T. aestivum* seeds with *A. tritici.*
Photos (A) © Howard Ferris, University of California, Davis, CA, United States of America, 1999; (B) courtesy Fera, United Kingdom; and (C) courtesy J. Swarup, India.

Figure 5. (A) Healthy *Triticum aestivum* seeds (left) and seeds infested with *Anguina tritici* (right).
Photo © Ulrich Zunke, University of Hamburg, Germany.

Figure 5. (B) Comparison of colour and shape of healthy *Triticum aestivum* seeds (left) and seeds infested with *Anguina tritici* (right).
Photo courtesy T. Kościuch, Poland.

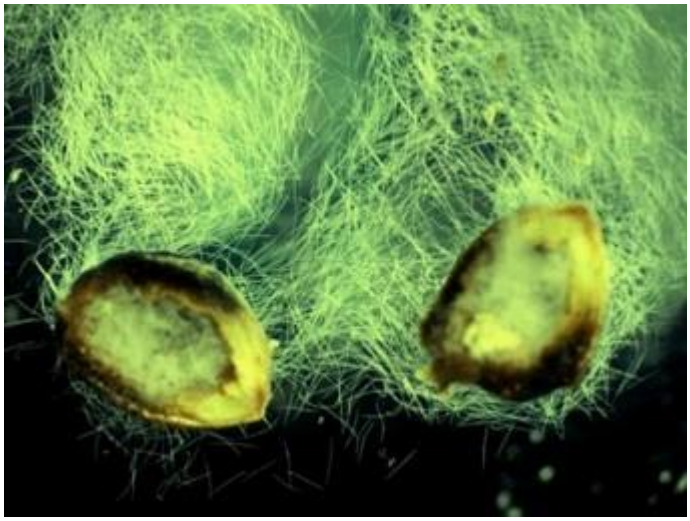

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Photo © Howard Ferris, University of California, Davis, CA, United States of America, 1999.

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Source: Pscheidt and Ocamb (2015, part of Ohio State University Extension Plant Pathology Slide).

Figure 8. Agrostis plants infested with *Anguina agrostis*.
Photo © Malcolm Storey, 2011–2015.

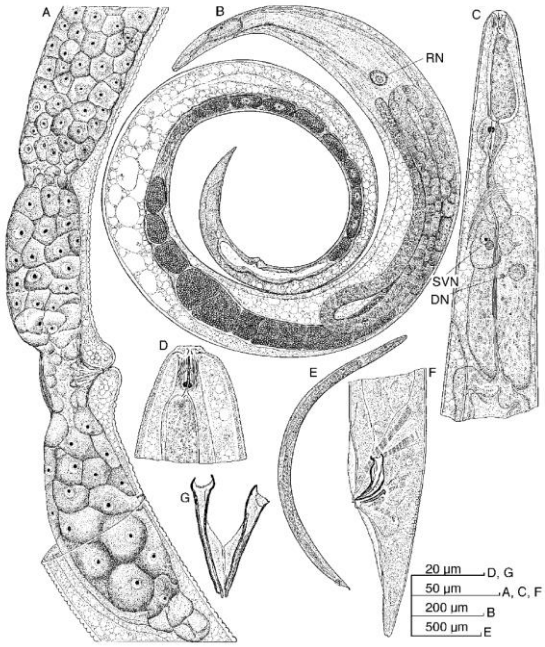
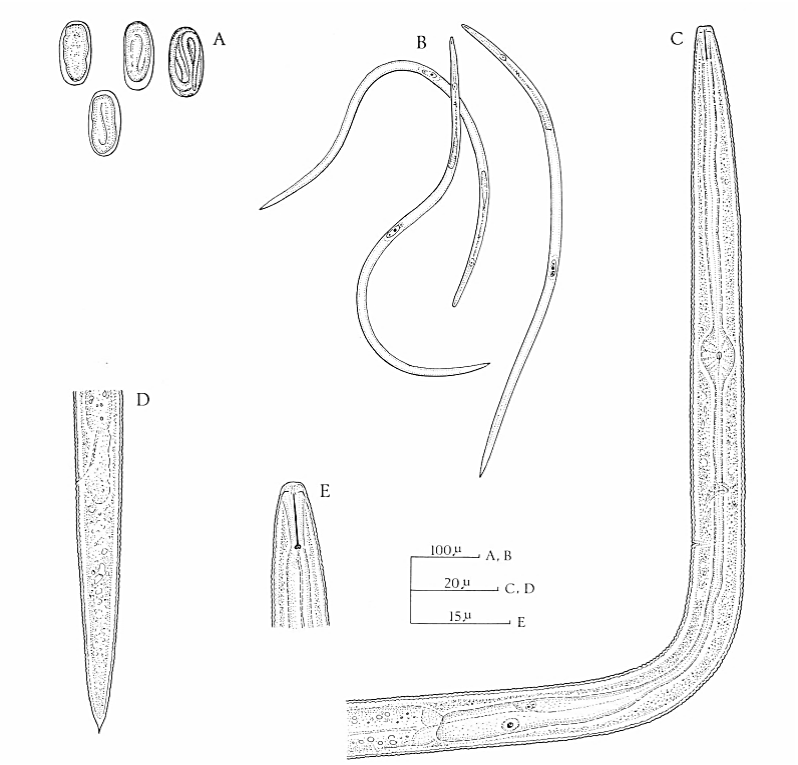


Figure 9. *Anguina tritici* from wheat grain: (A) vulval region showing a surface view of the uterus and postvulval uterine sac; (B) female; (C) oesophageal region of male; (D) head end of female; (E) male; (F) tail end of male; and (G) spicules. DN, nucleus of dorsal oesophageal gland; SVN, nuclei of subventral glands; RN, nucleus of renette cell.
Reproduced from Siddiqi (2000), courtesy CABI.



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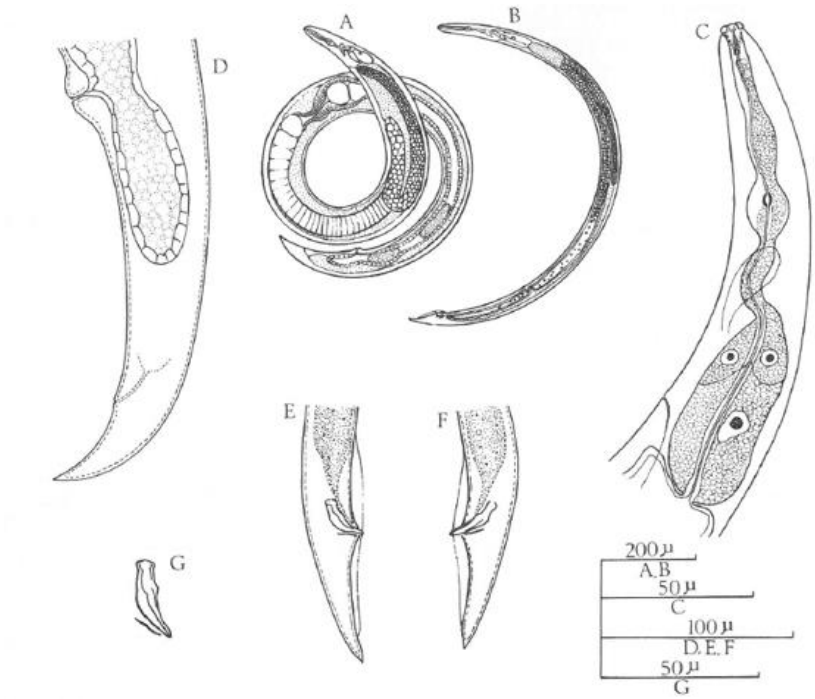
Reproduced from Southey (1972), courtesy CABI.



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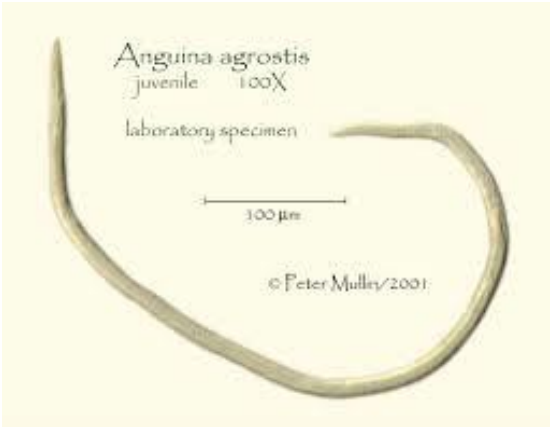
Reproduced from Southey (1973), courtesy CABI.

A



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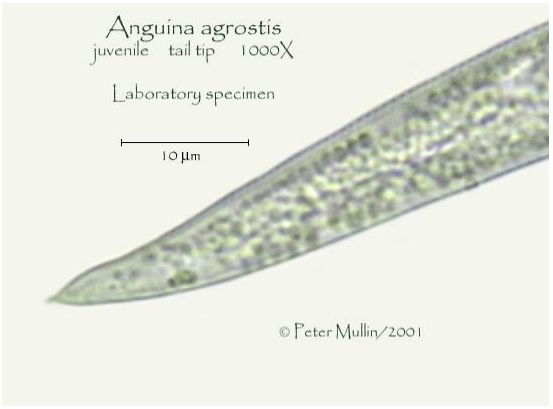

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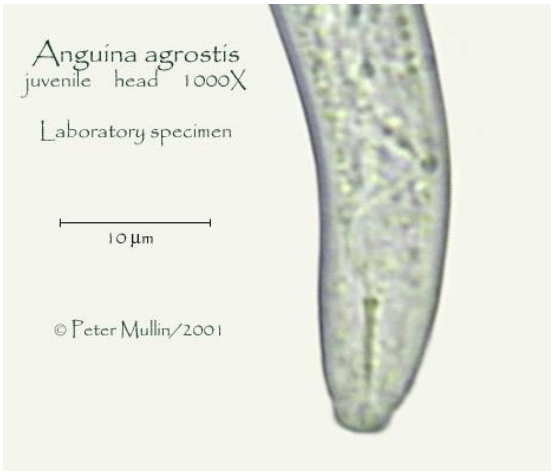

Figure 12. *Anguina agrostis* juvenile: (A) whole nematode; (B) tail; and (C) head.
Source: University of Nebraska-Lincoln Nematology Lab (n.d.). Photo © Peter Mullin, 2001.

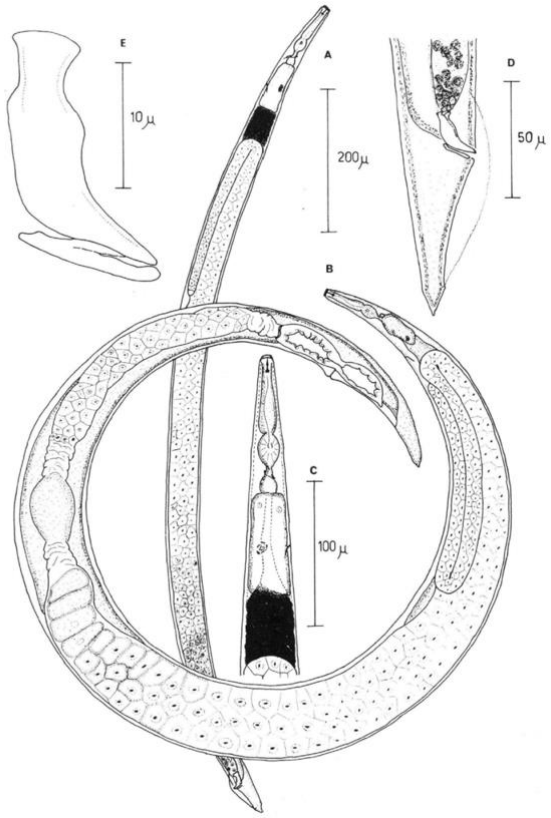

Figure 13. *Anguina funesta*: (A) adult male; (B) adult female; (C) male anterior; (D) male tail; and (E) spicule and gubernaculum.
Reproduced from Price *et al.* (1979), courtesy *Nematologica*.

Publication history

This is not an official part of the standard

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ISPM 27
Diagnostic protocols for regulated pests

DP 19: *Sorghum halepense*

Adopted 2017; published 2017

CONTENTS

1. Pest Information

Sorghum halepense (Johnsongrass) is a perennial grass with a ribbed leaf sheath, conspicuous midrib, large, purplish panicles, and far-reaching rhizomes (Figures 1 and 2). Its origin remains unclear, but some authors suggest that it originated from the hybridization of *Sorghum arundinaceum* and *Sorghum propinquum* through chromosome doubling (chromosomes: 2n = 4x = 40) (Ng'uni *et al.*, 2010). *S. halepense* is native to the Mediterranean area (Meredith, 1955) and has been introduced to other regions (Bor, 1960). It has become widespread, and is distributed from latitude 55° north to 45° south. It is best adapted to warm, humid areas with summer rainfall, areas with a high water table, and irrigated fields in subtropical zones. *S. halepense* is one of the most malignant weeds worldwide, impacting more than 30 cereal, vegetable and fruit crops (Holm *et al.*, 1977). It also threatens biodiversity in at least 50 countries in temperate and tropical areas throughout the world, including countries in which it is a native species (Holm *et al.*, 1977).

The main factors affecting the pest risk of *S. halepense* as a pest of plants are that: (1) it has a high asexual and sexual reproductive capacity; (2) its seeds can be dormant and are long-lived, and can move with traded commodities (Warwick and Black, 1983); (3) it has strong competitive ability and causes great yield loss in crops (Follak and Essl, 2012); (4) it is an alternate host of numerous pathogen species; (5) it has allelopathic effects and is toxic to livestock (da Nobrega *et al*., 2006); (6) it has developed resistance to a wide range of herbicide groups (Heap, n.d.); and (7) it has self-compatibility but readily crosses with related species, which may result in more invasive hybrids or cause gene introgression of crop species (Warwick and Black,1983; Arriola and Ellstrand, 1996).

S. halepense is able to reproduce by rhizomes or seeds. Rhizomes readily sprout and can be distributed by tillage. An individual *S. halepense* plant is able to produce as many as 28 000 seeds in a growing season. These seeds are able to survive and germinate under most environmental conditions. The seeds are caryopses and are brown, obovate, 3 × 1.6 mm in size, with an elliptic sessile spikelet that is appressed pubescent (Table 1).

Seeds are the main means of spread of *S. halepense*, and they are readily distributed naturally by wind and water as well as by birds and other animals. More importantly, the seeds are frequently disseminated by human activity as a contaminant of commodities traded around the world; in particular, crop seeds and raw grains, such as *Sorghum bicolor* (sorghum), *Glycine max* (soybean), *Zea mays* (maize), *Triticum aestivum* (wheat) and *Sesamum indicum* (sesame), as well as forage, *Gossypium* spp. (cotton) and birdseed mixes.

2. Taxonomic Information

Name: *Sorghum halepense* (L.) Pers., 1805

Synonyms: *Holcus halepensis* L., 1753

Taxonomic position: Plantae, Angiospermae, Monocotyledonae, Poales, Poaceae

Common names: Johnson grass, Johnsongrass (English)

3. Detection

Common survey methods for herbaceous species may be adopted for the detection of *S. halepense* in the field. In order to detect seeds of *S. halepense* in crop seeds, an inspection procedure should be followed in which a composite sample is prepared for laboratory analysis and sieve detection (ISTA, 2014).

3.1 Preparation of samples for laboratory analysis

General guidance on sampling methodologies is described in ISPM 31 (*Methodologies for sampling of consignments*). The sample for examination should be approximately 1 kg. Remaining sample material

should be labelled and conserved in paper bags or glassware free from moisture for possible further checking.

3.2 Sieve detection

A set of three sieves should be assembled with decreasing aperture sizes according to the seeds or grains being sampled, within an overall range of 2 mm to 10 mm. The largest aperture sieve is placed on top of the second largest sieve, with the smallest sieve on the bottom. The sample for examination is placed in the top sieve and the sieve set assembly is covered before sieving the sample through it. After sieving, the material remaining in each sieve layer is collected and placed onto white plates for visual examination. The suspected *S. halepense* seed fragments and seeds (resembling those shown in Figure 3) are selected for further identification.

4. Identification

Identification of *S. halepense* seeds is the main task and is commonly based on morphology. For suspected seeds with intact glumes and upper lemmas, morphological identification methods (section 4.1) are reliable. However, the fruits and seeds collected may be incomplete and parts of their characters unclear. In such cases, molecular (section 4.2) or biochemical (section 4.3) identification methods may need to be used. Seeds may also be sown and grown into seedlings and mature plants, either of which can be morphologically (section 4.4) or cytologically (section 4.5) examined for taxonomic traits and subsequently identified as a complement. Figure 4 presents a flow chart for the identification of *S. halepense*.

S. halepense is prone to be confused with five related species in the genus *Sorghum*:

- *S.* × *almum* Parodi (*S. bicolor* subsp. *drummondii* (Nees ex Steud.) de Wet ex Davidse), 1943
- *S. bicolor* (L.) Moench, 1794
- *S. propinquum* (Kunth) Hitchcock, 1929
- *Sorghum* spp. hybrid cv. Silk (silk sorghum), a hybrid between Krish hybrid sorghum (*S. halepense* × *S. roxburghii*) and *S. arundinaceum*, 1978 (CSIRO, 1978; Flora of China Editorial Committee, 1997, 2013; Ross, 1999; Barkworth, 2013).
- *S. sudanense* (Piper) Stapf, 1917.

This diagnostic protocol compares *S. halepense* with the above five closely related species. Detailed descriptions of plant morphological characteristics can be found for *S. halepense* in Holm *et al.* (1977) and Flora of China Editorial Committee (1997, 2013); for *S.* × *almum*, *S. bicolor*, *S. propinquum* and *S. sudanense* in Flora of China Editorial Committee (1997, 2013); and for *Sorghum* spp. hybrid cv. Silk in CSIRO (1978) and Ross (1999).

4.1 Morphological identification of seeds

The caryopsis of *S. halepense* is brown, obovate, 2.6–3.2 mm in length and 1.5–1.8 mm in width; obtuse in the apex with persistent style; hilum rotund, deep purple–brown; ventral side flat; embryo oval or obovate, with length approximately one-third to half of the caryopsis (Figures 2 and 3).

S. halepense seeds can be identified based on characteristics of the glume and upper lemma (Tables 1 and 2). A key for species identification can be used to distinguish similar species if a seed is not easily matched to the description of characteristics in Tables 1 and 2.

Table 1. Comparison of the sessile spikelet, caryopsis and seed weight in *Sorghum halepense* and five related species

Species	Sessile spikelet	Caryopsis	Weight of 1 000 seeds (g, approximate)
S. halepense	Elliptic or ovate, (3.8) 4–5 (6.5) mm in length, appressed pubescent	Dark brown, obovate or elliptic, 2.6–3.2 mm in length and 1.5–1.8 mm in width	4.9
S. × *almum*	Elliptic to oblong, 4.5–6.5 mm in length, short pubescent	Red–brown, broadly ovate or oval, 3.3–4 mm in length and 2–2.3 mm in width	6.6
S. propinquum	Ovate, or broadly ovate, 3.8–5 mm in length, bearded	Brown, broadly ovate or broadly oval, approximately 2 mm in length and 1.5 mm in width	3.8
S. sudanense	Elliptic, (5) 6–8 mm in length, sparsely pubescent	Red–brown, broadly ovate, 3.5–4.5 mm in length, 2.5–2.8 mm in width	10–15
S. bicolor	Elliptic to oblong or ovate, (3) 4.5– 6 (10) mm in length, densely hispid, or pubescent to glabrous	Pink to red–brown, ovate, 3.5–4 mm in length, 2.5–3 mm in width	>20
Sorghum spp. hybrid cv. Silk	Oval, approximately 3.8 mm in length, short pubescent	Yellow or yellow–brown, broadly ovate, 2.5–4 mm in length and 1.7–2.5 mm in width	4.2

Source: Based on Holm *et al.* (1977), Sun *et al.* (2002), Qiang (2009), Barkworth (2013), Flora of China Editorial Committee (2013) and Clayton et al (2016).

Table 2. Comparison of the glume and upper lemma of seeds in *Sorghum halepense* and five related species

	Glume	Lower glume	Upper glume	Upper lemma
S. halepense	Subleathery, tawny, red–brown or purple–black	Apex clearly tridenticulate, 5–7-veined, dorsum ciliary but the rest glabrous	3-veined	Triangular lanceolate, apex bilobed and awned or not; awn 10–16 mm
S. × *almum*	Chartaceous or subleathery, dark brown	Apex slightly tridenticulate, 5–7-veined, dorsum ciliary but the rest glabrous	3-veined	Lanceolate, apex obtuse or slightly acute, bilobed, awned; awn approximately 15 mm
S. propinquum	Subleathery, dark brown with inconspicuous crossveins	9–11-veined, apex acute to apiculate or tridenticulate, pubescent	7-veined	Lanceolate, approximately 3.5 mm in length, acute or emarginate, awnless

	Glume	Lower glume	Upper glume	Upper lemma
S. sudanense	Leathery, lemon yellow to red–brown	Apex bidenticulate, 11–13-veined, usually with crossveins, dorsum short ciliary	5–7-veined, with crossveins	Ovate or elliptic, apex bilobed, awned; awn 10–16 mm
S. bicolor	Leathery, pink to red–brown	Apex acute or tridenticulate, 12–16-veined with crossveins, dorsum dense ciliary	7–9-veined	Lanceolate to long oval, 2–4-veined, apex bilobed, awned; awn approximately 1 mm
Sorghum spp. hybrid cv. Silk	Leathery, tawny, red–brown or purple–black	Apex slightly tridenticulate, 5–7-veined, dorsum ciliary but the rest pubescent	3-veined	Broad lanceolate, apex slightly bilobed, awnless

Source: Based on Holm *et al.* (1977), Sun *et al.* (2002), Qiang (2009), Barkworth (2013) and Flora of China Editorial Committee (2013).

4.1.1 Key to the seed morphology of *Sorghum halepense* and five related species

Based on Holm *et al.* (1977), Qiang (2009) and Flora of China Editorial Committee (2013).

1. Glume with clear crossveins; lower glume with more than 11 veins; large seed weight (1 000-seed weight >10 g) **2**

– Glume with no clear crossveins; lower glume with 11 or fewer veins; small seed weight (1 000-seed weight <8 g) **3**

2. Lower glume 11–13-veined, with veins extending to the base; upper glume 5–7-veined, with clear ridge..... ***S. sudanense***

– Lower glume 12–16-veined, with veins not clear on the lower part; upper glume 7–9-veined, with inconspicuous ridge near the top ..... ***S. bicolor***

3. Lower glume 9–11-veined..... ***S. propinquum***

– Lower glume 5–7-veined..... **4**

4. Glume chartaceous or subleathery; upper lemma lanceolate, persistent rachilla rough in the fracture ..... ***S.* × *almum***

– Glume leathery; upper lemma broad lanceolate or triangular lanceolate, persistent rachilla neat in the fracture **5**

5. Lower glume with blurry tridenticulate apex; upper lemma broadly lanceolate..... ..... ***Sorghum* spp. hybrid cv. Silk**

– Lower glume with distinct tridenticulate apex; upper lemma triangular lanceolate ..... ***S. halepense***

4.2 Molecular identification of seeds

Two molecular tests have been referred to support or verify morphological identification of seeds of *S. halepense* in the case of uncertainty of visible morphological characters or for identifying partial seeds. For these methods, at least 0.05 g seeds is needed.

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

4.2.1 Methods based on DNA markers

For DNA extraction from seed samples, refer to the source paper of the molecular method for the specific technique used (Chen *et al.*, 2009). The method described by Moller *et al.* (1992) is recommended for DNA microextraction from seeds of *Sorghum* species[1]. If more than one seed is included in the extraction, the DNA may comprise a mixture of species. Under certain circumstances, DNA may be extracted from seedlings grown from seed samples.

4.2.1.1 ISSR markers

The method of Fang *et al.* (2008) is based on inter-simple sequence repeat (ISSR) markers. It was evaluated for discriminating the following *Sorghum* species (the origin of the samples used are given in parentheses): *S. saccharatum* (China); *Sorghum* hybrid *S. sudanense* × *S. bicolor*, *S. sudanense* or *S. halepense* (United States of America); *S. bicolor* (Afghanistan); and *S.* × *almum* (Australia). At least ten seeds are needed for each sample.

The ISSR method consists of two separate amplification procedures, each with a single polymerase chain reaction (PCR) primer. The primers are as described by Fang *et al.* (2008):

IR89: 5′-VBVATATATATATATAT-3′

IS16: 5′-AGAGAGAGAGAGACC-3′

Reactions are carried out in a reaction mixture made up to a volume of 20 µl with double-distilled (dd)H_2O and containing: 1× PCR buffer, 2.0 mM $MgCl_2$, 250 µM dNTPs, 400 nM primer, 30 ng DNA template and 1.5 U Taq DNA polymerase. The cycling parameters are 12 min at 94 °C, followed by 40 cycles of (30 s at 94 °C, 30 s at 48 °C and 1 min at 72 °C) and a final step of 12 min at 72 °C. The PCR products are analysed by gel electrophoresis.

The IR89 primer produces 1 500 base pair (bp) and 100 bp amplicons, and the IS16 primer produces 1 200 bp, 1 100 bp, 850 bp and 400 bp amplicons. The *Sorghum* species considered in this diagnostic protocol have the following band patterns:

- *S. halepense:* a single band, 1 500 bp
- *S.* × *almum:* two bands, 1 500 bp and 400 bp
- *S. bicolor:* four bands, 1 200 bp, 1 100 bp, 400 bp and 100 bp
- *Sorghum* hybrid (*S. bicolor* × *S. sudanense*): five bands, 1 200 bp, 1 100 bp, 400 bp, 850 bp and 100 bp
- *S. saccharatum:* three bands, 1 200 bp, 400 bp and 100 bp
- *S. sudanense:* two bands, 400 bp and 100 bp.

4.2.1.2 SCAR markers

The method of Zhang *et al.* (2013) is based on sequence characterized amplified region (SCAR) markers. It was evaluated for discriminating *S. halepense* from 11 other *Sorghum* species, as follows (the origin of the samples used are given in parentheses): *S. halepense* (Argentina, Australia, China and United States of America); *S.* × *almum* (Argentina, Australia, Ethiopia and United States of America); *S. bicolor* (Argentina, Brazil, China, France, United States of America, and two from an unknown area); *S. vulgare* (unknown); *S. verticilliflorum* (unknown); *S. saccharatum* (China, and

[1] Laboratories may find that alternative DNA extraction techniques work equally well.

three from an unknown area); *S. nitidum* (Australia and China); *S. arundinaceum* (Australia); *S. drummondii* (Democratic Republic of the Congo, Ethiopia, Kenya and Portugal); *S. sudanense* (Argentina and China); *Sorghum* spp. hybrid cv. Silk (Australia); and S. *propinquum* (China). At least ten seeds are needed for each sample.

The PCR primers used in this assay are as described by Zhang *et al.* (2013):

SH1: 5′-AGATTGAGTCTCAGGTGC-3′

SH2: 5′-GAGTCTCAGGGTATGATCT-3′

Each 20 µl amplification reaction contains 2 µl 10× PCR buffer, 0.4 mM dNTPs, 0.25 mM of each primer, 1 U Taq DNA polymerase and 25 ng DNA (made up to volume with ddH_2O). The thermocycler is programmed for 35 cycles of 30 s at 94 °C, 40 s at 55 °C and 80 s at 72 °C. The PCR products are analysed by gel electrophoresis.

The primers produce a diagnostic band of 500 bp, which is found in *S. halepense* samples and some *S.* × *almum* samples from Australia. No bands are produced by *S. bicolor*, *S. vulgare*, *S. verticilliflorum*, *S. saccharatum*, *S. nitidum*, *S. arundinaceum*, *S. drummondii*, *S. sudanense*, *Sorghum* spp. hybrid cv. Silk and *S. propinquum*.

4.2.2 Controls for molecular tests

For the test result obtained to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid isolation and amplification of the target pest or target nucleic acid. For ISSR and SCAR PCR a positive nucleic acid control, a positive extraction control, an internal control and a negative amplification control (no template control) are the minimum controls that should be used.

Positive nucleic acid control. This control is used to monitor the efficiency of the test method (apart from the extraction). Pre-prepared (stored) genomic DNA of *S. halepense* may be used.

Internal control. For ISSR and SCAR PCR, plant internal controls *matK-trnK* or other suitable targets should be incorporated into the protocol to eliminate the possibility of PCR false negatives due to nucleic acid extraction failure or degradation or the presence of PCR inhibitors. Preferably, these internal control primers should be used:

CP3: 5′-ACGAATTCATGGTCCGGTGAAGTGTTCG-3′

CP4: 5′-TAGAATTCCCCGGTTCGCTCGCCGTAC-3′

The length of the PCR product is 750 bp (Zhang *et al.*, 2013). The laboratory should choose an internal control and validate it.

Negative amplification control (no template control). This control is necessary for PCR to rule out false positives due to contamination during preparation of the reaction mixture. PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage.

Positive extraction control. This control is used to ensure that target nucleic acid extracted is of sufficient quantity and quality for PCR.

The positive control should be approximately one-tenth of the amount of DNA extracted.

For PCR, care needs to be taken to avoid cross-contamination due to aerosols from the positive control or from positive samples. The positive control used in the laboratory should be sequenced so that this sequence can be readily compared with sequences obtained from PCR amplicons of the correct size. Alternatively, synthetic positive controls can be made with a known sequence that, again, can be compared with PCR amplicons of the correct size.

Negative extraction control. This control is used to monitor contamination during nucleic acid extraction and/or cross-reaction with other plants. The control comprises nucleic acid that is extracted

from the plant that caused contamination and subsequently amplified. It is recommended that multiple controls be included when large numbers of positive samples are expected.

4.3 Biochemical identification of seeds

The sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) for seed prolamin method of Fang *et al.* (2007) is used to distinguish *S. halepense* from the closely related species *S. bicolor*, *S. sudanense* and *S.* × *almum*. *Hordeum vulgare* is used as a control.

For each sample, at least 10 but preferably 30 mature, plump seeds are ground into a fine powder, and 0.1 g of the powder is transferred to a 1.5 ml microtube. Solvent (600 µl) mixed with the 60% mass fraction of n-propanol, glycol, isopropanol and tert-butanol is added to the sample powder in the tube. The slurry is incubated at 37 °C for 10 h, then is centrifuged at 7 100 *g* for 15 min. The supernatant, which is the prolamin extract, is transferred to a clean tube and stored at 4 °C until it is needed.

Prolamin extract (500 µl) is added to the same volume of cold acetone. The mixture is incubated at 4 °C for 10 min, then is centrifuged at 7 100 *g* for 15 min. The supernatant is discarded, and the prolamin pellet is air-dried at room temperature, then dissolved in 100 µl resuspension buffer containing 6 M urea, 30% glycerine and 25 mM acetic acid.

A 25 µl volume of the final prolamin sample is loaded onto a 15% acid (A)-PAGE gel for a run at 150 V for 4.5 h. The electrophoretic buffer is acetic acid–glycine solution (pH 3.2–3.5). Protein bands are stained with Coomassie Brilliant Blue G-250, then analysed with a gel imaging system.

The prolamins from seeds of different species show different numbers of bands in different (α, β and γ) areas by A-PAGE, as follows (see also Figure 5 for a diagrammatic representation):

- *S. halepense:* one band in the γ area
- *S. bicolor:* three bands in the α area, one band in the β area and two bands in the γ area
- *Sorghum* hybrid (*S. sudanense* × *S. bicolor*): two bands in the β area and two bands in the γ area
- *S. sudanense:* two bands in the γ area
- *S.* × *almum:* no bands.

4.4 Morphological identification of plants

Seeds can be grown for more than 100 days into mature plants for the identification of *S. halepense*. This method allows rhizomes to be one of the determining factors for the identification. Seeds are incubated for seven days on moistened filter papers in Petri dishes (9 mm in diameter) under a 12 h photoperiod at 25 °C. Seedlings with roots and leaves are transplanted into 10 cm diameter plastic pots containing a sterilized soil mix of 1:1:1 sand, soil and peat. The pots with the transplanted seedlings are placed in a greenhouse under natural light and with 28 °C/20 °C day/night temperatures.

There are many resources in the literature on plants and weeds that may be used to identify the family Poaceae, genus *Sorghum* and species *S. halepense*. In this diagnostic protocol the characters used to identify *S. halepense* are from Holm *et al.* (1977) and Flora of China Editorial Committee (2013). Figures 1 and 2 show the morphological characters of *S. halepense*. Additional photos are available at USDA (n.d.a).

Seedling: Coleoptile approximately 13 mm, primary leaves linear, 28 mm × 3 mm; hypocotyl 16–18 mm in length and epicotyl 4–6 mm (Guo and Huang, 1992). Seedlings are the earliest stage at which an identification can be made.

Mature plant: Perennial with vigorous, spreading rhizomes. Culms 0.5–1.5 (–3.0) m tall, 4–6 (–20) mm in diameter; nodes puberulous. Leaf sheaths glabrous; leaf blades linear or linear-lanceolate, (10–) 25–80 (–90) × (0.5–) 1–4 cm, glabrous; ligule 0.5–1 (2–6) mm, glabrous ciliolate membrane.

Inflorescence: Panicle lanceolate to pyramidal in outline, (10–) 20–40 (–55) cm, soft white hairs in basal axil; primary branches solitary or whorled, spreading, lower part bare, upper part branched, the secondary branches tipped by racemes; racemes fragile, composed of (1–) 2–5 spikelet pairs.

Spikelet: Usually in pairs although towards the tip of the inflorescence they may occur in threes; when the spikelet is in pairs, the lower is sessile and perfect with the upper pedicelled, narrow, long and stamen-bearing; when the spikelet is in threes, one is sessile and perfect, the others are pedicelled and staminate. Sessile spikelet elliptic, (3.8–) 4–5 (–6.5) mm; callus obtuse, bearded; lower glume subleathery, often pale yellow or yellowish brown at maturity, shortly pubescent or glabrescent, 5–7-veined, veins distinct in upper part, apex tridenticulate; upper lemma acute and mucronate or bilobed and awned or not; awn 1–1.6 cm. Pedicelled spikelet staminate, narrowly lanceolate, (3.6–) 4.5–7 mm, often violet-purple.

The following keys can be used to discriminate individual plants of *S. halepense* from the five related *Sorghum* species.

4.4.1 Key to the morphological characters of vegetative organs of *Sorghum halepense* and five related species

Based on Kang *et al.* (2000), Sun *et al.* (2002) and Flora of China Editorial Committee (2013).

1. Annual, without rhizome; usually cultivated or occasionally wild **2**

– Perennial, developed rhizome; usually wild or occasionally cultivated **3**

2. Culm base 20–50 mm in diameter ..... ***S. bicolor***

– Culm base 3–9 mm in diameter ..... ***S. sudanense***

3. Rhizome thick and short **4**

– Rhizome thin and developed **5**

4. Culm base 10–30 mm in diameter, node with grey short pubescent, ligule an eciliolate membrane, 0.1–1 mm long, with clear hair in the apex ..... ***S. propinquum***

– Culm base less than 10 mm in diameter, node glabrous, ligule a ciliolate membrane, 2.5–3.5 mm long, without clear hair in the apex ..... ***S. × almum***

5. Culm robust with base approximately 10 mm in diameter, thicker than rhizome; leaf with trichome or shell-like trace on the epidermis ..... ***Sorghum* spp. hybrid cv. Silk**

– Culm fragile with base 4–6 mm in diameter, thinner than rhizome; leaf glabrous ..... ***S. halepense***

4.4.2 Key to the morphological characters of reproductive organs of *Sorghum halepense* and five related species

Based on Flora of China Editorial Committee (2013).

1. Racemes robust and not easily fractured ..... ***S. bicolor***

– Racemes fragile **2**

2. Pedicelled spikelet persistent **3**

– Pedicelled spikelet deciduous **4**

3. Panicle 30–50 cm long, dark magenta; caryopsis deep red–brown ..... ***S. × almum***

– Panicle 15–30 cm long, magenta; caryopsis red–brown ..... ***S. sudanense***

4. Racemes loosely arranged ..... ***Sorghum* spp. hybrid cv. Silk**

– Racemes tightly arranged **5**

5. Panicle ovate; sessile spikelet ovate ..... ***S. propinquum***

– Panicle lanceolate; sessile spikelet elliptic ..... ***S. halepense***

4.5 Cytological identification of plants

Chromosome counts and flow cytometry techniques may be used for the identification of *S. halepense* (Price *et al.*, 2005; Li *et al.*, 2009; Jessup *et al.*, 2012). The chromosome number of *S. halepense* (2n = 4x = 40) is greater than that of four of its relatives: *S. Propinquum* (2n = 2x = 20), *S. sudanense* (2n = 2x = 20), *S. bicolor* (2n = 2x = 20) and flowering *Sorghum* spp. hybrid cv. Silk (2n = 3x = 30). The chromosome number of *S.* × *almum* and non-flowering *Sorghum* spp. hybrid cv. Silk (2n = 4x = 40) is the same as for *S. halepense*.

4.5.1 Chromosome counts

Chromosome counts may be made following the method of Price *et al*. (2005). Root tips (approximately 4 mm long) are removed from plants, treated with an aqueous 0.4% 8-hydroxyquinoline solution for 5 h at room temperature, fixed in 95% ethanol–glacial acetic acid (4:1 v/v), rinsed several times with distilled water, hydrolysed for 5 min in 0.1 M hydrochloric acid, rinsed for 5 min with distilled water and washed for 5 min in citrate buffer (pH 4.5). To digest the cell wall, root tips are treated for 15–50 min at 37 ℃ with aqueous 5% cellulase (pH 4.5) and 1.0% pectolyase Y-23, and rinsed three times with distilled water. Rinsed meristems are placed on a clean glass slide with a drop of ethanol–acetic acid (3:1 v/v), teased apart with a fine-tipped pair of tweezers, and allowed to air-dry at room temperature for two days. The chromosomes are stained with Azure Blue. Chromosomes from two or more root tips of each plant are counted.

Samples with more than 30 chromosomes can be suspected to be *S. halepense*, or *S.* × *almum* or non-flowering *Sorghum* spp. hybrid cv. Silk. *S. propinquum*, *S. sudanense*, *S. bicolor* and flowering *Sorghum* spp. hybrid cv. Silk can be excluded. Furthermore, samples with 40 chromosomes can be identified as *S. halepense* when *S.* × *almum* and non-flowering *Sorghum* spp. hybrid cv. Silk are excluded on the basis of being non-flowering and having a short rhizome.

4.5.2 Flow cytometry

Flow cytometry may be carried out following the method of Li *et al*. (2009) and Jessup *et al*. (2012). Newly expanded leaf material from seedling plants which the suspected *S. halepense* seeds have grown into is collected aseptically, kept on ice, chopped finely using a standard razor blade and macerated in 0.25 ml Galbraith's buffer (pH 7.2) in a Petri dish. The chopped leaves are filtered through a 53 µm nylon mesh. An additional 1.0 ml Galbraith's buffer is added and the material is then strained through a filter into a 2.0 ml microtube. Propidium iodide is added to the microtube to a final volume of 50 µl and the mixture is allowed to incubate for 15 min at 0°C.

The mean fluorescence of nuclei is quantified using a flow cytometer (Coulter Electronics[2]) equipped with a water-cooled laser tuned at 514 nm and 500 mW. Fluorescence at >615 nm is detected with a photomultiplier screened by a long pass filter. The mean 2C DNA content of each target species is calculated by comparing its mean nuclear fluorescence with the mean nuclear fluorescence of an

[2] In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

internal standard. Because of the variation of *Sorghum* DNA content, one of two different internal standards is used to avoid overlap of the standard and target species. One standard, *Arabidopsis thaliana* ecotype Columbia, has a genome size of 157 Mb or 1C = 0.16 pg. The DNA content of *A. thaliana* and *S. bicolo*r Tx623 (2C DNA content = 1.67 pg) is determined from 15 replicates of leaf samples from *S. bicolor* and *A. thaliana* Columbia. At least three replicates for each test sample are analysed to obtain the mean DNA content (Price *et al*., 2005; Jessup *et al*., 2012).

Samples with more than 30 chromosomes can be suspected to be *S. halepense*, or *S.* × *almum* or non-flowering *Sorghum* spp. hybrid cv. Silk. *S. propinquum*, *S. sudanense*, *S. bicolor* and flowering *Sorghum* spp. hybrid cv. Silk can be excluded. Furthermore, samples with 40 chromosomes can be identified as *S. halepense* when *S.* × *almum* and non-flowering *Sorghum* spp. hybrid cv. Silk are excluded on the basis of being non-flowering and having a short rhizome.

4.6 Comparison of the confidence level of the identification methods

The seed identification method based on seed morphology is the preferred and most reliable of the five methods described for the identification of *S. halepense*. Identification based on morphological traits of vegetative organs and sexual reproductive organs of mature plants is also reliable. Molecular and biochemical methods are conditional and limited because they have been based on regional and limited samples of *S. halepense*. If there is a lack of confidence in seed identification, molecular, biochemical, cytological and morphology of mature plant identification methods may be used as complementary methods. A comparison of the confidence level of the identification methods is presented in Table 3.

Table 3. Confidence levels for the identification methods for *Sorghum halepense*

Method		**Sample source**	**Reliability**	**Sample sources in making the methods**
Morphological identification of seeds		Seeds	Reliable	Large number of samples, worldwide
Molecular identification	Inter-simple sequence repeat (ISSR) markers	Seeds or parts of plants	Limited or regional	30 individuals in each sample of six species
	Sequence characterized amplified region (SCAR) markers	Seeds or parts of plants	Limited or regional	65 samples of 12 species from Argentina, Australia, Brazil, China, Democratic Republic of the Congo, Ethiopia, France, Kenya, Portugal, United States
Biochemical identification		Seeds	Limited or regional	Each sample of five *Sorghum* species from involved eight species
Morphological identification of plants		Mature plants	Reliable	Large number of samples, worldwide

Method	Sample source	Reliability	Sample sources in making the methods
Cytological identification	Mature plants	Limited or regional	2-8 individuals from United States of America

5. Records

Records and evidence should be retained as described in section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*). In cases where other contracting parties may be affected by the results of the diagnosis, the records and evidence and additional material should be kept for at least one year in a manner that ensures traceability.

6. Contact Points for Further Information

Further information on this protocol can be obtained from:

Weed Research Laboratory, Nanjing Agricultural University, Tongwei Rd 6, Weigang, Nanjing 210095, China (Sheng Qiang; e-mail: qiangs@njau.edu.cn or wrl@njau.edu.cn; tel. and fax: +86 25 84395117).

United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Plant Protection and Quarantine (PPQ), Seed Examination Facility, Building 308, Room 319, BARC-East, Beltsville, MD 20705, United States of America (Rodney W. Young; e-mail: rodney.w.young@aphis.usda.gov; tel.: +1 301 313 9333; fax: +1 301 504 9840).

Department of Plant Protection, Faculty of Agriculture, Çanakkale Onsekiz Mart University, Çanakkale, Turkey (Ahmet Uludag; e-mail: ahuludag@yahoo.com; tel.: +90 537 578 1211).

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will in turn forward it to the Technical Panel on Diagnostic Protocols (TPDP).

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8. References

The present annex refers to ISPMs. ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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9. Figures

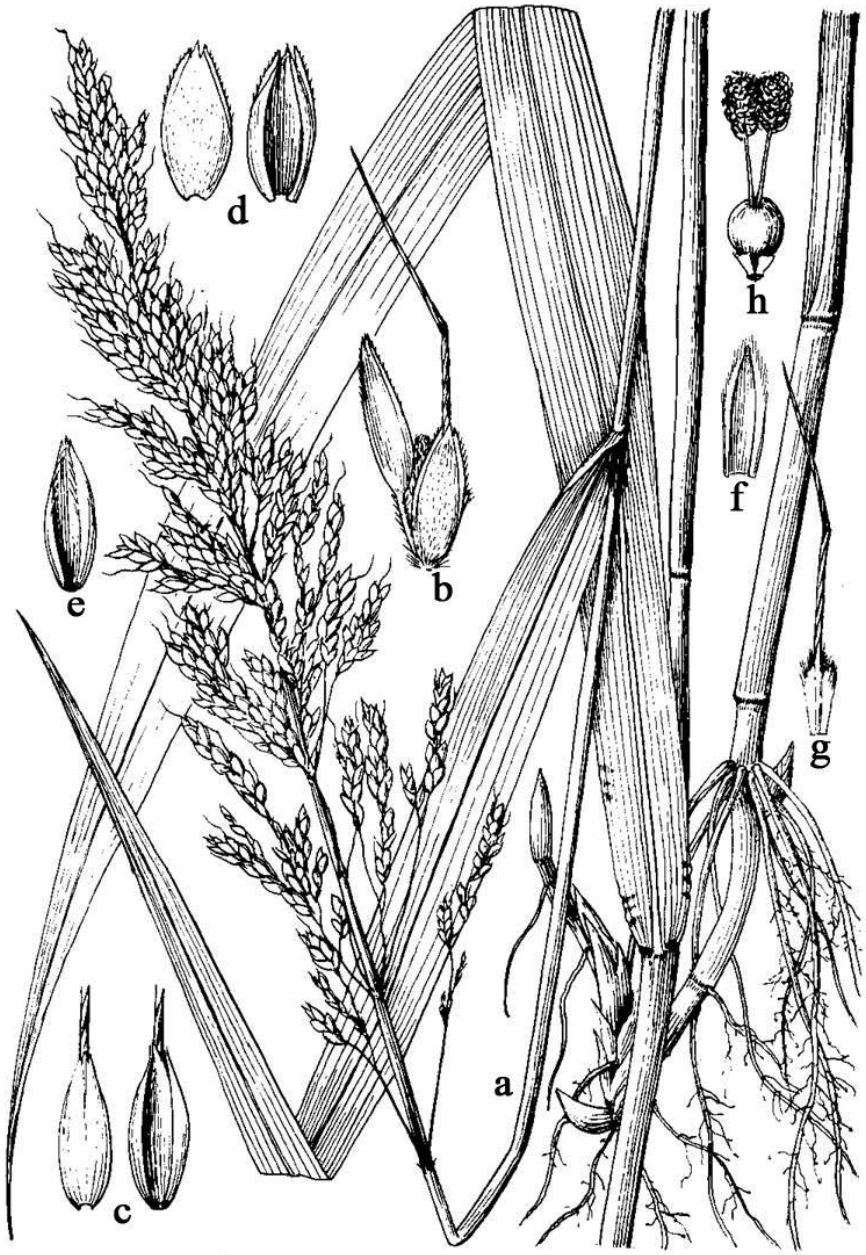

Figure 1. Parts of a *Sorghum halepense* plant: (a) whole plant; (b) spikelet; (c) abaxial and adaxial views of sessile spikelet; (d) lower glume of sessile spikelet; (e) upper glume of sessile spikelet; (f) lower lemma of sessile spikelet; (g) upper lemma of sessile spikelet; and (h) lodicules and pistil.
Source: Flora of China Editorial Committee (1997; plate 28, 1–8).

Figure 2. Morphological characteristics of *Sorghum halepense*: (a) above-ground parts; (b) rhizome; (c) sheath mouth; (d) part of panicle; and (e) perfect spikelet with two pedicelled, staminate spikelets.
Photo courtesy Sheng Qiang, Nanjing Agricultural University, China.

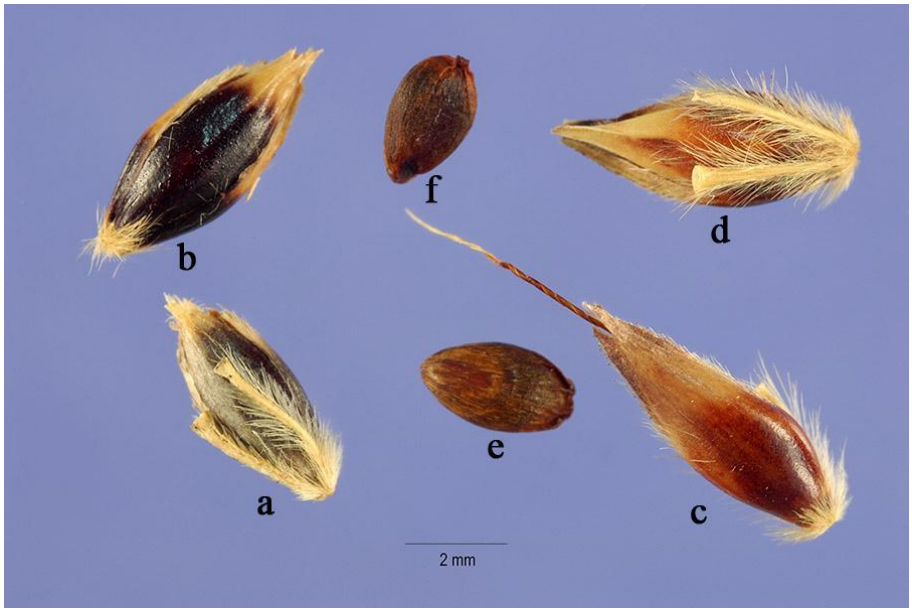

Figure 3. Spikelets and caryopses of *Sorghum halepense*: (a) and (d) adaxial view of sessile spikelet with residual rachilla; (b) abaxial view of sessile spikelet; (c) abaxial view of sessile spikelet with awn; (e) abaxial view of caryopsis; and (f) adaxial view of caryopsis.
Source: United States Department of Agriculture (n.d.b).

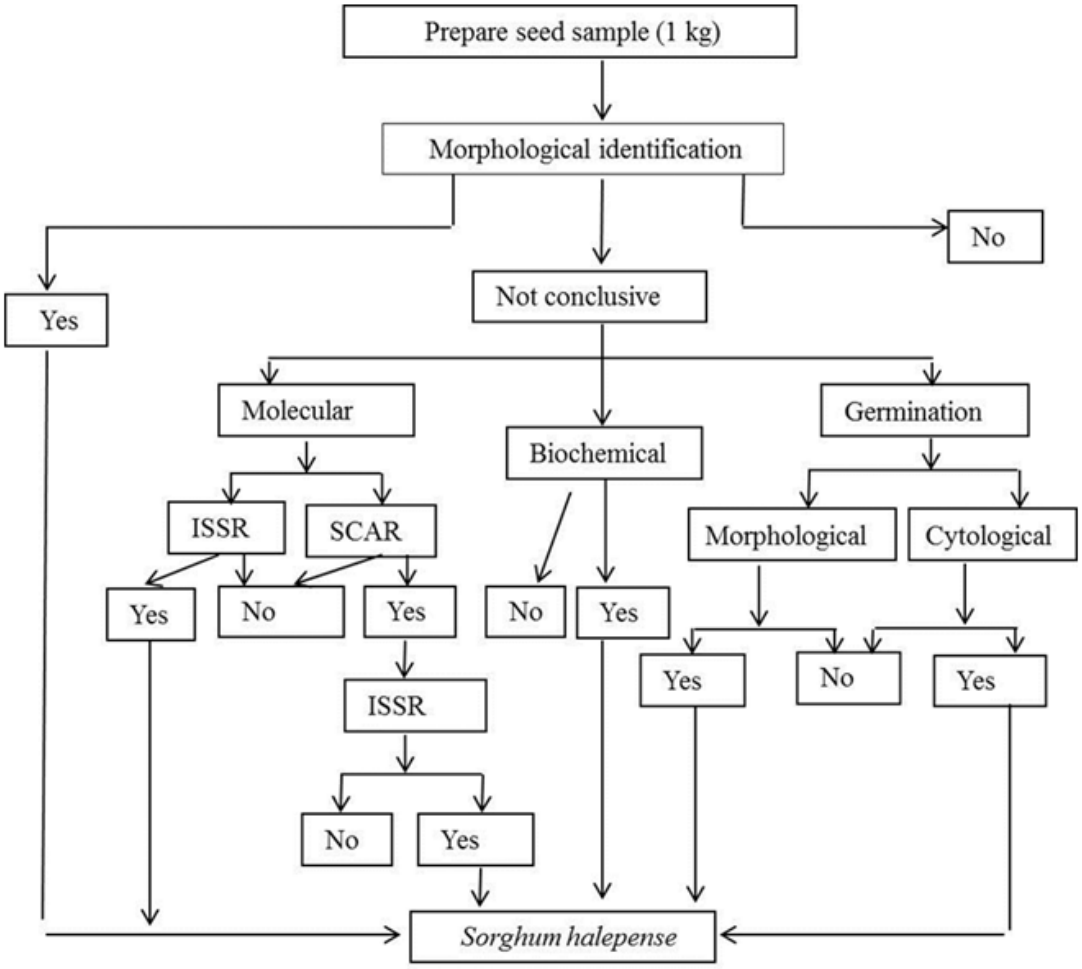

Figure 4. Flow chart for the identification of *Sorghum halepense*.
ISSR, inter-simple sequence repeat; SCAR, sequence characterized amplified region.

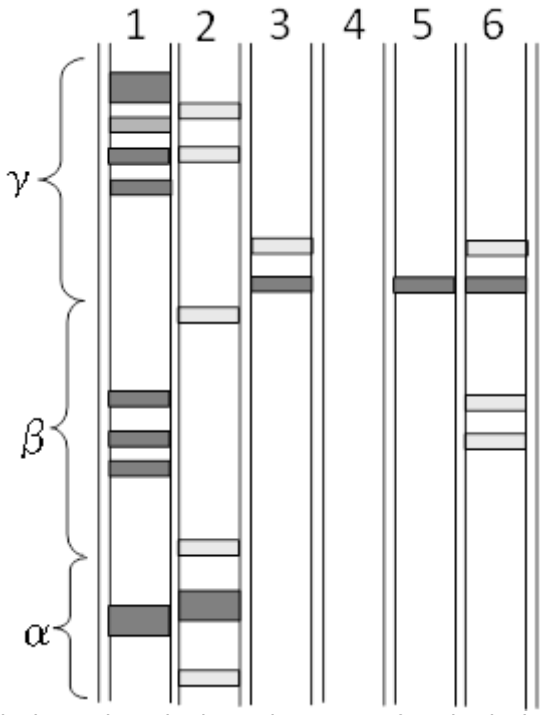

Figure 5. A-polyacrylamide gel electrophoresis (PAGE) pattern of prolamin bands from seeds of different *Sorghum* species: (1) *Hordeum vulgare* (control); (2) *S. bicolor*; (3) *S. sudanense*; (4) *S.* × *almum*; (5) *S. halepense*; and (6) *S. sudanense* × *S. bicolor*.

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ISPM 27
Diagnostic protocols for regulated pests

DP 20: *Dendroctonus ponderosae*

Adopted 2017; published 2017

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1. Pest Information

There are 20 *Dendroctonus* species that have been recognized worldwide (Table 1) and described in the literature (Armendáriz-Toledano *et al*., 2015). These beetles are phloeophagous (bark-feeding) insects that live beneath the bark layer of trees where the adults and larvae form characteristic galleries (Wood, 1982). The galleries may be engraved in both the inner bark layer and the wood. Most of the species are present only in North and Central America, but two species are native or endemic to Europe and Asia (Six and Bracewell, 2015; CABI, 2016a). The species *Dendroctonus valens* LeConte has been recently introduced to Asia (CABI, 2016b).

Dendroctonus ponderosae Hopkins, 1902 is a destructive pest of pine trees (Keeling *et al*., 2013). Adults and larvae kill the trees by directly feeding on and girdling them. In addition, *D. ponderosae* acts as a vector of at least three species of blue stain fungi, including *Ceratocystis montium* (Rumbold) J. Hunt, *Grosmannia clavigera* (Robinson-Jeffery and Davidson) Zipfel, de Beer and Wingfield (=*Ophiostoma clavigerum* (Robinson-Jeffery and Davidson) Harrington) and *Leptographium longiclavatum* Lee, Kim and Breuil (Figure 1), which block nutrient and water flow, contributing to the decline of the health of infected trees (Amman and Cole, 1983; Rice *et al*., 2008; CABI, 2015; Six and Bracewell, 2015).

D. ponderosae has been known to cause up to 85 percent tree mortality in pine tree stands and can kill many healthy pine trees (CABI, 2015). Serious outbreaks can cause millions of hectares of damage to pine forests and have a devastating ecological and economic impact (Keeling *et al*., 2013; Janes *et al*., 2014). *D. ponderosae* spreads within its native range by flight; each generation can migrate several kilometres. The beetles usually attack older trees or weakened pines, but during epidemics, *D. ponderosae* may attack trees with rapid growth rates and non-*Pinus* coniferous species. Janes *et al*. (2014) identified four oscillating phases of *D. ponderosae* population dynamics: (1) small population size, with the beetles restricted to "low-quality hosts" or trees in poor health; (2) incipient epidemics, during which the beetles mainly attack trees with a large diameter; (3) epidemics, during which the beetles attack healthy trees; and (4) post-epidemic collapse. Historically, cycles of these phases occur every 20–40 years in areas where *D. ponderosae* is endemic, with epidemics lasting on average for five years.

D. ponderosae is known to be present in North America (CABI, 2015). Major tree hosts for this pest include *Pinus contorta* (lodgepole pine), *Pinus lambertiana* (sugar pine), *Pinus monticola* (western white pine) and *Pinus ponderosa* (ponderosa pine). Other minor host species from which *D. ponderosae* has been collected include *Picea engelmannii* (Engelmann spruce), *Pinus aristata* (Rocky Mountain bristle cone pine), *Pinus albicaulis* (whitebark pine), *Pinus balfouriana* (foxtail pine), *Pinus coulteri* (big-cone pine), *Pinus edulis* (pinyon pine), *Pinus flexilis* (limber pine), *Pinus monophylla* (single-leaf pinyon pine), *Pinus strobiformis* (southwestern white pine) and *Pinus sylvestris* (Scots pine) (CABI & EPPO, 1997). Some of these records are incidental.

Attack on a pine tree is initiated by unmated adult female *D. ponderosae*, which release pheromones to attract males (CABI, 2015). Females bore straight vertical egg galleries along the phloem or inner bark. Male beetles help build the galleries by removing frass and later plug the entrance with frass. Eggs are laid individually or in clusters along the gallery, surrounded by tightly packed frass, and hatch 7 to 14 days after laying (CABI & EPPO, 1997). Females begin laying eggs about seven days after the initial attack on a tree and cease when temperatures become too cold to continue. Egg laying occurs during summer up to early autumn in North America (CABI, 2015). *D. ponderosae* has four larval instars (CABI & EPPO 1997). The larvae feed on phloem, together constructing a radiating series of feeding galleries (Figures 2 and 3). Infestation usually occurs over a five-week period, but may happen in as little as three or four days. In most cases the adults remain within the galleries and die after the production of one brood (CABI & EPPO 1997). However, some adult beetles may instead re-emerge to begin a new gallery. It sometimes takes only one generation of *D. ponderosae* to kill a healthy tree.

D. ponderosae is typically univoltine (one generation per year), but can be semivoltine (one generation every two years) where the climate is cooler, such as at higher elevations (Bentz *et al*., 2013). Mitton and Ferenberg (2012) reported that there were up to two generations per year in warm climates on certain pine species. Adults can be found on or under bark or in flight searching for a new host. Eggs, larvae and pupae are found internally under bark of shoots, or in branches and trunks. The species usually overwinters as second or third instar larvae but occasionally other life stages can be found along with larvae in colder months.

Table 1. *Dendroctonus* species

Species	Author	Distribution
Dendroctonus adjunctus	Blandford	North America
Dendroctonus approximatus	Dietz	North America
Dendroctonus armandi	Tsai and Li	Asia
Dendroctonus brevicomis	LeConte	North America
Dendroctonus frontalis	Zimmermann	North and Central America
Dendroctonus jeffreyi	Hopkins	North America
Dendroctonus mesoamericanus	Armendáriz-Toledano & Sullivan	Central America
Dendroctonus mexicanus	Hopkins	North and Central America
Dendroctonus micans	Erichson	Europe and Asia
Dendroctonus murrayanae	Hopkins	North America
Dendroctonus parallelocollis	Chapuis	North America
Dendroctonus ponderosae	Hopkins	North America
Dendroctonus pseudotsugae	Hopkins	North America
Dendroctonus punctatus	LeConte	North America
Dendroctonus rhizophagus	Thomas & Bright	North America
Dendroctonus rufipennis	(Kirby)	North America
Dendroctonus simplex	LeConte	North America
Dendroctonus terebrans	(Olivier)	North America
Dendroctonus valens	LeConte	North America and Asia
Dendroctonus vitei	Wood	North America

Source: Based on Wood (1982), Bright (2014), Armendáriz-Toledano *et al.* (2015) and CABI (2016a, 2016b).

2. Taxonomic Information

Name: *Dendroctonus ponderosae* Hopkins, 1902

Synonym: *Dendroctonus monticolae* Hopkins, 1905

Taxonomic position: Insecta, Coleoptera, Curculionidae, Scolytinae, Hylurgini

Common names: Mountain pine beetle, Black Hills beetle (in English only)

Scolytinae Latreille, 1804, the subfamily to which *Dendroctonus* Erichson, 1836 belongs, was long treated as a family of the Curculionoida. Recently Bright (2014) continued to refer to the group as the family Scolytidae following Wood (1982, 1986), while Jordal *et al*. (2014) presented phylogenetic evidence for subfamily status for the Scolytinae. In Alonso-Zarazaga and Lyal (2009) and Hulcr *et al.*

(2015), *Dendroctonus* belongs to the tribe Hylurgini. Diagnostic characters for adult Hylurgini (=Tomicini) are presented in Wood (1986).

This diagnostic protocol follows the classification set out in Bouchard *et al*. (2011), where the group is treated as a subfamily of the Curculionidae – the Scolytinae – and is divided into 26 extant tribes (Hulcr *et al*., 2015), one of which is Hylurgini Gistel, 1848, to which *Dendroctonus* belongs.

3. Detection

It is useful when examining wood to look for evidence of circular holes (1.5–3.5 mm in diameter) or frass in suspected material.

Larvae and pupae are found in the host plant or wood products only under bark or in the phloem, not in the wood or xylem. Trees can be examined externally for symptoms of infestation while pine or other coniferous wood products, particularly unprocessed logs, dunnage, crates or pallets with bark, should be examined for galleries and beetles (adults and larvae).

The entrance tunnel of galleries created by this pest is short and perpendicular to the trunk. The egg gallery, which initially ascends diagonally from the entrance tunnel, is vertical and only slightly wider than the adult beetle. The egg tunnel may be 32 to 50 cm long. The egg niche pattern is characteristic for *D. ponderosae*, alternating between groups of one to five niches (CABI & EPPO, 1997). Larvae mine tunnels that begin more or less parallel to the egg gallery and then widen and diverge from it. These tunnels are 1–4 cm long, terminating in a pupal chamber. Eventually the beetles exit the tunnel through the bark to the outside.

3.1 Symptoms of infestation in the field

Four general symptoms indicate possible attack of *D. ponderosae* in living pine trees:

- yellowing, dying needles at the base or top of the crown
- entrance tunnels on trunks, often with popcorn-sized, dark red–orange to cream-coloured exudate (pitch tubes) (Figure 4)
- visible frass (reddish boring dust in bark crevices)
- vertical, J-shaped maternal galleries ranging in length from 10 to 122 cm (average about 25 cm) (CABI, 2015) and lateral larval galleries under the outer bark layer (Figures 2 and 3).

After a few months to a year (depending on location and temperature) the attacked tree will change leaf colour from yellowish green to red. Attacks on *P. ponderosa* and *P. contorta* cause needles to change from yellowish green in spring to bright orange by mid-summer.

3.2 Samples from plants and wood products

The bark can be removed from affected plant or wood products using a sharp, strong knife or a small axe (Kelley and Farrell, 1998). The wood underneath the bark layer and the inner bark should be visually examined for vertical galleries. A 40× magnifying lens can be used to inspect for galleries and for adults, larvae and eggs. If gallery engravings are present, some of the bark or affected material should be collected and, if possible, photographed. Infested material can be transported in a sealed bag or container. Double bagging is advisable to prevent escape of the beetles.

Detected adults, larvae, pupae or eggs can be removed using forceps. Larvae can be placed in boiling water to fix them. Specimens should then be placed into a glass vial containing 70–80% ethanol. Adults can also be placed into a dry tube and then in a freezer at –20 °C for at least 24 h or at –80 °C for at least 6 h before point- or card-mounting. Doing so will preserve the reference material well for morphological identification (section 4.1).

It is important to collect any adults that are present because adults have more reliable characters for identification; it is not possible to identify juveniles to species based on morphology alone. In the laboratory, adult specimens should be pinned for examination while larvae, pupae and eggs should be

placed in ethanol. See section 4.1.1 for details on preparation of specimens for morphological examination.

4. Identification

D. ponderosae can be identified by examination of the adult external morphological characters. Features of the adult body are illustrated and labelled in Figures 5 and 6. Descriptions of and keys to bark beetle species based on adult life stage are available. Generic keys of Scolytinae larvae are available in Thomas (1957, 1965) but juvenile stages are difficult to identify as there are fewer distinct characters to differentiate between the species. Currently, it is not possible to identify *Dendroctonus* larvae to the genus or species level with confidence. The shape and form of the galleries may be useful in detection and identification but are not sufficient for identification without adult specimens for confirmation.

As yet, no reliable molecular tests can be recommended to distinguish between *D. ponderosae* and other *Dendroctonus* species with similar morphology. At present, there are no protocols using universally adapted polymerase chain reaction methods for the identification of *D. ponderosae* to the exclusion of the closely related species *D. jeffreyi*. Hence there remains the need to rely on morphological identification.

4.1 Morphological identification of adults

4.1.1 Preparation of adults for morphological examination

The ethanol-preserved specimens (section 3.2) are transferred to a small Petri dish filled with 70–80% ethanol to be cleaned from dirt, debris and frass. Specimens can be cleaned by gentle brushing with a fine hair brush. Adult specimens preserved in ethanol to be point- or card-mounted should first be dried by removing the specimen from ethanol, blotting it with paper towel and allowing it to air-dry for 2–5 min. Specimens removed from –20 or –80 °C freezers should be placed on blotting paper and thawed for 10–20 min or until any visible condensation evaporates from the specimen. A triangular card mount is most appropriate for small beetles and attaching the beetle to the card along the right lateral side of its thorax is common practice. Ideally the left lateral, dorsal and ventral aspects should be free and visible to facilitate comparison with other pinned specimens and images. Once adults are pinned, they may be examined under a dissecting microscope of at least 40× magnification (a higher magnification may be preferable). Strong, diffuse lighting is very important for examination of adult bark beetles as their surface sculpturing is characteristic. As adult bark beetles can be very shiny, light reflected from specimens may sometimes interfere with examination of characters. The sheen can be reduced by placing tracing paper or drafting film over the microscope's light source.

4.1.2 Diagnostic characters of adults of the subfamily Scolytinae

The main diagnostic features of Scolytinae are presented below and are based on key characters highlighted in Wood (1982, 1986), Anderson (2002), Rabaglia (2002) and Hulcr *et al*. (2015). Wood (1986) also provided a key to the world genera of Scolytinae and Anderson (2002) included a key to the Scolytinae genera of North America. The main diagnostic features of Scolytinae are:

- body cylindrical
- head enlarged
- snout or rostrum very short or non-existent (Figure 7)
- antennae elbowed and clubbed
- antennae geniculate with a single to seven-segmented funicle; the three- or four-segmented antennal club has apical sutures (Figure 8), which may be transverse, sinuate, recurved or procurved
- pregular sclerite (=submentum) ventrally distinctly visible, with pregular suture (Figure 9)
- legs and antennae short and retractable

- at least one pair of tibiae usually with stout spines or denticles (teeth) along the lateral outer margins (Figure 10); lateral denticles on foretibiae usually socketed
- tarsi with four visible segments (Figure 10)
- length of the first tarsal segment not more than that of the second or third tarsal segments (Figure 10).

4.1.3 Diagnostic characters of adults of the genus *Dendroctonus*

Following Wood (1982 and 1986), the following characters are diagnostic for *Dendroctonus*:

- body length between 2.5 and 9.0 mm
- male frons evenly convex but slightly impressed medially (Figure 11)
- anterior margin of pronotum distinctly emarginate (Figure 12)
- pronotum punctate (Figure 12), without asperities, to micro-asperate
- scutellum visible (Figure 13(a)), small, rounded or depressed
- anterior margin of elytra procurved with crenulations (Figure 13(a)); elytral bases slightly notched at scutellum in dorsal view
- procoxae contiguous (Figure 14(b))
- lateral precoxal prothoracic ridge not developed (Figure 14(b))
- antennal funicle five-segmented (Figure 8)
- antennal club symmetrical, strongly flattened, subcircular, with three sutures that are transverse to slightly procurved (Figure 8).

4.1.3.1 Key to distinguish Dendroctonus *adults in Scolytinae*

The following key, adapted from diagnostic characters listed in Rabaglia (2002), can be used to distinguish *Dendroctonus* from other genera commonly encountered in *Pinus* spp.

1. Anterior margin of elytra straight or transverse, without crenulations (Figure 13(b)); pronotum usually armed with asperities and head usually concealed dorsally by declivous pronotum (Figure 15).....**not *Dendroctonus***

– Anterior margin of elytra procurved (in dorsal view) and/or armed with crenulations (Figure 13(a)); head usually visible dorsally (Figures 16 and 12).....**2**

[1] 2. Prothoracic precoxal area longer than diameter of forecoxa, and lateral margin with a distinct ridge (Figure 14(a)).....**not *Dendroctonus***

– Prothoracic precoxal area shorter than diameter of forecoxa, and lateral margin with a weakly developed ridge or no ridge (Figure 14(b)).....**3**

3. Antennal club not symmetrical, with sutures fused and oblique or obsolete .....**not *Dendroctonus***

– Antennal club symmetrical, with sutures transverse or slightly procurved (Figure 8).....**4**

[2] 4. Anterior margin of pronotum transverse and no medial notch visible; antennal funicle six- or seven-segmented.....**not *Dendroctonus***

– Anterior margin of pronotum distinctly emarginate or slightly notched medially (Figures 16 and 12); antennal funicle five-segmented, club sutures slightly procurved (Figure 8) ..... ***Dendroctonus***

4.1.4 Identification of adults of *Dendroctonus ponderosae*

4.1.4.1 Diagnostic characters

Diagnostic characters of *D. ponderosae* adults are based on key characters and descriptions in Wood (1982).

Male 3.5–6.8 mm (average male: 5.5 mm). Approximately 2.2 times as long as wide. Mature adults black, pterothorax brown, some teneral stages light brown. Females appear externally similar to males except epistomal process less distinct and crenulations on elytra and granulations on declivity larger. Frons: convex with a narrow median line only lightly impressed, not deeply grooved (Figure 11). Epistomal process half as wide as width between the eyes (measured between posterior inner lateral margin of eye), epistomal process with oblique lateral arms; brush of yellow setae present beneath (Figure 11). Pronotum widest at the base, constricted anteriorly, with surface shining between closely spaced granulose punctures (Figure 12(a)). Pronotum without callus. Elytral declivity dull with interstria 1 strongly elevated; interstria 2 impressed; interstriae bear granules in one row (not random) (Figure 17).

4.1.4.2 Similar species

D. ponderosae is morphologically similar to *D. jeffreyi* (Figure 18), which makes them difficult to distinguish. The pronotal punctures are separated by a distance not greater than the diameter of the puncture in *D. ponderosae*, while *D. jeffreyi* has finer pronotal punctures more widely separated (at least twice the diameter of one puncture), and they are not as deep (Figure 12). The body length of *D. ponderosae* (average male body length 5.5 mm) is slightly less than that of *D. jeffreyi* (average male body length 6 mm). *D. jeffreyi* has a distribution range only from South Oregon (the United States of America) to North Baja California (the United Mexican States), while *D. ponderosae* is present throughout the middle and western parts of North America. The host plant range of *D. jeffreyi* is more restricted than that of *D. ponderosae*, with *D. jeffreyi* usually attacking only *Pinus jeffreyi* and rarely *P. ponderosa*. *D. ponderosae* is distinguished from *D. frontalis* by its larger size: the latter species has males that are only 2.0–3.2 mm (average 2.8 mm) long. Unlike *D. frontalis*, specimens of *D. ponderosae* lack a pronotal callus in the female and distinct groove in the middle of the frons.

In the United States of America, *Ips pini* (Say) is often found in the same tree with *D. ponderosae* during non-outbreak periods, but these two species are not easily confused as they have different parent galleries and adult *Ips* have distinct teeth around the lateral margin of the elytral declivity. However, larvae of these species and their galleries may appear very similar. Confirmation of beetle species should be specimen-based and for morphological identification, adult specimens should be examined.

4.1.4.3 Simplified key to adults of **Dendroctonus** *species*

The following key, a simplified version of a key from Wood (1982), can be used to differentiate *D. ponderosae* from 16 *Dendroctonus* species from North America. Three species – *D. armandi*, *D. mesoamericanus* and *D. micans* found in Asia, Central America, and Europe and Asia respectively – are not included in the key. See Table 1 for a complete list of described species worldwide.

1. Frons with narrow deep median groove (Figure 19) extending from just above epistomal process to upper level of eyes (Figure 20); epistomal process laterally reaching margin of eyes, lateral margin raised (Figure 20); males with one or two tubercles on lateral area of frons (except *D. adjunctus*); if protuberance obscure in female then a transverse elevated callus (collar-like sculpturing) on lateral and dorsal pronotum is present (obscure in *D. adjunctus*) (Figure 7); usually smaller than 7.5 mm (in *Pinus* spp.) ..... **not *D. ponderosae***

– Frons without deep median groove (Figure 11); epistomal process relatively narrower and less prominent; males without raised tubercles on frons; pronotal transverse elevated callus not present in either sex; larger species, usually about 5.0–9.0 mm in body length (in *Pinus* spp. and other conifers) **2**

2. Coarse granulation around the episternal area; coarse pronotal punctures separated by a distance less than the diameter of one puncture and some punctures bearing a small granule; elytral declivity with relatively large granules forming a single line (uniserial) along each interstria; elytral interstria 2 strongly impressed, interstria 1 strongly elevated, interstria 3 weakly elevated; surface of declivity dull and minutely rugulose (Figure 17) ..... ***D. ponderosae***

– Not with the above combination of characters ..... **not *D. ponderosae* (and not the similar species *D. jeffreyi*)**

4.2 Morphological identification of larvae

While adult specimens (in good condition) are the only way to confirm the identification of *D. ponderosae* using morphological methods, in the absence of an adult specimen, it is useful to examine larvae. However, there is potential for confusion among Scolytinae species, which may appear very similar at the larval stage. Larvae of *D. ponderosae* share morphological features with other species in the genus *Dendroctonus* and with species in other genera of Scolytinae. Nevertheless, examination of larvae may be useful in determining if the specimen is consistent with the known morphology of the species, and may help to support diagnosis of a specimen as either not *D. ponderosae* or suspected or possible *D. ponderosae*.

4.2.1 Preparation of larvae for morphological examination

The ethanol-preserved specimens (section 3.2) are transferred to a small Petri dish filled with 70% ethanol for morphological examination. Specimens should be clean of dirt, debris and frass for examination. Specimens can be cleaned by gentle brushing with a fine hair brush. They may be examined under a dissecting microscope of at least 40× magnification (a higher magnification may be preferable).

4.2.2 Diagnostic characters of larvae of the subfamily Scolytinae

Mature larvae of this subfamily are 4–6 mm long, and have no legs. The body is C-shaped and subcylindrical (Figure 21), with three thoracic and ten abdominal segments. Larvae have white bodies with dark brown chewing mouthparts (mandibles). The head capsule is lightly sclerotized, usually amber or light brown, and as long as broad; the antennae have only one segment; and the cranium has a Y-shaped ecdysial suture (Figure 22). The thorax bears three pairs of pedal lobes that each have two to four setae. Each abdominal segment has two or three tergal folds. The prothorax and the first eight segments of the abdomen bear spiracles (Bright, 1991). Eggs are smooth, oval, white and translucent (CABI & EPPO, 1997).

4.2.3 Diagnostic characters of larvae of the genus *Dendroctonus*

The following characters are based on Thomas (1957).

The general appearance of these larvae is as for other Scolytinae. They are C-shaped, white or cream-coloured larvae with lightly sclerotized heads. Mature specimens are large (2–9 mm). The diagnostic characters of *Dendroctonus* larvae include having head free (almost entirely visible dorsally), anterior margin of frons without tubercles, and mandibles with three incisorial teeth (Figure 23). The postlabium has a posterior pair of setae closer together than the median pair (Figure 24(a)), each lateral cluster of post-labial setae is triangular in configuration, and the shape of the premental sclerite is triangular and proximally abruptly narrowed – it appears as a distinct projection from the main body of the sclerite (Figure 24(a)). The pedal lobes are smooth but may be surrounded by spinules (but not inside the pedal lobe area), with three or four setae on each lobe (Figure 25).

4.2.3.1 Key to distinguish Dendroctonus *larvae in Scolytinae*

This key is based on work by Thomas (1957), with only 15 genera examined from mostly North American fauna. It serves as a guide to highlight further some distinctive larval characters of *Dendroctonus*.

1. Premental sclerite of labium proximally triangular in shape, or with distinct narrow projection (Figure 24(a), (b)); postlabial setae in triangular arrangement, middle pair furthest apart; no tubercle on anterior margin of frons ..... ***Dendroctonus***

– Premental sclerite proximally broadly rectangular (Figure 24(c)) or if triangular then postlabial setae linear (Figure 24(b)) or not as above; tubercle may or may not be present on anterior margin of frons (Figure 26).....**not *Dendroctonus***

4.2.4 Identification of larvae of *Dendroctonus ponderosae*

4.2.4.1 Diagnostic characters

The following key characters are based on Thomas (1965).

Mature larvae are between 4.3 and 5.2 mm long. The head capsule is evenly pigmented, light amber, and as long as broad. Diagnostic characters include frons with a prominent pair of elevations posterior of mid frons (Figure 22(a)), and tubercle on the inner basal angle of the mandible (Figure 23).

4.2.4.2 Key to distinguish larvae of **Dendroctonus ponderosae** *from the larvae of other* **Dendroctonus** *species*

The following key is based on Thomas (1965) and is restricted to North American species.

1. Spiracular tubercles absent; dorsal surface without sclerotized plates on abdominal segments 8 or 9; frons bearing either two rounded protuberances (elevations) (Figure 22(a)) or a single rounded protuberance (Figure 22(b)) **2**

– Spiracular tubercles sclerotized; sclerotized dorsal plates present on either abdominal segments 8 or 9 or both segments (Figure 27) **or** if spiracular tubercles and abdominal plates absent, then frons smooth, without protuberance or with a transverse ridge..... **not *D. ponderosae***

2. Paired protuberances posterior on upper frons (Figure 22(a)); mandibles with dorsal tubercle near inner basal angle (Figure 23).....***D. ponderosae* and *D. jeffreyi***

– Single protuberance on frons, not paired; mandibles without tubercle ..... **not *D. ponderosae***

D. ponderosae and *D. jeffreyi* larvae are morphologically distinct from other *Dendroctonus* species but cannot be separated from each other. It is important to note that specimens which match the above morphological characters for *D. ponderosae* or which are suspect should ideally be sent to a Scolytinae specialist as identification cannot be readily confirmed based on larvae alone.

5. Records

Records and evidence should be retained as described in section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*).

In cases where other contracting parties may be affected by the results of the diagnosis, in particular in cases of non-compliance (ISPM 13 (*Guidelines for the notification of non-compliance and emergency action*)) and where *D. ponderosae* is found in an area for the first time, the following records and evidence and additional material should be kept for at least one year in a manner that ensures traceability: preserved pinned or slide-mounted specimens, and photographs of distinctive taxonomic structures.

6. Contact Points for Further Information

Further information on this protocol can be obtained from:

State Government of Victoria, Department of Economic Development, Jobs, Transport and Resources (DEDJTR), AgriBio, La Trobe University, 5 Ring Road, Bundoora, VIC 3083, Australia (Linda Semeraro; e-mail: Linda.Semeraro@ecodev.vic.gov.au).

Embrapa Florestas, Empresa Brasileira de Pesquisa Agropecuaria (EMBRAPA), Estrada da Ribeira, km 111, Colombo, PR, Brazil (Caixa Postal 319, CEP 83411-000, Brazil) (Edson Tadeu Iede; e-mail: iedeet@cnpf.embrapa.br).

Plant Protection Service, PO Box 9102, 6700 HC Wageningen, Netherlands (Brigitta Wessels-Berk; e-mail: b.f.wessels-berk@minlnv.nl).

Embrapa Florestas, Empresa Brasileira de Pesquisa Agropecuaria (EMBRAPA), Estrada da Ribeira, km 111, Colombo, PR, Brazil (Caixa Postal 319, CEP 83411-000, Brazil) (Guilherme Schnell e Schühli; e-mail: guilherme.schuhli@embrapa.br).

Canadian National Collection of Insects, Arachnids and Nematodes, Agriculture and Agri-Food Canada, K.W. Neatby Building, 960 Carling Avenue, Ottawa, Ontario K1A0C6, Canada (Hume Douglas; e-mail:hume.douglas@canada.ca).

Anses-Laboratoire de la Santé des Végétaux, station de Montpellier, CBGP campus international de Baillarguet CS 30016, FR-34988 Montferrier-sur-Lez, France (Jean-François Germain; e-mail: jean-francois.germain@anses.fr; tel.: +33 4 67 02 25 68).

School of Forest Resources and Conservation, University of Florida, Gainesville, FL 32611, United States of America (Jiri Hulcr; e-mail: hulcr@ufl.edu; tel.: +1 352 273 0299).

Forest Health Protection, United States Department of Agriculture (USDA) US Forest Service, 3CE, 201 14th St, SW, Washington, DC 20250, United States of America (Robert J. Rabaglia; e-mail: brabaglia@fs.fed.us; tel.: +1 703 605 5338).

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will in turn forward it to the Technical Panel on Diagnostic Protocols (TPDP).

7. Acknowledgements

The first draft of this protocol was written by Linda Semeraro (DEDJTR, Australia (see preceding section)), Hume Douglas (Canadian National Collection of Insects, Arachnids and Nematodes, Agriculture and Agri-Food Canada, Canada (see preceding section)), Brigitta Wessels-Berk (Plant Protection Service, the Netherlands (see preceding section)), Jean-François Germain (Anses-Laboratoire de la Santé des Végétaux, France (see preceding section)), Edson Tadeu Iede (EMBRAPA, Brazil (see preceding section)) and Norman Barr (USDA Animal and Plant Health Inspection Service, United States of America).

Mallik Malipatil (DEDJTR, Australia) provided initial advice and guidance during the preparation of the protocol.

The following experts reviewed the protocol: Mark Blacket (DEDJTR, Australia), Jiri Hulcr (University of Florida, United States of America), Christopher Lyal (Natural History Museum, United Kingdom), Dorothy Opondo (Kenya Plant Health Inspectorate Service, Kenya), Robert Rabaglia (USDA US Forest Service, United States of America), and Ramona Vaitkevica (State Plant Protection Service of Latvia, Latvia).

8. References

The present annex may refer to International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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9. Figures

Figure 1. Blue stain fungus affecting pine wood.
Photo R.F. Billings, Texas A&M Forest Service, United States of America, Bugwood.org.

Figure 2. *Dendroctonus ponderosae* galleries.
Photo USDA Forest Service - Ogden , USDA Forest Service, Bugwood.org

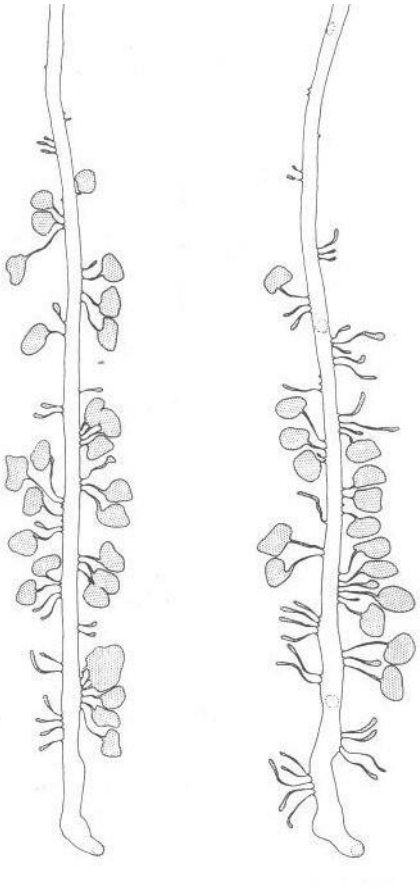

Figure 3. *Dendroctonus ponderosae*, egg and larval galleries.
Source: Wood (1982; p. 172).

Figure 4. Pitch tube, evidence of attack by *Dendroctonus ponderosae* on ponderosa pine. Photo W. Cranshaw, Colorado State University, United States of America, Bugwood.org.

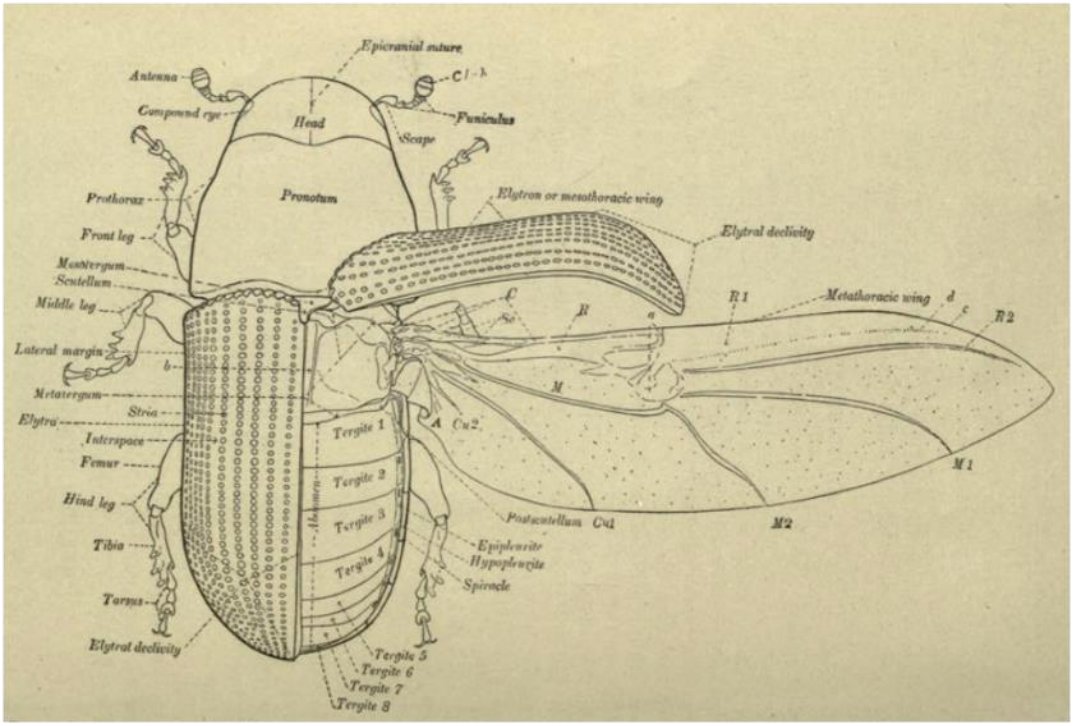

Figure 5. *Dendroctonus valens*, showing adult beetle in dorsal aspect.
Source: Hopkins (1909; p. 6).

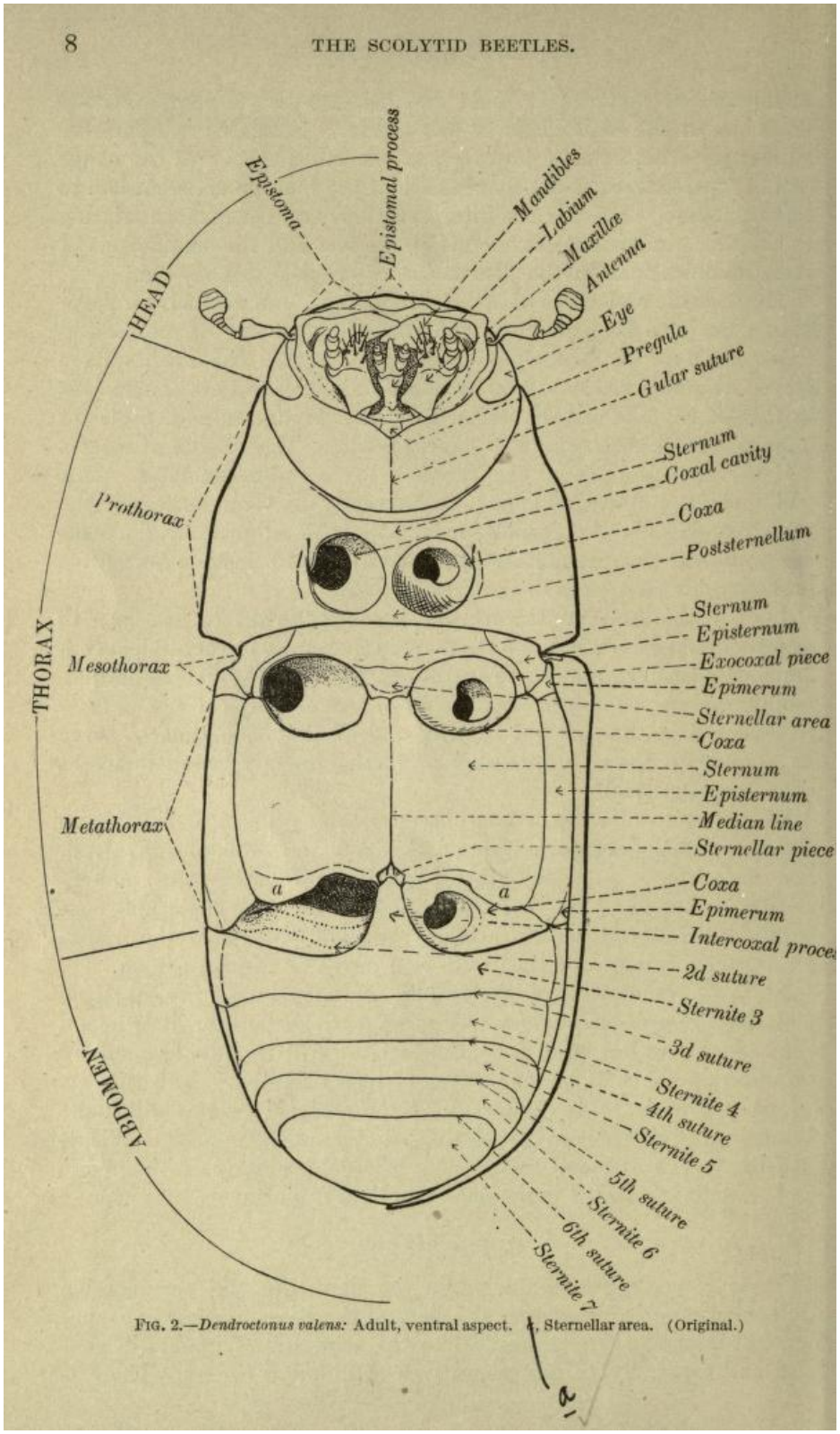

Figure 6. *Dendroctonus valens*, showing adult beetle in ventral aspect.
Source: Hopkins (1909; p. 8).

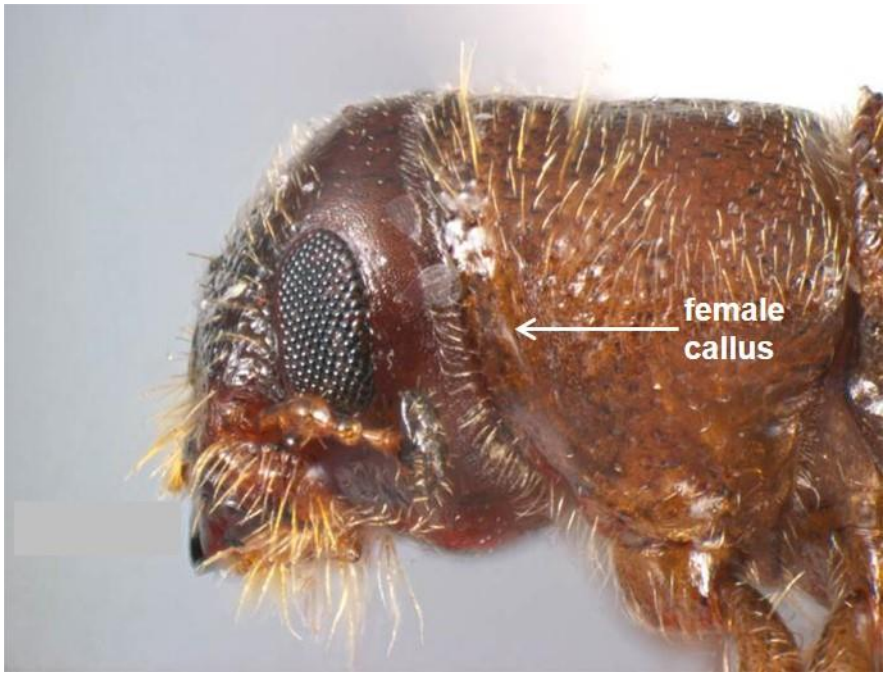

Figure 7. Dendroctonus frontalis, lateral head and pronotum.
Photo S. Hinkley and K. Walker, Museum Victoria, Australia, PaDIL.

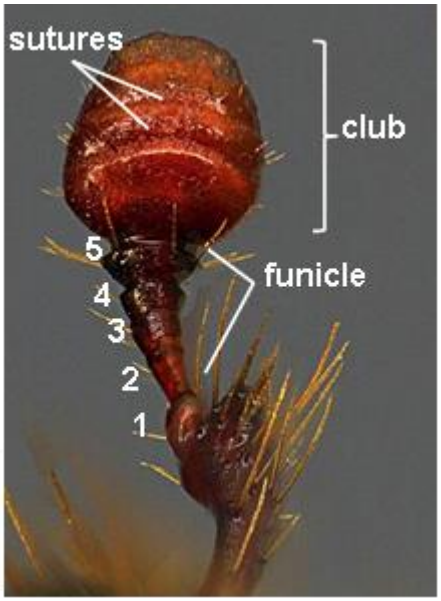

Figure 8. Scolytinae antenna
Photo courtesy of S.A. Valley, Oregon Department of Agriculture, United States of America.

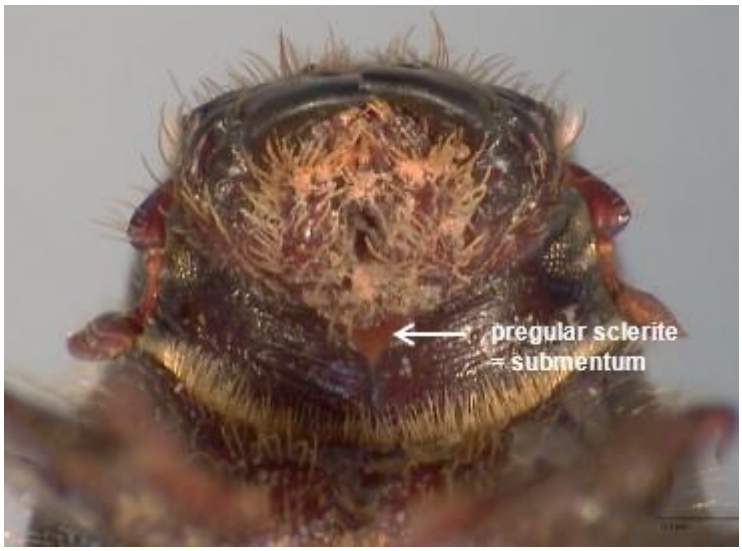

Figure 9. *Dendroctonus terebrans* ventral aspect of head showing pregular sclerite (=submentum).
Photo courtesy of L. Semeraro, Agriculture Victoria, Department of Economic Development, Jobs, Transport and Resources, Australia.

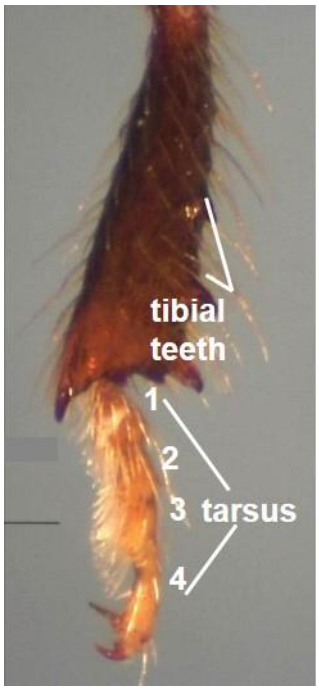

Figure 10. *Dendroctonus frontalis*, hind tibia.
Photo courtesy of L. Semeraro, Agriculture Victoria, State Government of Victoria Department of Economic Development, Jobs, Transport and Resources, Australia.

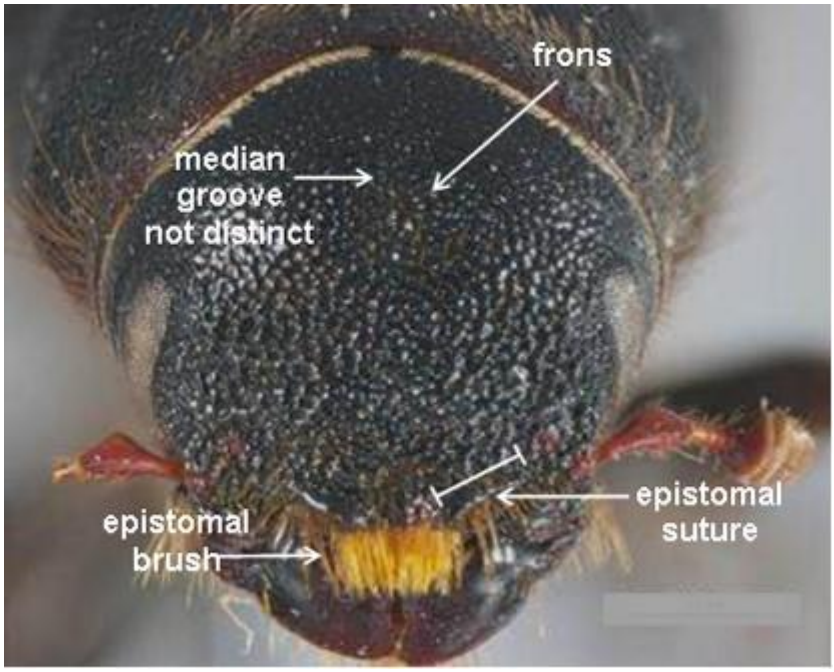

Figure 11. *Dendroctonus ponderosae,* dorsal aspect of face and frons area.
Photo S. Hinkley and K. Walker, Museum Victoria, Australia, PaDIL.

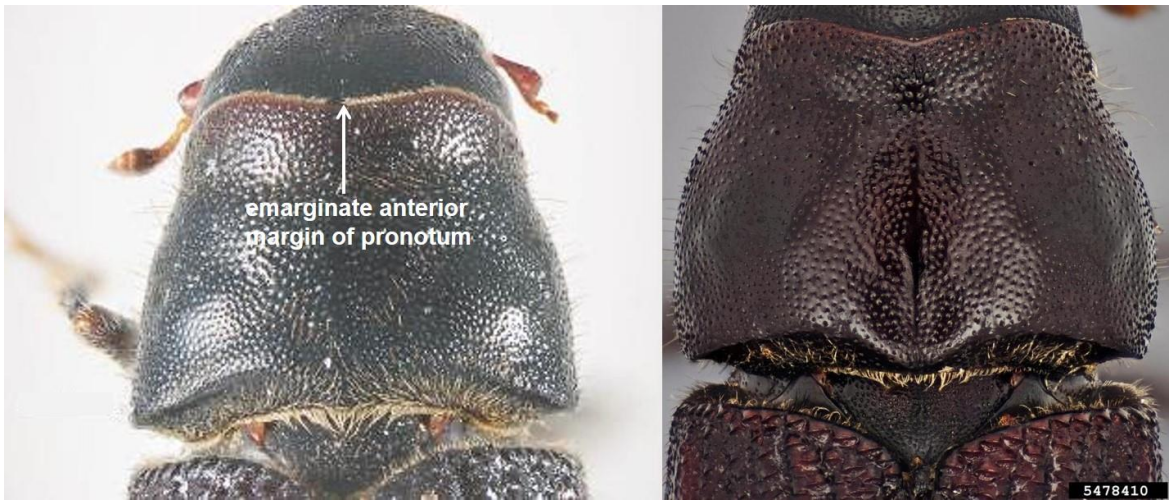

Figure 12. Dorsal aspect of head and pronotum in (a) *Dendroctonus ponderosae* (figure to the left) and (b) *Dendroctonus jeffreyi* (figure to the right).
Photos (a) S. Hinkley and K. Walker, Museum Victoria, Australia, PaDIL and (b) S.A. Valley, Oregon Department of Agriculture, United States of America, Bugwood.org.

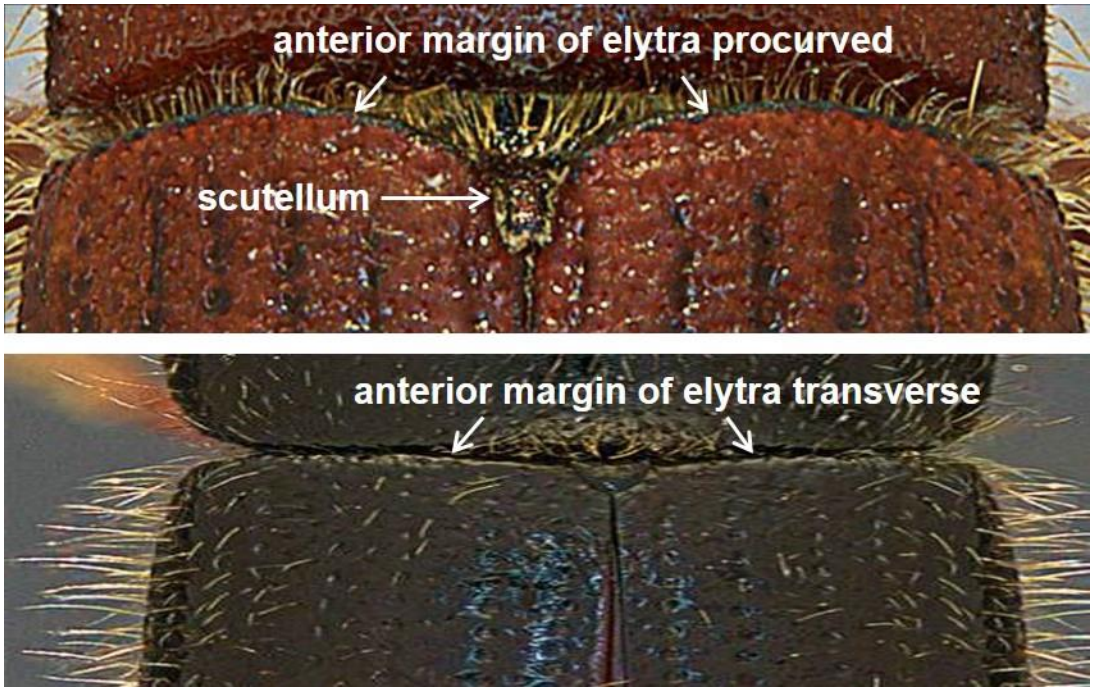

Figure 13. Shape of elytral base (not *Dendroctonus ponderosae*): (a) procurved (upper figure) and (b) transverse (down figure).
Photos courtesy of S.A. Valley, Oregon Department of Agriculture, United States of America.

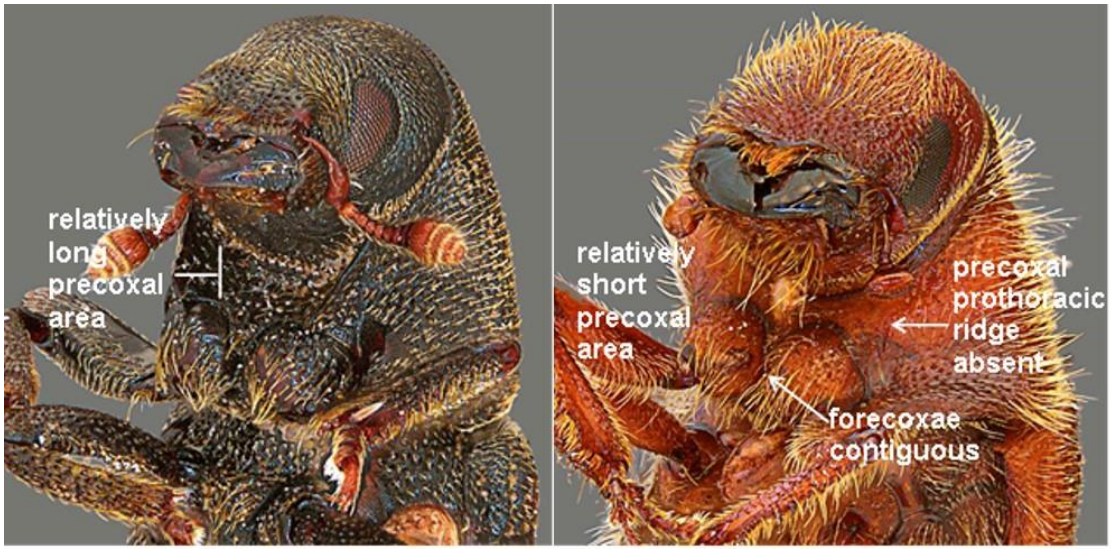

Figure 14. Anteroventral aspect (not *Dendroctonus ponderosae*): (a) presence of precoxal ridge, contiguous forecoxae (=procoxae) (figure to the left) and (b) absence of precoxal ridge, separate forecoxae (figure to the right).
Photos courtesy of S.A. Valley, Oregon Department of Agriculture, United States of America.

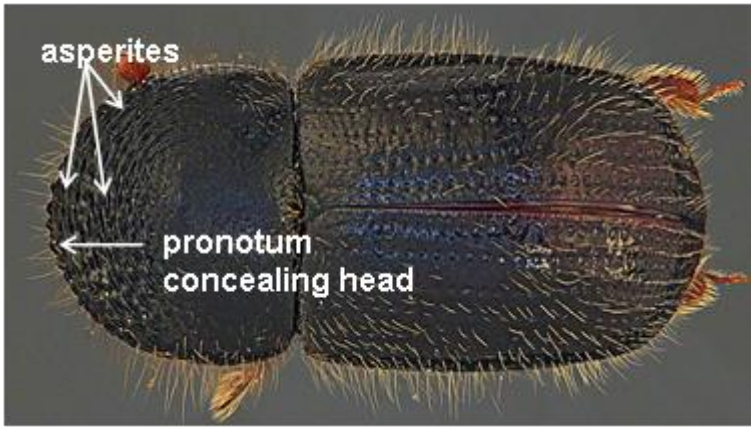

Figure 15. Scolytinae (not *Dendroctonus ponderosae*), dorsal aspect of habitus showing basic body division.
Photo courtesy of S.A. Valley, Oregon Department of Agriculture, United States of America.

Figure 16. *Dendroctonus ponderosae*, dorsal habitus aspect showing entire specimen (shape, surface sculpturing and coloration)
Photo courtesy of K. Bolte, Canadian Forest Service, Canada.

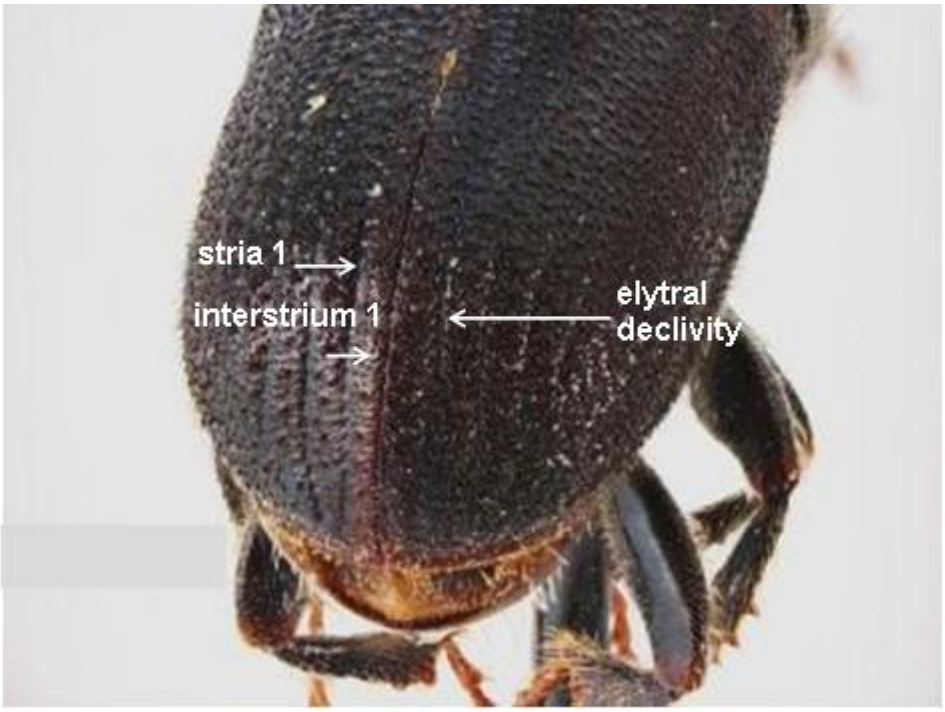

Figure 17. *Dendroctonus ponderosae*, caudal aspect showing elytral declivity.
Photo S. Hinkley and K. Walker, Museum Victoria, Australia, PaDIL.

Figure 18. *Dendroctonus jeffreyi*: (a) dorsal aspect, habitus (upper figure) and (b) lateral view (down figure). Photos S.A. Valley, Oregon Department of Agriculture, United States of America, Bugwood.org

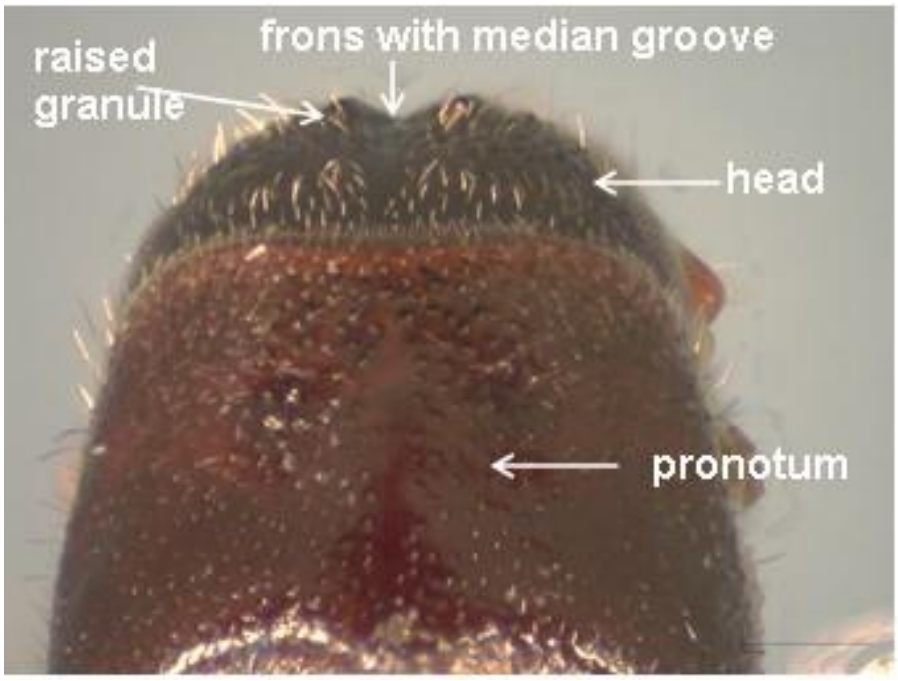

Figure 19. *Dendroctonus frontalis*, dorsal aspect of head and pronotum.
Photo S. Hinkley and K. Walker, Museum Victoria, Australia, PaDIL.

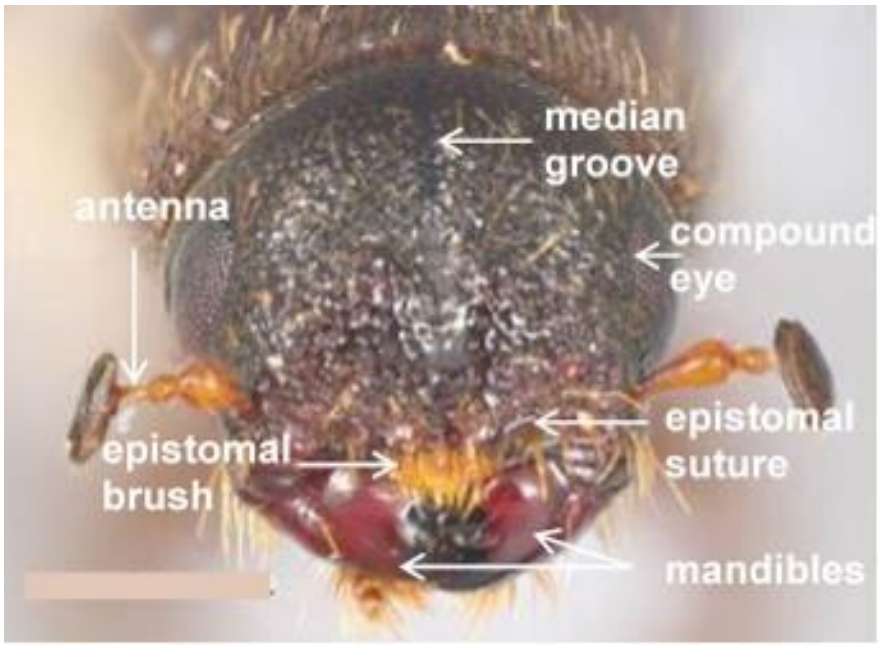

Figure 20. *Dendroctonus frontalis*, face.
Photo State Government of Victoria Department of Economic Development, Jobs, Transport and Resources, Australia.

Figure 21. *Dendroctonus terebrans* larva and pupa.
Photo G.J. Lenhard, Louisiana State University Agriculture Center, United States of America, Bugwood.org.

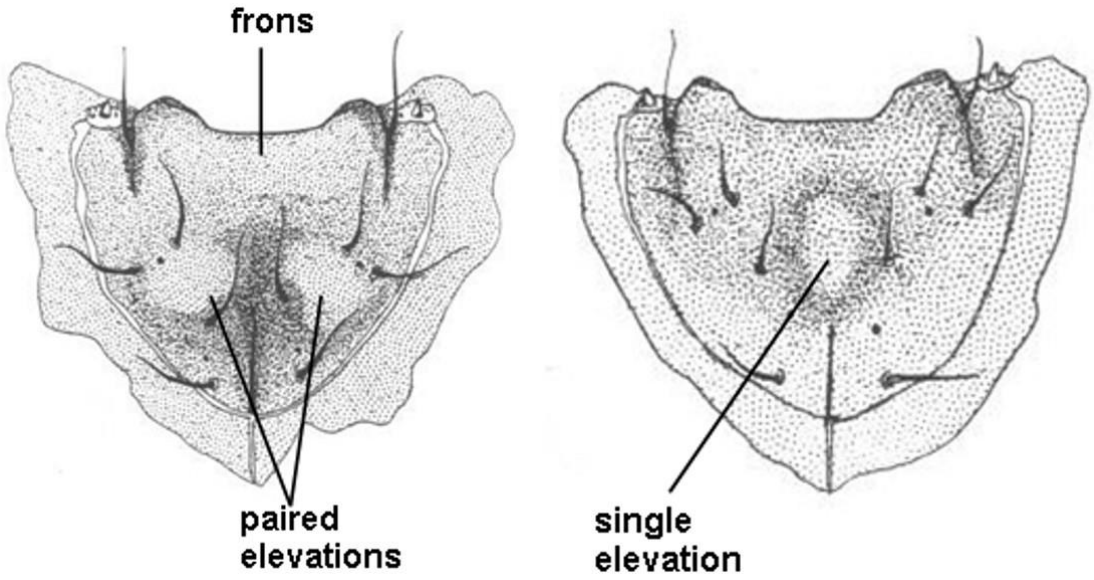

Figure 22. Dorsal aspect of larval head showing frons with (a) paired elevations in *Dendroctonus ponderosae* and (b) a single elevation in *Dendroctonus frontalis*.
Source: Thomas (1965; p. 381).

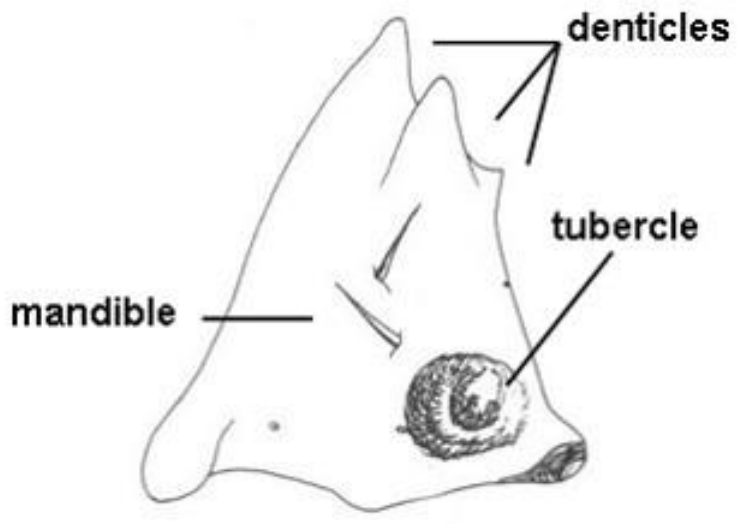

Figure 23. Larva of *Dendroctonus ponderosae*, mandible.
Source: Thomas (1965; p. 385).

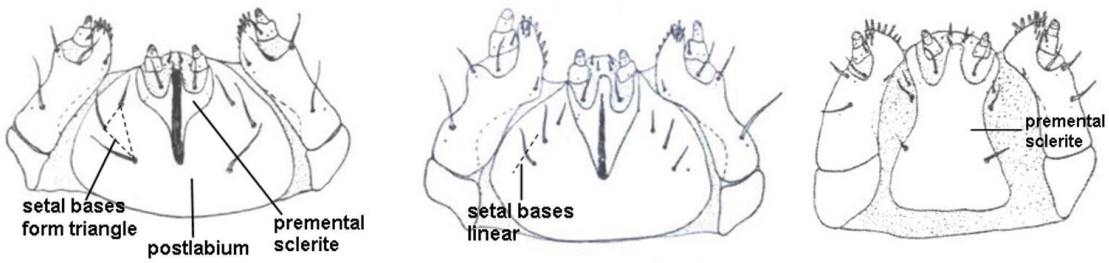

Figure 24. Scolytinae larvae, ventral aspect of head, showing (a) triangular premental sclerite and pattern of postlabial setal bases, (b) linear postlabial setal bases and (c) rectangular premental sclerite.
Source: Thomas (1965; pp. 39–40).

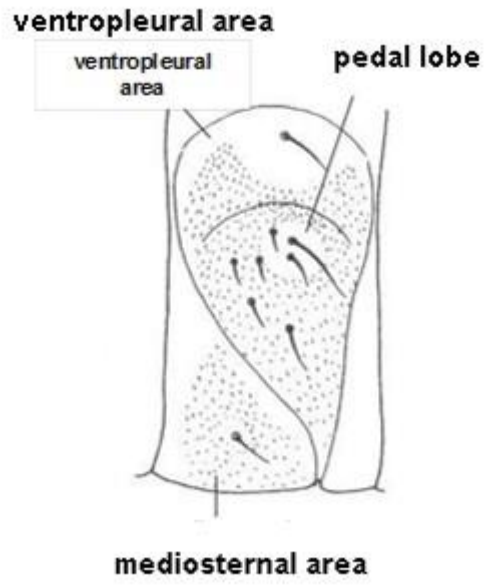

Figure 25. Scolytinae larva, pedal lobe.
Source: Zimmerman (1994; p. 672).

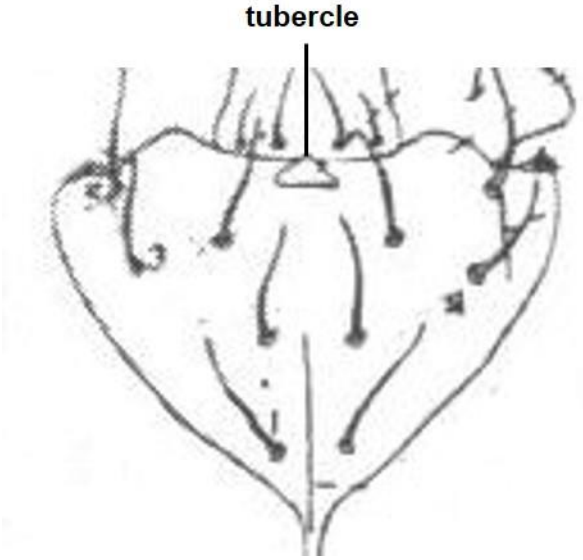

Figure 26. Larva (*Hylurgops pinifex* – not *Dendroctonus* species), frons with anterior median tubercle. Source: Modified from Thomas (1957; p. 33).

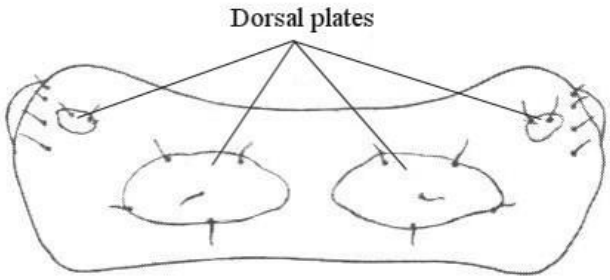

Figure 27. *Dendroctonus valens,* dorsal aspect of abdominal segments showing sclerotized plates. Source: Thomas (1957; p. 43).

Publication history

This is not an official part of the standard

2006-05 SC added subject *Dendroctonus ponderosae* syn*. Scolytus scolytus* (2006-019).

2015-03 Expert consultation on draft DPs.

2015-06 TPDP revised draft DP.

2015-11 SC noted title change from "*Dendroctonus ponderosae* syn. *Scolytus scolytus* (2006-019)" to "*Dendroctonus ponderosae* (2006-019)".

2015-12 SC approved draft DP for first consultation (2016_eSC_May_02).

2016-02 First consultation.

2016-11 TPDP recommended to SC for adoption (2016_eTPDP_Nov_03).

2016-11 SC approved draft to be submitted to the 45 day DP notification period (2017_eSC_May_04).

2017-01 SC adopted DP on behalf of CPM (no formal objections received).

Publication history last updated: 2017-03

This diagnostic protocol was adopted by the Standards Committee on behalf of the Commission on Phytosanitary Measures in January 2017.
The annex is a prescriptive part of ISPM 27.

ISPM 27
Diagnostic protocols for regulated pests

DP 21: '*Candidatus* Liberibacter solanacearum'

Adopted 2017; published 2017

CONTENTS

1. Pest Information

'*Candidatus* Liberibacter solanacearum' is a phloem-limited, Gram-negative, unculturable bacterium that is associated with several emerging diseases. '*Ca*. L. solanacearum' was first identified in 2008 from the psyllid *Bactericera cockerelli* by Hansen *et al*. (2008) and from potatoes, tomatoes and peppers by Liefting *et al*. (2008, 2009a, 2009b), and later from carrot and the carrot psyllid *Trioza apicalis* by Munyaneza *et al*. (2010). The bacterium has a rod-shaped morphology and is about 0.2 µm wide and 4 µm long (Liefting *et al*., 2009a; Secor *et al*., 2009).

Other '*Ca*. Liberibacter' species include those associated with citrus Huanglongbing (also known as citrus greening disease): '*Ca*. L. africanus', '*Ca*. L. americanus' and '*Ca*. L. asiaticus' (Nelson *et al*., 2013a). Several new '*Ca*. Liberibacter' species have recently been discovered such as '*Ca*. L. europaeus' (Raddadi *et al*., 2011), '*Ca*. L. caribbeanus' (Keremane *et al*., 2015) and the first cultured species from this bacterial clade, *Liberibacter crescens* (Fagen *et al*., 2014). It is unclear if these new '*Ca.* Liberibacter' species are associated with plant disease. The discovery of additional '*Ca*. Liberibacter' species is likely to continue with the application of new technologies such as next-generation sequencing.

In North and Central America and Oceania, '*Ca*. L. solanacearum' primarily infects solanaceous crops and weeds, including *Solanum tuberosum* (potato), *Solanum lycopersicum* (tomato), *Capsicum annuum* (pepper), *Solanum betaceum* (tamarillo), *Nicotiana tabacum* (tobacco), *Solanum melongena* (eggplant), *Physalis peruviana* (cape gooseberry), *Solanum elaeagnifolium* (silverleaf nightshade), *Solanum ptycanthum* (eastern black nightshade) and *Lycium barbarum* (wolfberry) (EPPO 2013; Haapalainen, 2014). In Europe and North Africa, '*Ca*. L. solanacearum' has been associated with symptoms in species of the family Apiaceae, including *Daucus carota* subsp. *sativus* (carrot), *Apium graveolens* (celery) and *Pastinaca sativa* (parsnip) (EPPO 2013; Teresani *et al*., 2014).

In solanaceous plants, '*Ca*. L. solanacearum' is primarily spread from infected to healthy plants by the tomato and potato psyllid *B. cockerelli* (Munyaneza *et al*., 2007; Munyaneza, 2012; EPPO, 2013). Horizontal transmission between plants from the family Apiaceae has been reported to occur by the psyllids *T. apicalis* (Nissinen *et al*., 2014) and *Bactericera trigonica* (Teresani *et al*., 2014, 2015). The bacterium is found in several organs and tissues of its psyllid host, including the alimentary canal, salivary glands, haemolymph and bacteriomes (Cooper *et al*., 2013), and is transmitted in a propagative, circulative and persistent manner (Sengoda *et al*., 2014). Vertical (transovarial) transmission of '*Ca*. L. solanacearum' has been reported in *B. cockerelli* (Hansen *et al*., 2008). '*Ca*. L. solanacearum' can also be transmitted by grafting and via dodder (Crosslin and Munyaneza, 2009; Secor *et al*., 2009; Munyaneza, 2012; Haapalainen, 2014; Munyaneza, 2015). Although transmitted through seed potato tubers, '*Ca*. L. solanacearum' transmission has not been shown through true potato seed or seed from other solanaceous plants (Munyaneza, 2012). It has been demonstrated that the bacterium can be disseminated with infected carrot seeds, although vertical transmission through seed has been reported only once (Bertolini *et al*., 2014).

Five haplotypes of '*Ca*. L. solanacearum' have so far been described (Nelson *et al*., 2011, 2013b; Teresani *et al*., 2014). Two haplotypes (A and B) are associated with diseases in potato and other solanaceous species in America and Oceania, whereas the other three haplotypes (C, D and E) are associated with carrot and celery crops in Europe and North Africa. The haplotypes were differentiated by single nucleotide polymorphisms (SNPs) in the 16S ribosomal (r)RNA gene, 16S-23S rRNA intergenic spacer (IGS) region, and 50S *rplJ* and *rplL* ribosomal protein genes.

Further information on '*Ca*. L. solanacearum', including its insect vectors, disease epidemiology, vector biology, and management, can be found in reviews by Secor *et al*. (2009), Munyaneza (2012, 2015), Nelson *et al*. (2013a) and Haapalainen (2014).

2. Taxonomic Information

Name: '*Candidatus* Liberibacter solanacearum' (Liefting *et al*., 2009b)

Synonym: '*Candidatus* Liberibacter psyllaurous' (Hansen *et al*., 2008)

Taxonomic position: Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, '*Candidatus* Liberibacter'

Common names: Zebra chip, zebra complex

3. Detection

Plants infected with '*Ca*. L. solanacearum' may be asymptomatic or exhibit symptoms that may be similar to those associated with other phloem-limited bacteria and physiological disorders. Specific tests are therefore required for the detection and identification of '*Ca*. L. solanacearum'. Because of the inability to culture '*Ca*. L. solanacearum' and the overall low titre in which this bacterium occurs in its host plants, molecular tests are required for detection and identification.

3.1 Symptoms

The above-ground plant symptoms associated with '*Ca*. L. solanacearum' infection in potato and other solanaceous species (Figures 1 to 3) resemble those associated with phytoplasmas and include stunting, erectness of new foliage, chlorosis and purpling of foliage, upward rolling of leaves, shortened and thickened terminal internodes resulting in plant rosetting, enlarged nodes, axillary branches or aerial tuber formation, leaf scorching, disruption of fruit-set, and production of numerous small, misshapen, poor quality fruit. In potato, the below-ground symptoms characteristic of '*Ca*. L. solanacearum' include collapsed stolons and browning of vascular tissue concomitant with necrotic flecking of internal tissues and streaking of the medullary ray tissues, all of which can affect the entire tuber. Freshly cut tubers, when infected, show in minutes necrotic browning in medullary ray tissue throughout the tuber (Figure 4). Upon frying, these symptoms become more pronounced and chips or fries processed from affected tubers show very dark blotches, stripes or streaks, rendering them commercially unacceptable (Figure 4). Symptoms in carrots associated with '*Ca*. L. solanacearum' infection include leaf curling, yellowish, bronze and purplish discoloration of leaves, stunting of the shoots and roots, and proliferation of secondary roots (Figure 5) (Munyaneza *et al*., 2010; Nissinen *et al*., 2014). These symptoms resemble those associated with phytoplasmas and *Spiroplasma citri* in carrots (Lee et al., 2006; Cebrián *et al*., 2010; Munyaneza *et al*., 2011). In celery, vegetative disorders associated with the pathogen include an abnormal number of shoots per plant and curled stems (Figure 6) (Teresani *et al*., 2014).

3.2 Sampling

General guidance on sampling methodologies is provided in ISPM 31 (*Methodologies for sampling of consignments*).

3.2.1 Plants

The within-plant distribution of '*Ca*. L. solanacearum' is highly variable; careful sampling is therefore required to improve the accuracy of diagnosis. Sampling protocols should consider that '*Ca*. L. solanacearum' may not be detectable by polymerase chain reaction (PCR) until three weeks after infective psyllids have fed on the plants (Levy *et al*., 2011). If typical foliar symptoms are present, three to five leaves and/or stems should be collected from symptomatic parts of the plant. In asymptomatic plants, leaves and/or stems from five to ten different parts of the plant should be sampled and should include newly developing leaves (Levy *et al*., 2011; Cooper *et al*., 2015). Below-ground plant parts such as tubers, roots and stolons can also be used to detect '*Ca*. L. solanacearum'. Potato tubers showing obvious zebra chip symptoms will result in reliable detection. Detection from asymptomatic potato tubers will be less reliable and is not recommended, even if above-ground symptoms are present, as not all tubers from an infected plant will become infected by

'*Ca*. L. solanacearum' (Buchman *et al*., 2011). The basal end (heel) of the tuber, the end which attaches to the stolon, is the recommended tissue to sample. Before extraction, all plant material is subsampled so that the material used contains as much vascular tissue as possible (e.g. petioles, leaf midribs, cambium, and the heel end or vascular ring of potato tubers).

3.2.2 Carrot seeds

Insufficient data exist to recommend a sample size and bulking rate for seed testing. The single study of seed transmission in carrot by Bertolini *et al*. (2014) detected '*Ca*. L. solanacearum' in samples of 500 carrot seeds. The International Seed Federation (ISF) recommends testing samples of 20 000 carrot seeds composed of two subsamples of 10 000 seeds.

3.2.3 Psyllids

Crosslin *et al*. (2011) determined that '*Ca*. L. solanacearum' can be reliably detected by conventional and real-time PCR in bulks of 30 laboratory-reared adult *B. cockerelli*. However, it is best to limit bulking to ten psyllids if they are sampled from the field by either sticky traps or hand collection. If the insects are collected from sticky traps, it is not necessary to remove the glue before DNA extraction. But if desired, the glue may be removed before testing as described by Bertolini *et al*. (2014) and Teresani *et al*. (2014). '*Ca*. L. solanacearum' can be reliably detected in infected psyllids for up to ten months on sticky traps stored inside at room temperature (Crosslin *et al*., 2011). For long-term storage before testing, psyllids are preserved in 70% ethanol.

3.3 Molecular detection

PCR is the method of choice for the detection of '*Ca*. L. solanacearum'. Conventional PCR can be used, but real-time PCR is recommended because of its better sensitivity.

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

3.3.1 Sample preparation

Plant material may be homogenized using one of a variety of methods. The method chosen is dependent on the nature of the plant material. Soft plant tissue can be ground using homogenizers (e.g. Bioreba HOMEX 6[1], handheld homogenizer) or bead beater machines (e.g. Roche MagNA Lyser Instrument[1], BioSpec BeadBeater[1]). Alternatively, homogenization can be carried out by hammering plant material contained in a stomacher bag with a rubber or wooden hammer. Hard plant tissue will need to be ground in a mortar with a pestle and if the tissue is very hard, the grinding will need to be aided with the addition of liquid nitrogen. Whichever grinding method is used, it is important that complete disruption of the plant vascular tissue is achieved in order to release any '*Ca*. L. solanacearum' present.

Seeds may be crushed with a pestle in a mortar, in a coffee grinder or inside a plastic bag using a hammer. The ISF protocol for carrot seed recommends bag-mixing (stomaching) rather than grinding. To remove fungicide treatments and to facilitate seed crushing, seeds are washed by shaking for 30 min in 1:10 (w/v) 0.5% Triton X-100 and, after several rinses, are left to soften in water overnight.

[1]In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

Psyllids are easily homogenized in microfuge tubes with micropestles.

3.3.2 Nucleic acid extraction

A wide range of methods are available for nucleic acid extraction. The following nucleic acid extraction kits, buffers and procedures have been used successfully for the extraction of '*Ca*. L. solanacearum' nucleic acid from plants and insects.

Samples may contain compounds that are inhibitory to PCR depending on the host species, plant tissue, age of the tissue and any treatments. It is important therefore to check the PCR competency of the DNA extractions using internal control primers that amplify a gene from the host. Inhibitory effects of the host can be overcome by further purifying the DNA through a sephacryl spin column (e.g. GE Healthcare illustra MicroSpin S-300 HR Columns[1]) or by adding bovine serum albumin to the PCR mixture at a final concentration of 0.5 mg/ml (Kreader, 1996).

3.3.2.1 CTAB extraction

DNA extraction from plant tissue is performed according to Munyaneza *et al*. (2010). In this method, 500 mg plant tissue is homogenized in 1 ml extraction buffer (100 mM Tris-HCl, pH 8.0, 50 mM ethylenediaminetetraacetic acid (EDTA), 500 mM NaCl and 10 mM 2-mercaptoethanol). The homogenate (300 µl) is mixed with 80 µl lysozyme (50 mg/ml in 10 mM Tris-HCl, pH 8.0) and incubated at 37 °C for 30 min. After incubation, 500 µl cetyl trimethylammonium bromide (CTAB) buffer (100 mM Tris, pH 8.0, 1.4 M NaCl, 50 mM EDTA, pH 8.0, 2% (w/v) CTAB, 1% (w/v) polyvinylpyrrolidone (PVP)-40 and 0.2% (v/v) 2-mercaptoethanol) is added to the homogenate and incubated at 65 °C for 30 min. The sample is allowed to cool at room temperature for 3 min before the addition of 500 µl ice-cold chloroform. Samples are mixed by vortexing and then centrifuged at 13 000 *g* for 10 min. The upper aqueous layer is transferred to a new microfuge tube, 0.6 volume of isopropanol is added and the tube is placed on ice for 20 min to precipitate the DNA. DNA is recovered by centrifugation as described above. The pellet is washed with ice-cold 75% ethanol and centrifuged at 13 000 *g* for 2 min. After removal of ethanol, the pellet is air-dried and resuspended in 100 µl sterile water.

DNA extraction from insects is described by Goodwin *et al*. (1994), where individual insects are homogenized in 125 µl CTAB extraction buffer (100 mM Tris, pH 8.0, 1.4 M NaCl, 20 mM EDTA, pH 8.0, 2% (w/v) CTAB and 1% (w/v) PVP-40). The homogenate is briefly vortexed and then incubated at 65 °C for 5 min. The suspension is extracted once with an equal volume of chloroform:isoamyl alcohol (24:1 (v/v)) and the DNA precipitated by adding 0.1 volume of 3 M sodium acetate, pH 5.2, and 2.5 volumes of ice-cold ethanol and incubating at –20 °C for at least 1 h. After centrifuging at 13 000 *g* for 15 min, the pellet is washed with 70% ice-cold ethanol, air-dried and resuspended in 15 µl sterile water.

3.3.2.2 Commercial kits

Commercial kits based on silica spin columns (e.g. Qiagen DNeasy Plant Mini Kit[1] for plants, Qiagen DNeasy Blood and Tissue Kit[1] for insects) (Li *et al*., 2009) or magnetic beads (e.g. InviMag Plant DNA Mini Kit[1]) are used according to the manufacturer's instructions. The advantage of using magnetic beads is that the extractions can be performed on an automated workstation (e.g. Thermo Scientific KingFisher Magnetic Particle Processors[1]). For plant tissue that contains high levels of polyphenolic compounds (e.g. *S. betaceum*, *S. elaeagnifolium* and *S. ptycanthum*) a modified lysis step as described by Green *et al*. (1999) is recommended. The plant material is homogenized in CTAB extraction buffer (100 mM Tris, pH 8.0, 1.4 M NaCl, 50 mM EDTA, pH 8.0, 2.5% (w/v) CTAB, 1% (w/v) PVP-40 and 0.2% (v/v) 2-mercaptoethanol added just before use). The homogenate (0.5 ml) is transferred to a microcentrifuge tube, mixed by inversion with 22 µl ribonuclease (RNase) A (20 mg/ml) and incubated at 65 °C with intermittent shaking for 25–35 min. The homogenate is then processed according to the manufacturer's instructions from the commercial kit being used.

3.3.2.3 Tissue print or squash

For high-throughput screening of plant or psyllid samples, the tissue print or squash method described by Bertolini *et al*. (2014) and Teresani *et al*. (2014) may be used instead of DNA extraction. Fresh or frozen plant material or psyllids are immobilized by spotting 5 µl crude extract onto small pieces of positively charged nylon membranes or Whatman 3MM[1] filter paper held inside microfuge tubes. Spotted extracts are left to dry for 5 min and then stored at room temperature in the dark until required. The DNA is released by adding 100 µl distilled water, vortexing and placing on ice, and 3 µl is used as the template in PCR. This method is less sensitive than testing DNA extracts; these samples can therefore be tested only by real-time PCR, and the method is not recommended when a reliable result is critical.

3.3.3 Real-time PCR

Real-time PCR is performed using the assay of Li *et al*. (2009) or Teresani *et al*. (2014). Both assays are designed to target the same region of the16S rRNA gene. The assay of Li *et al*. (2009) is based on the real-time PCR of Li *et al*. (2006) designed to detect the three citrus-infecting '*Ca.* Liberibacter' species (Huanglongbing). All liberibacter species use the same reverse primer and probe, whereas the forward primer is specific to each liberibacter species. The assay was specific as no cross-reactivity was observed with phytoplasmas, viruses, *Xylella fastidiosa*, the citrus-infecting liberibacters and 64 DNA extracts from healthy potato plants both when run as a simplex reaction and when multiplexed with internal control primers that target the *cytochrome oxidase* (COX) gene (Li *et al*., 2009). The detection limit of the real-time PCR when multiplexed with the COX internal control primers was about 20 copies of the 16S rDNA templates of '*Ca*. L. solanacearum' for field-collected potato samples, and it was about tenfold more sensitive than conventional PCR with the LsoF/OI2c primer pair (Li *et al*., 2009).

The primers and probe for the '*Ca*. L. solanacearum' real-time PCR are:

LsoF (forward primer): 5′-GTC GAG CGC TTA TTT TTA ATA GGA-3′ (Li *et al*., 2009)

HLBr (reverse primer): 5′-GCG TTA TCC CGT AGA AAA AGG TAG-3′ (Li *et al*., 2006)

HLBp (TaqMan probe): 5′-FAM-AGA CGG GTG AGT AAC GCG-BHQ-3′ (Li *et al*., 2006)

The 25 µl reaction mixture consists of a final concentration of 1× TaqMan real-time PCR master mix, 250 nM of each primer, 120 nM probe and 2 µl DNA template. Depending on the master mix used, additional $MgCl_2$ may need to be added to ensure that the final concentration is 6.0 mM. All samples are tested in duplicate. The amplification conditions are an initial denaturation step of 95 °C for 20 s followed by 40 cycles of 95 °C for 1 s and 58 °C for 40 s. Cycling conditions may vary depending on the type of master mix and machine used (e.g. some mixes require a polymerase activation step of 95 °C for 10 min, mixes that contain uracil-DNA glycosylase (UDG) require an initial hold at 50 °C for 2 min, and the cycling may require longer than 1 s at 95 °C). Real-time PCR results are analysed with the manufacturer's software.

The presence of amplifiable DNA in the plant extracts can be confirmed using the COX primers and probe of Weller *et al*. (2000):

COX-F (forward primer): 5′-CGT CGC ATT CCA GAT TAT CCA-3′

COX-R (reverse primer): 5′-CAA CTA CGG ATA TAT AAG AGC CAA AAC TG-3′

COX-P (TaqMan probe): 5′-FAM-TGC TTA CGC TGG ATG GAA TGC CCT-BHQ-3′

The 25 µl reaction mixture consists of a final concentration of 1× TaqMan real-time PCR master mix, 100 nM of each primer, 50 nM probe and 2 µl DNA template. The amplification conditions are an initial hold step at 50 °C for 2 min and an initial polymerase activation step of 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 1 min. Cycling conditions may vary depending on the type of master mix and machine used (e.g. some mixes do not require the UDG hold or polymerase activation steps described).

3.3.4 Conventional PCR

Conventional PCR is performed using the primers of Ravindran *et al*. (2011) that amplify the 16S-23S rRNA IGS region. These primers are specific to '*Ca*. L. solanacearum' and are more sensitive than the LsoF/OI2c primers (section 4.1.1). DNA extracted from a symptomatic potato plant was detected down to a dilution of 0.65 ng by Ravindran *et al*. (2011).

The primers for the '*Ca*. L. solanacearum' conventional PCR are:

Lso TX 16/23F (forward primer): 5′-AAT TTT AGC AAG TTC TAA GGG-3′

Lso TX 16/23R (reverse primer): 5′-GGT ACC TCC CAT ATC GC-3′

The 25 µl reaction mixture consists of a final concentration of 1× Taq DNA polymerase buffer containing 2 mM $MgCl_2$, 500 nM of each primer, 200 µM dNTPs, 0.5 U Taq DNA polymerase and 2 µl DNA template. The amplification conditions are an initial denaturation step of 98 °C for 30 s followed by 35 cycles of 98 °C for 10 s, 55 °C for 20 s and 72 °C for 30 s, and a final extension step of 72 °C for 7 min. Cycling conditions may vary depending on the type of master mix and machine used. The amplicon size is 383 base pairs (bp).

The presence of amplifiable DNA in the extracts can be confirmed using the general eukaryotic 28S rRNA gene primers of Werren *et al*. (1995):

28Sf (forward primer): 5′-CCC TGT TGA GCT TGA CTC TAG TCT GGC-3′

28Sr (reverse primer): 5′-AAG AGC CGA CAT CGA AGG ATC-3′

The reaction mixture for the 28S rRNA assay has the same components and is cycled under the same conditions as the '*Ca*. L. solanacearum' conventional PCR so the two assays can be run simultaneously in separate tubes. The 28Sf/28Sr primer pair produces a 500–600 bp amplicon: the size of the amplicon will vary depending on the presence of expansion domains.

3.3.5 Controls for molecular tests

For the test result obtained to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid isolation and amplification of the target pest or target nucleic acid. For PCR a positive nucleic acid control, an internal control and a negative amplification control (no template control) are the minimum controls that should be used.

Positive nucleic acid control. This control is used to monitor the efficiency of the test method (apart from the extraction), and specifically the amplification. Pre-prepared (stored) DNA extracted from an infected host or a synthetic control (e.g. cloned PCR product) may be used.

Internal control. For conventional and real-time PCR, plant internal controls such as the general eukaryotic 28S rRNA gene (Werren *et al*., 1995) or the COX gene (Weller *et al*., 2000) should be incorporated into the protocol to eliminate the possibility of PCR false negatives due to nucleic acid extraction failure or degradation or the presence of PCR inhibitors.

Negative amplification control (no template control). This control is necessary for conventional and real-time PCR to rule out false positives due to contamination during preparation of the reaction mixture. PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage.

Additional controls that could be considered for each series of nucleic acid extractions from the test samples are described below.

Negative extraction control. This control is used to monitor contamination during nucleic acid extraction and/or cross-reaction with the host tissue. The control comprises nucleic acid that is extracted from uninfected host tissue and subsequently amplified. It is recommended that multiple controls be included when large numbers of positive samples are expected.

3.3.6 Interpretation of results from PCR

3.3.6.1 Real-time PCR

The real-time PCR will be considered valid only if the following criteria are met:

- the positive control produces an exponential amplification curve with the pathogen-specific primers
- the negative extraction control and the negative amplification control do not produce an amplification curve with the pathogen-specific primers.

For the COX internal control assay, the negative extraction control (if used), positive control and each of the test samples must produce an amplification curve. Failure of the samples to produce an amplification curve with the internal control primers suggests, for example, that the DNA extraction has failed, the DNA has not been included in the reaction mixture, compounds inhibitory to PCR are present in the DNA extract or the DNA has degraded.

A sample will be considered positive if it produces an exponential amplification curve. The cycle threshold (Ct) cut-off value needs to be verified in each laboratory when implementing the test for the first time.

3.3.6.2 Conventional PCR

The conventional PCR will be considered valid only if the following criteria are met:

- the positive control produces the correct size amplicon with the pathogen-specific primers
- the negative extraction control (if used) and the negative amplification control do not produce amplicons of the correct size with the pathogen-specific primers.

For the 28S rRNA internal control assay, the negative extraction control (if used), positive control and each of the test samples must produce an amplicon of the correct size. Note that synthetic and plasmid positive controls will not produce an amplicon. Failure of the samples to amplify with the internal control primers suggests, for example, that the DNA extraction has failed, the nucleic acid has not been included in the reaction mixture, compounds inhibitory to PCR are present in the DNA extract or the DNA has degraded.

A sample will be considered positive if it produces an amplicon of the correct size.

4. Identification

The minimum identification requirement for '*Ca*. L. solanacearum' is a positive result from one of the PCR tests described in this diagnostic protocol. Both tests are specific to '*Ca*. L. solanacearum', but if the outcome is critical (e.g. post-entry quarantine sample, new host record, new distribution), the conventional PCR (section 3.3.4) should be performed and the product should be sequenced. For the sequence to be considered as the same species as '*Ca*. L. solanacearum', it should be ≥98% identical to the sequence from the reference isolate (GenBank accession number EU834130).

4.1 Haplotype identification

The known haplotype can be determined by amplifying and sequencing three genomic regions, as described in the sections below.

4.1.1 16S rRNA gene

A 1 163 bp region of the 16S rRNA gene is amplified using the same forward primer as for the real-time PCR designed by Li *et al*. (2009) to a region of the 16S rRNA gene that is unique to '*Ca*. L. solanacearum'. The forward primer is used in combination with the universal liberibacter reverse primer of Jagoueix *et al*. (1996).

The primers for the '*Ca*. L. solanacearum' conventional PCR are:

LsoF (forward primer): 5′-GTC GAG CGC TTA TTT TTA ATA GGA-3′ (Li *et al*., 2009)

OI2c (reverse primer): 5′-GCC TCG CGA CTT CGC AAC CCA T-3′ (Jagoueix *et al*., 1996)

The 25 µl reaction mixture consists of a final concentration of 1× Taq DNA polymerase buffer containing 2.5 mM $MgCl_2$, 200 nM of each primer, 200 µM dNTPs, 1 U Taq DNA polymerase and 2 µl DNA template. The amplification conditions are an initial denaturation step of 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 62 °C for 30 s and 72 °C for 1 min, and a final extension step of 72 °C for 10 min. Cycling conditions may vary depending on the type of master mix and machine used.

4.1.2 16S-23S rRNA IGS region

The 16S-23S rRNA IGS region is amplified using the Lso TX 16/23F / Lso TX 16/23R primer pair as described in section 3.3.4. These primers will fail to amplify the 16S-23S rRNA IGS region containing the last five SNP differences between haplotypes.

4.1.3 *rplJ-rplL* ribosomal protein genes

The partial 50S *rplJ* and *rplL* ribosomal protein genes are amplified using the primers of Munyaneza *et al*. (2009):

CL514F (forward primer): 5′-CTC TAA GAT TTC GGT TGG TT-3′

CL514R (reverse primer): 5′-TAT ATC TAT CGT TGC ACC AG-3′

The 25 µl reaction mixture consists of a final concentration of 1× Taq DNA polymerase buffer containing 2 mM $MgCl_2$, 400 nM of each primer, 400 µM dNTPs, 1 U Taq DNA polymerase and 2 µl DNA template. The amplification conditions are an initial denaturation step of 94 °C for 30 s followed by 40 cycles of 94 °C for 30 s, 53 °C for 30 s and 72 °C for 30 s, and a final extension step of 72 °C for 7 min. Cycling conditions may vary depending on the type of master mix and machine used. The amplicon size is 669 bp.

4.1.4 Haplotype sequence analysis

The sequence from the unknown haplotype is aligned with the reference sequences for the 16S rRNA gene and the 16S-23S rRNA IGS region (GenBank accession number EU812559) and the 50S *rplJ* and *rplL* ribosomal protein genes (GenBank accession number EU834131). The haplotype is determined by comparing the sequence at each of the nucleotide positions listed in Table 1.

Table 1. Single-nucleotide polymorphism differences between haplotypes of '*Candidatus* Liberibacter solanacearum'

Region (gene / position)	**Haplotype†**				
	A	B	C	D	E
16S rRNA / 116	C	C	C	T	C
16S rRNA / 151	A	A	A	A	G
16S rRNA / 212	T	G	T	T	T
16S rRNA / 581	T	C	T	T	T
16S rRNA / 959	C	C	C	C	T
16S rRNA / 1049	A	A	G	G	A
16S rRNA / 1073	G	G	G	A	G
16S-23S rRNA IGS / 1620	A	A	A	A	G
16S-23S rRNA IGS / 1632	A	A	A	A	G
16S-23S rRNA IGS / 1648	G	G	G	G	A

Region (gene / position)	Haplotype†				
16S-23S rRNA IGS / 1742	A	A	A	G	A
16S-23S rRNA IGS / 1748	C	C	C	T	C
16S-23S rRNA IGS / 1858	–	G	G	–	–
16S-23S rRNA IGS / 1859	–	T	–	–	–
16S-23S rRNA IGS / 1860	T	T	T	–	T
16S-23S rRNA IGS / 1873	A	A	A	A	G
16S-23S rRNA IGS / 1920	T	T	C	T	T
16S-23S rRNA IGS / 1943	G	A	G	G	Unknown
16S-23S rRNA IGS / 2055	C	T	C	C	Unknown
16S-23S rRNA IGS / 2081	G	G	G	A	Unknown
16S-23S rRNA IGS / 2218	G	A	G	G	Unknown
16S-23S rRNA IGS / 2260	C	T	C	C	Unknown
50S *rplJ* and *rplL* / 583	G	G	C	G	G
50S *rplJ* and *rplL* / 622	A	A	A	G	A
50S *rplJ* and *rplL* / 640	C	C	T	C	C
50S *rplJ* and *rplL* / 669	G	C	G	G	G
50S *rplJ* and *rplL* / 689	C	C	C	T	T
50S *rplJ* and *rplL* / 691	G	T	T	G	G
50S *rplJ* and *rplL* / 700	A	A	A	G	A
50S *rplJ* and *rplL* / 712	G	T	G	G	G
50S *rplJ* and *rplL* / 722	G	G	G	G	A
50S *rplJ* and *rplL* / 749	C	C	C	A	C
50S *rplJ* and *rplL* / 780	–	–	A	A	A
50S *rplJ* and *rplL* / 786	G	A	G	G	G
50S *rplJ* and *rplL* / 850	T	T	T	C	C
50S *rplJ* and *rplL* / 909	T	C	C	C	C
50S *rplJ* and *rplL* / 920	T	C	C	T	T
50S *rplJ* and *rplL* / 922	–	–	TGT	–	–
50S *rplJ* and *rplL* / 955	G	G	T	G	G
50S *rplJ* and *rplL* / 987	T	G	G	G	G
50S *rplJ* and *rplL* / 993	A	A	G	A	A
50S *rplJ* and *rplL* / 1041	G	A	A	G	G
50S *rplJ* and *rplL* / 1049	A	G	A	A	A
50S *rplJ* and *rplL* / 1072	C	C	C	T	C
50S *rplJ* and *rplL* / 1107	G	A	G	G	G
50S *rplJ* and *rplL* / 1110	–	–	C	–	–
50S *rplJ* and *rplL* / 1122	G	A	A	A	A
50S *rplJ* and *rplL* / 1143	G	A	G	G	G

Source: Adapted from Nelson *et al.* (2013b) and Teresani *et al*. (2014).

IGS, intergenic spacer (region); rRNA, ribosomal RNA.

† Dashes represent a deletion at that position.

5. Records

Records and evidence should be retained as described in section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*).

In cases where other contracting parties may be affected by the results of the diagnosis, in particular in cases of non-compliance (ISPM 13 (*Guidelines for the notification of non-compliance and emergency action*)) and where '*Ca*. L. solanacearum' is found in an area for the first time, the following records and evidence and additional material should be kept for at least one year in a manner that ensures traceability:

- the original sample should be kept frozen at −80 °C or freeze-dried, or dried over calcium chloride and kept at 4 °C
- if relevant, DNA extractions should be kept at −20 °C or at −80 °C, and plant extracts spotted on membranes should be kept at room temperature
- if relevant, PCR amplification products should be kept at −20 °C or at −80 °C.

6. Contact Points for Further Information

Further information on this protocol can be obtained from:

Plant Health and Environment Laboratory, Ministry for Primary Industries, PO Box 2095, Auckland 1140, New Zealand (Lia W. Liefting; e-mail: lia.liefting@mpi.govt.nz; tel.: +64 9 909 5726; fax: +64 9 909 5739).

Centro de Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias (IVIA), Carretera Moncada-Náquera km 4.5, 46113 Moncada (Valencia), Spain (María M. López; e-mail: mlopez@ivia.es; tel.: +34 963 424000; fax: +34 963 424001).

United States Department of Agriculture, Agricultural Research Service (USDA-ARS), Yakima Agricultural Research Laboratory, 5230 Konnowac Pass Road, Wapato, WA 98901, United States of America (Joseph E. Munyaneza; e-mail: joseph.munyaneza@ars.usda.gov; tel.: +1 509 454 6564; fax: +1 509 454 5646).

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will in turn forward it to the Technical Panel on Diagnostic Protocols (TPDP).

7. Acknowledgements

The first draft of this protocol was written by L.W. Liefting (Plant Health and Environment Laboratory, Ministry for Primary Industries, New Zealand (see preceding section)), M.M. López (Centro de Protección Vegetal y Biotecnología, IVIA, Spain (see preceding section)) and J.E. Munyaneza (USDA-ARS, United States of America (see preceding section)).

8. References

The present annex refers to ISPMs. ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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9. Figures

Figure 1. Early infection of '*Candidatus* Liberibacter solanacearum' in *Solanum tuberosum* (potato).
Photo courtesy J.E. Munyaneza, United States Department of Agriculture, Agricultural Research Service, Wapato, WA, United States of America.

Figure 2. '*Candidatus* Liberibacter solanacearum' infection in *Solanum lycopersicum* (tomato).
Photo courtesy L.W. Liefting, Plant Health and Environment Laboratory, Ministry for Primary Industries, Auckland, New Zealand.

Figure 3. '*Candidatus* Liberibacter solanacearum' infection in *Capsicum annuum* (pepper).
Photo courtesy J.E. Munyaneza, United States Department of Agriculture, Agricultural Research Service, Wapato, WA, United States of America.

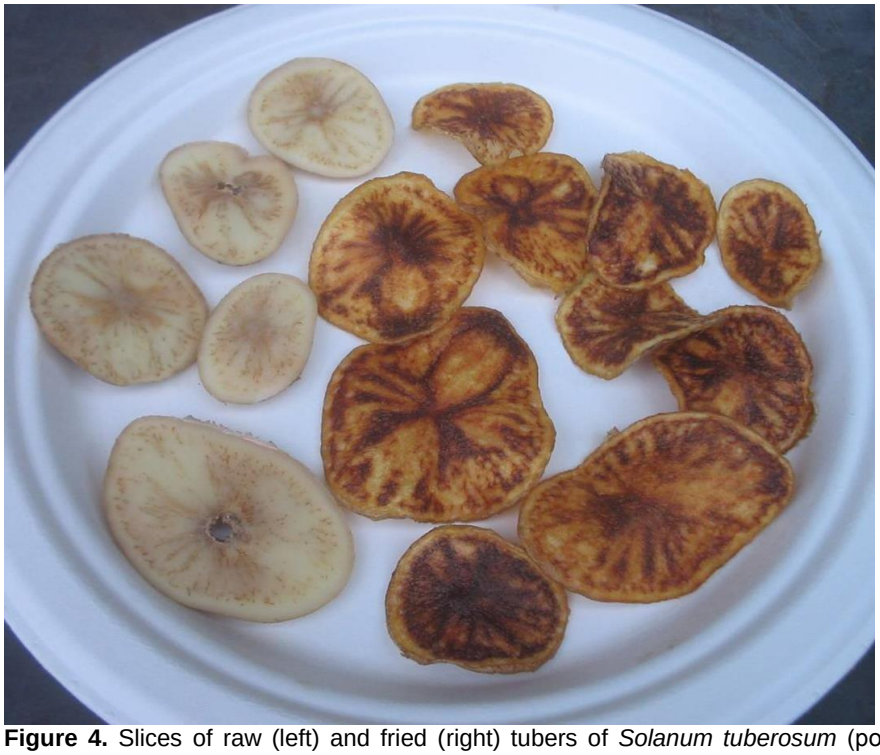

Figure 4. Slices of raw (left) and fried (right) tubers of *Solanum tuberosum* (potato) infected with '*Candidatus* Liberibacter solanacearum'.
Photo courtesy J.E. Munyaneza, United States Department of Agriculture, Agricultural Research Service, Wapato, WA, United States of America.

Figure 5. '*Candidatus* Liberibacter solanacearum' infection in *Daucus carota* subsp. *sativus* (carrot), showing leaf discoloration, leaf curling and reduced root size (left and middle), compared with uninfected control plants (right).
Photo courtesy J.E. Munyaneza, United States Department of Agriculture, Agricultural Research service, Wapato, WA, United States of America.

Figure 6. '*Candidatus* Liberibacter solanacearum' infection in *Apium graveolens* (celery), showing an abnormal number of shoots and curling of stems.
Photo courtesy M.M. López, Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain.

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ISPM 27
Diagnostic protocols for regulated pests

DP 22: *Fusarium circinatum*

Adopted 2017; published 2017

CONTENTS

1. Pest Information

Fusarium circinatum is an ascomycete fungus formerly described as the anamorph of *Gibberella circinata* (Geiser *et al.*, 2013) and it is the causal agent of pitch canker disease. The disease almost exclusively affects *Pinus* spp., but has also been described on *Pseudotsuga menziesii* (Douglas fir). *F. circinatum* has been found in asymptomatic grasses (Poaceae) near native stands of pine trees with symptoms of the disease (Swett and Gordon, 2012; Swett *et al.*, 2014). The disease affects plantations and nurseries in several countries worldwide and is a serious threat to pine forests wherever it occurs (especially on *Pinus radiata*) as it results in extensive tree mortality, reduced tree growth and reduced timber quality. *F. circinatum* causes cankers that girdle branches, aerial roots and even trunks of *Pinus* spp. Cankers are often associated with conspicuous resin exudates ("pitch"). Multiple-branch infections may cause severe crown dieback and eventually lead to the death of the tree. This fungus may also infect *Pinus* spp. seeds and may cause damping off in seedlings in nurseries. It has been found in regions of North, Central and South America, Asia and South Africa and has been officially reported in parts of southern Europe. Information on its distribution, updated regularly, is available in the European and Mediterranean Plant Protection Organization (EPPO) Global Database (https://gd.eppo.int/) and the CABI Invasive Species Compendium (http://www.cabi.org/isc/datasheet/25153).

F. circinatum is predominantly a wound pathogen that enters the host tree through mechanical wounds or the feeding holes of wood-boring insects. If a wound is not deep enough for the pathogen to reach water within host tissues, ambient moisture or very high relative humidity is required for spore germination. Conidia of *F. circinatum* germinate over a wide range of temperatures; slowly at 10 °C and progressively faster with increasing temperature, up to an optimum around 20 °C (Inman *et al.*, 2008). In nature *F. circinatum* is known to propagate only asexually, through production of microconidia and macroconidia. Both spore types are borne in a viscous liquid and appear better suited to dispersal by splashing water or attachment to motile organisms than to aerial dispersal. However, microconidia and macroconidia can become airborne and they are presumably the primary propagules recovered by air sampling in areas where pitch canker is found (Correll *et al.*, 1991). The fungus may be spread from tree to tree by aerial dispersal of the conidia or through vectors (Gordon *et al.*, 2001; Schweigkofler *et al.*, 2004). However, long-range dispersal of the pathogen from affected areas to disease-free areas may be driven by the movement of infected seeds or infected plant material (Storer *et al.*, 1998) or via vectors associated with logs and other unmanufactured wood articles (Tkacz *et al.*, 1998). Conifer seeds can be colonized by *F. circinatum* internally (where it can remain dormant until seed germination) and externally on the seed coat (Storer *et al.*, 1998). In many pine species, seed contamination may be restricted to the seed coat (Dwinell, 1999).

F. circinatum may also produce perithecia, which contain meiotically derived spores (ascospores). However, perithecia are rarely produced on culture media under laboratory conditions and they have not been observed in nature.

2. Taxonomic Information

Name: *Fusarium circinatum* Nirenberg & O'Donnell, 1998

Synonyms: *Fusarium subglutinans* f.sp. *pini* J.C. Correll, T.R. Gordon, A.H. McCain, J.W. Fox, C.S. Koehler, D.L. Wood & M.E. Schultz, 1991; *Gibberella circinata* Nirenberg & O'Donnell ex Britz, T.A. Cout., M.J. Wingf. & Marasas, 2002

Taxonomic position: Eukaryota, Fungi, Dikarya, Ascomycota, Pezizomycotina, Sordariomycetes, Hypocreomycetidae, Hypocreales, Nectriaceae

Common name: Pine pitch canker (in English only)

MycoBank: MB#444883

3. Detection

Although they may exhibit different levels of susceptibility to *F. circinatum*, all *Pinus* spp., along with *P. menziesii*, may be affected by the fungus, and the symptoms can be observed at any time of year. In addition, *F. circinatum* can affect plants of different ages, ranging from seedlings to mature trees, and it can be detected on all plant parts (roots, trunk, branches, shoots, cones and seeds). *F. circinatum* may also be soil-borne. There are no published methods for the isolation of *F. circinatum* from soil. This protocol describes the identification of *F. circinatum* on asymptomatic and symptomatic plant tissue and on seeds. The requirement for detection of *F. circinatum* is outlined in the flow chart in Figure 1. Plants and trees should be inspected for any typical symptoms of pine pitch canker (section 3.1.1) whereas seeds may be analysed by random sampling (section 3.2.2). Diagnostic method A, isolation and culture (section 3.3) and diagnostic method B, molecular tests (section 3.4), may both be used for plant tissue and seeds.

Because of the high diversity and complexity of the *Fusarium* genus, and challenges in using morphological characters to distinguish *F. circinatum* from other members of the *Fusarium fujikuroi* species complex, it is recommended that diagnosis by both method A and method B is confirmed by DNA sequence analysis of the isolated fungus, particularly if the outcome is critical (e.g. post-entry quarantine sample, new host record, new country record). PCR cross-reaction might occur with phylogenetically close *Fusarium* spp., such as the *Fusarium* species recently described from Colombia (Herron *et al.*, 2015).

3.1 Symptoms

3.1.1 Trees

Root infection. Symptoms – brown discoloration and disintegration of the cortex – are similar to symptoms caused by other root rot pathogens. Root symptoms may lead to above-ground symptoms, which are generally not apparent until the pathogen reaches the crown after it girdles the stem, causing yellowing of the foliage. Resin-soaked tissue may then be observed after removal of the bark on the lower part of the stem.

Aerial infection. Symptoms include yellowing of the needles, which turn red in time and finally drop, and dieback of the shoots. Multiple branch tip dieback, a result of repeated infections, may lead to a significant crown dieback. Cankers might appear on the shoots, on the main branches and even on the trunk, associated with conspicuous resin exudates (pitch) in response to the fungal infection (Figure 2). The cankers can girdle branches and even trunks.

Symptoms in older trees can be mistaken for those caused by *Sphaeropsis sapinea* (Fr.) Dyco & B. Sutton (synonym *Diplodia pinea*) (Sutton, 1980) or feeding damage caused by wood-boring insects. Therefore, the diagnosis should be based on laboratory testing. The resin bleeding sometimes coats the trunk and lower branches for several metres below the level of the infection. Stem cankers are flat or slightly sunken and sometimes affect large areas of cortical and subcortical tissue of the trunk. Removal of the bark reveals subcortical lesions with brown and resin-impregnated tissues (Figure 3).

Female cones. On infected branches female cones may also become affected and abort before reaching full size. However, depending on the timing and severity of infection, an infected cone may remain symptomless.

3.1.2 Seedlings and seed contamination

Seeds can be infected (Storer *et al.*, 1998). Infected seedlings usually show damping off symptoms: the needles turn red, brown or chlorotic and die from the base up, or the seedling dies (Figure 4). In some cases affected seedlings may show brown discoloration on roots and the lower part of stems. However, *F. circinatum* may infect seedlings without apparent symptoms.

It is reported in the literature that *F. circinatum* may sometimes be present in a quiescent form that cannot be detected in seeds by isolation (Storer *et al.*, 1998). Therefore, the absence of *F. circinatum*

cannot be ascertained by isolation from seeds. In contrast, non-viable propagules of *F. circinatum* may generate positive results using the molecular tests.

3.2 Sampling and sample preparation

3.2.1 Plant tissue (except seeds)

Whole seedlings should be placed in plastic bags that are then sealed and kept under cool conditions (4 °C) until they are sent to the laboratory. In the laboratory, the samples should be kept in a refrigerator at 4 °C until analysis, which should be preferably within two days of arrival.

For trunk or branch cankers, the inner bark of the area directly around the visible lesion should be cut repeatedly with a sterile blade until a canker margin is observed. Pieces of tissue, including phloem and xylem, should be removed in order to collect portions of the lesion edge, where the fungus is most active. The pieces of tissue should be wrapped in sheets of paper and placed in a plastic bag that is then sealed. All samples of plant material should be sent to the laboratory as soon as possible after sampling, and kept under cool conditions (4 °C) until transfer. In the laboratory, the samples must be kept in a refrigerator at 4 °C, to be analysed within two days of arrival.

3.2.2 Seeds

As no symptoms can be observed on seeds, the lot should be sampled randomly. As counting of seeds may be laborious, the sampled seeds may be weighed instead of counted. Depending on the method chosen for the identification, the total number of seeds to be tested per lot in order to detect the pathogen at different levels of infection in the lot may be different and needs to be determined statistically (useful guidance is given in tables 1 and 2 of ISPM 31 (*Methodologies for sampling of consignments*)). Sample size recommended by the International Seed Testing Association (ISTA) is 400 seeds for plating (ISTA, 2016). However, larger samples (e.g. 1 000 seeds) can easily be processed by biological enrichment before DNA analysis (Ioos *et al.*, 2009).

Seeds may be analysed by isolation and culture (section 3.3.2) or by conventional or real-time polymerase chain reaction (PCR) after a biological enrichment step (section 3.4.1.2). These methods have been compared in the framework of a European collaborative study, and performance values have been calculated for each of the methods (Ioos *et al.*, 2013).

3.3 Diagnostic method A: Isolation and culture

3.3.1 Plant tissue (except seeds)

For symptomatic seedlings the pathogen is isolated from the lower part of the stem or from the roots. The roots and the lower part of the stem are washed thoroughly with water and isolations are made from the leading edge of the lesions.

On mature trees, isolations are made from cankers. The cankers are washed thoroughly with water, and isolations are made from wood chips taken from the edge of the lesion found beneath the affected bark.

Plant material should be surface-sterilized for up to 1 min in a 1.5% solution of active sodium hypochlorite or 50% alcohol, and rinsed twice in sterile distilled water (Pérez-Sierra *et al.*, 2007). Selective media, such as dichloran chloramphenicol peptone agar (DCPA) or Komada's medium, are recommended for isolations. Potato dextrose agar supplemented with 0.5 mg/ml streptomycin sulphate salt (775 units/mg solid) (PDAS) can also be used (EPPO, 2005) (section 3.3.3).

Plates are incubated at 22 °C ± 6 °C under near ultraviolet (UV) light or in daylight. During incubation, the plates are observed daily and all the *Fusarium* spp. colonies are transferred to potato dextrose agar (PDA) and to Spezieller Nährstoffarmer agar (SNA) and incubated at 22 °C ± 6 °C under near UV light or in daylight for ten days.

3.3.2 Seeds

Seeds are analysed without any surface disinfection as *F. circinatum* may be present on the seed husk as well as inside the seed. Seeds are plated directly onto DCPA, Komada's medium or PDAS.

Plates are incubated at 22 °C ± 6 °C under near UV light or in daylight. During incubation, the plates are observed daily and all the *Fusarium* spp. colonies are transferred to PDA and to SNA (section 3.3.3) for morphological identification (section 4.1).

Although this method is time- and space-consuming when serial analyses are conducted, it does not require expensive equipment and it is efficient and reliable for isolating any *Fusarium* spp. from seeds. However, Storer *et al.* (1998) demonstrated that agar plating of pine seeds may not be able to detect dormant (quiescent) propagules of *F. circinatum.*

3.3.3 Culture media

Dichloran chloramphenicol peptone agar. DCPA is suitable for isolation of *Fusarium* spp. from plant tissue, including seeds, but not for identification. The medium, slightly modified by Ioos *et al.* (2004) after Andrews and Pitt (1986), contains 15.0 g bacteriological peptone, 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.2 g chloramphenicol, 2 mg 2,6-dichloro-4-nitroaniline (dichloran) (0.2% (w/v) in ethanol, 1.0 ml), 0.0005 g crystal violet (0.05% (w/v) in water, 1.0 ml) and 20.0 g technical agar made up to 1 litre with distilled water.

Komada's medium. This medium is suitable for isolation of *Fusarium* spp. from plant tissue, including seeds, but not for identification. The base medium contains 1.0 g K_2HPO_4, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 10 mg Fe-Na-ethylenediaminetetraacetic acid (EDTA), 2.0 g L-asparagine, 20.0 g D-galactose and 15.0 g technical agar, made up to 1 litre with distilled water. The pH is adjusted to 3.8 ± 0.2 with 10% phosphoric acid. The medium is autoclaved at 121 °C for 15 min and slightly cooled before adding the following filter-sterilized supplements: 1.0 g pentachloronitrobenzene (PNCB) (75% (w/w)), 0.5 g ox-gall, 1.0 g $Na_2B_4O_7 \cdot 10H_2O$ and 6 ml/litre stock solution streptomycin (5 g streptomycin in 100 ml distilled water) (Komada, 1975).

Potato dextrose agar. PDA is used to study *Fusarium* spp. colony morphology and pigmentation. The medium contains 15 g dextrose, 20 g agar and the broth from 200 g white potatoes made up to 1 litre with distilled water (Hawksworth *et al.*, 1995). Commercially available preparations of PDA are as suitable as those made in the laboratory. PDAS can be used for isolation.

Spezieller Nährstoffarmer agar. SNA should be used to study the formation and type of microconidia, macroconidia and conidiogenous cells, but it is not recommended for isolation. The medium contains 1.0 g KH_2PO_4, 1.0 g KNO_3, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose and 20.0 g technical agar made up to 1 litre with distilled water. Optionally, one or two 1 cm^2 pieces of sterile filter paper may be laid on the surface of the agar – *Fusarium* sporodochia are sometimes more likely to be produced at the edge of the paper (Gerlach and Nirenberg, 1982).

3.4 Diagnostic method B: Molecular tests

There are several molecular methods currently available for confirming the identity of *F. circinatum* isolates (identification by sequence analysis) or to detect and/or identify it directly *in planta* (conventional PCR, SYBR Green real-time PCR or real-time PCR using a hydrolysis probe). These methods are fast, efficient and reliable in detecting *F. circinatum* specifically, without agar plating, thus saving a lot of space and time, but they require facilities equipped for molecular testing. In addition, as these techniques target the DNA of the fungus, viable and non-viable cells of the pathogen are equally detected.

The real-time PCR using a hydrolysis probe offers enhanced specificity over the conventional PCR and the SYBR Green real-time PCR. Positive results obtained following real-time PCR using a hydrolysis probe are conclusive, whereas positive results obtained following conventional PCR or SYBR Green real-time PCR should be confirmed by sequence analysis.

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

3.4.1 Preparation of material

3.4.1.1 Plant tissue (except seeds)

Potentially infected plant tissues (symptomatic and asymptomatic) are picked from the sample and first cut roughly using a sterile scalpel blade, without a prior surface disinfection step. Small pieces of approximately 0.5–1.0 cm^2 should be first collected then subsequently cut into smaller pieces (<2–3 mm^2, each side) into a sterile plastic Petri dish. The amount of tissue required for each reaction is recommended in the manufacturer's instructions for the DNA extraction kit being used. The sample, corresponding to approximately 200 µl, is transferred to a 2 ml microcentrifuge tube and ground for 2 min with two 3 mm steel or tungsten carbide beads and the quantity of lysis buffer recommended by the manufacturer and provided in the DNA extraction kit, at a frequency of 30 Hz with a bead beater (TissueLyser from Qiagen[1], or equivalent). The samples may also be ground in a mortar by a pestle with liquid nitrogen, or by using other efficient grinding techniques, such as a FastPrep homogenizer (MP Biomedicals[1]).

3.4.1.2 Seeds

A preliminary biological enrichment procedure should be followed when the presence of *F. circinatum* is tested by conventional or real-time PCR carried out directly on a seed DNA extract. The purpose of this preliminary biological enrichment step is to increase the biomass of viable *F. circinatum* propagules before DNA extraction and molecular testing. At least 400 seeds per seed lot are incubated at 22 °C ± 3 °C for 72 h in a cell culture flask with Difco Potato Dextrose Broth (PDB) (Becton, Dickinson and Company[1]) (ISTA, 2016). Depending on the species of *Pinus*, the average size of the seed may vary greatly and the quantity of PDB per flask should be manually adjusted so that the seed layer is almost completely overlaid by the liquid medium. After incubation, the entire contents of the flask (seeds and PDB) are transferred aseptically to a decontaminated mixer bowl of appropriate volume and are ground with a mixer mill until a homogenous solution is obtained. Sterile water or sterile PDB may be added at this step if the ground sample is too dense for pipetting. Two subsamples of approximately 500 µl are collected and transferred aseptically to individual 2 ml microcentrifuge tubes for DNA extraction.

3.4.1.3 Fungal culture

Fungal material is harvested from a pure culture grown for seven days on PDA by scraping the aerial mycelium using a sterile scalpel blade or a sterile needle. A pellet of approximately 2–3 mm diameter may be used directly for DNA extraction. It is recommended that before extraction the fungal material is ground in a mortar by a pestle with extraction buffer, or by using other efficient grinding techniques, such as a FastPrep homogenizer (MP Biomedicals[1]).

3.4.2 Nucleic acid extraction

Total DNA from plant tissue, seeds or fungal culture should be extracted preferably following the extraction protocol described by Ioos *et al.* (2009) using a commercial plant DNA extraction kit such as the NucleoSpin Plant II kit (Macherey-Nagel[1]), which has been proved to be efficient. Total DNA is extracted following the manufacturer's instructions with slight modifications. First, the chemical lysis incubation step (with lysis buffer) is extended to 20 min. After this incubation, the sample is centrifuged

[1]In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

for 5 min at approximately 11 000 *g* to compact the debris and only the supernatant is recovered to be further processed following the manufacturer's instructions. Total DNA is finally eluted with 100 µl of the elution buffer provided in the kit and stored frozen until analysis. Total DNA or a 1:10 dilution, depending on the presence of inhibiting compounds, is used as a template for conventional or real-time PCR.

A quick DNA extraction method modified from Truett *et al.* (2000) can be used for fungal material. In this method, a small amount of aerial mycelium is disrupted in 40 µl of 25 mM NaOH, pH 12, in a 1.5 ml tube and 20 µl of the resulting solution is incubated in a 0.2 ml microcentrifuge tube for 15 min at 100 ºC and 5 min at 5 ºC in a thermocycler, then 20 µl of 40 mM Tris-HCl, pH 5, is added. The resulting lysate can be used directly as a template for PCR.

3.4.3 Detection of *Fusarium circinatum* by conventional PCR

A conventional PCR test with CIRC1A/CIRC4A primers, from the ribosomal (r)DNA intergenic spacer (IGS) region, designed by Schweigkofler *et al.* (2004), can be used for direct detection of the pathogen in plant tissue or seeds as well for identification of the fungus in pure culture. In all cases, the nature of the PCR amplicon should be verified by sequencing. Infection by other *Fusarium* spp. is frequent and cryptic speciation has been reported in the *Fusarium fujikuroi* species complex (Steenkamp *et al.*, 2002). In addition, PCR cross-reaction might occur with phylogenetically close *Fusarium* spp., especially when a large amount of *Fusarium* template DNA is used.

The primers are:

CIRC1A (forward): 5′-CTT GGC TCG AGA AGG G-3′

CIRC4A (reverse): 5′-ACC TAC CCT ACA CCT CTC ACT-3′

Using these primers and the PCR detailed in Table 1, a region of *F. circinatum*-specific IGS is amplified.

Table 1. CIRC1A/CIRC4A conventional PCR master mix composition, cycling parameters and amplicons

Reagent	Final concentration
PCR-grade water	–[†]
PCR buffer	1×
MgCl2	2 mM
dNTPs	250 µM
Primer CIRC1A	0.5 µM
Primer CIRC4A	0.5 µM
DNA polymerase	1 U
DNA (volume)	6.25 µl
Cycling parameters	
Initial denaturation[‡]	94 °C for 3 min
Number of cycles	45
Denaturation	94 °C for 35 s
Annealing	66 °C for 55 s
Elongation	72 °C for 50 s
Final elongation	72 °C for 12 min
Expected amplicons	
Size	360 bp

[†] For a final reaction volume of 25 µl.

[‡] According to the DNA polymerase manufacturer's instruction.

bp, base pairs; PCR, polymerase chain reaction.

The PCR products are separated by electrophoresis in a 1–2% agarose gel and visualized under UV light after staining.

3.4.3.1 Interpretation of results from conventional PCR

A sample will be considered positive if it produces a 360 base pair (bp) PCR product whose sequence shows 99–100% identity with a *F. circinatum* reference sequence (section 4.2), provided that the negative amplification control and negative extraction control are negative.

A sample will be considered negative if it does not produce a 360 bp PCR product, provided that the positive nucleic acid control and internal control are positive, or if it produces a 360 bp PCR product whose sequence does not show 99–100% identity with a *F. circinatum* reference sequence.

3.4.4 Detection of *Fusarium circinatum* by SYBR Green real-time PCR

A SYBR Green real-time PCR test with CIRC1A/CIRC4A primers designed by Schweigkofler *et al.* (2004) (see section 3.4.3 for their sequence) can be used for direct detection of the pathogen in plant tissue or seeds as well as for identification of the fungus in pure culture. In all cases, the nature of the PCR amplicon should be verified by sequencing for the same reasons as those presented in section 3.4.3.

Using these primers and the PCR detailed in Table 2, a region of *F. circinatum*-specific IGS is amplified.

Table 2. CIRC1A/CIRC4A SYBR Green real-time PCR master mix composition, cycling parameters and amplicons

Reagent	Final concentration
PCR-grade water	–†
PCR buffer	1×
MgCl2	2 mM
dNTPs	250 µM
Primer CIRC1A	0.5 µM
Primer CIRC4A	0.5 µM
SYBR Green	X‡
DNA polymerase	1 U
DNA (volume)	6.25 µl
Cycling parameters	
Initial denaturation*	94 °C for 3 min
Number of cycles	45
Denaturation	94 °C for 35 s
Annealing	66 °C for 55 s
Elongation	72 °C for 50 s
Expected amplicons	
Size	360 bp

† For a final reaction volume of 25 µl.

‡ Following the manufacturer's recommendation. May be directly included in a ready-to-use SYBR Green master mix.

* According to the DNA polymerase manufacturer's instruction.

bp, base pairs; PCR, polymerase chain reaction.

3.4.4.1 Interpretation of results from SYBR Green real-time PCR

The nature of the amplicons should be checked by the melting curves yielded at the end of the amplification and by comparison with the melting curves yielded with the positive nucleic acid control.

A sample will be considered positive if it produces a PCR product with a melting peak temperature identical to that of the positive nucleic acid control and whose sequence shows 99–100% identity with a *F. circinatum* reference sequence (section 4.2), provided that the amplification curve is exponential and that the negative amplification control and negative extraction control are negative.

A sample will be considered negative if it does not produce a PCR product with a melting peak temperature identical to that of the positive nucleic acid control, provided that the positive nucleic acid control and internal control are positive, or if it produces a PCR product whose sequence does not show 99–100% identity with a *F. circinatum* reference sequence.

3.4.5 Detection and identification of *Fusarium circinatum* by real-time PCR using a hydrolysis probe

Ioos *et al.* (2009) described a technique based on a real-time PCR using a hydrolysis probe designed from the rDNA IGS region to identify the anamorphic stage of *F. circinatum* in pure culture or directly in plant samples. This PCR test produces a 149 bp amplicon for *F. circinatum* (sequences of the IGS region for *F. circinatum* may be retrieved from GenBank, accession numbers AY249397 to AY249403). A *F. circinatum*-specific region of IGS is amplified using the primer pair FCIR-F/FCIR-R and is detected by a fluorescent hydrolysis probe, FCIR-P. This method has proved to be more sensitive than the conventional CIRC1A/CIRC4A PCR by detecting as little as 8 fg target DNA per reaction, and its specificity is higher (Ioos *et al.*, 2009).

The primers and probe are:

FCIR-F (forward primer): 5′-TCG ATG TGT CGT CTC TGG AC-3′

FCIR-R (reverse primer): 5′-CGA TCC TCA AAT CGA CCA AGA-3′

FCIR-P (probe): 5′-FAM-CGA GTC TGG CGG GAC TTT GTG C-BHQ1-3′

Using these primers and the PCR detailed in Table 3, a region of *F. circinatum* IGS is amplified.

Table 3. FCIR-F/-R/-P real-time PCR using a hydrolysis probe master mix composition and cycling parameters

Reagent	Final concentration
PCR-grade water	–[†]
PCR buffer	1×
MgCl2	5 mM
dNTPs	200 µM
Primer FCIR-F	0.3 µM
Primer FCIR-R	0.3 µM
Probe FCIR-P	0.1 µM
DNA polymerase	0.5 U
DNA (volume)	2 µl
Cycling parameters	
Initial denaturation[‡]	95 °C for 10 min
Number of cycles	40
Denaturation	95 °C for 15 s
Annealing-elongation[‡]	70 °C for 55 s
Expected amplicons	
Size	149 bp

[†] For a final reaction volume of 20 µl.

[‡] According to the DNA polymerase manufacturer's instruction.

3.4.5.1 Interpretation of results from real-time PCR using a hydrolysis probe

The fluorescence of the reporter dye is monitored at the end of each annealing-elongation step. The accumulation of *F. circinatum* PCR amplicons is monitored in real time by the measurement of the specific fluorescence of the reporter dye cleaved from the FCIR-P probe. A DNA template containing amplifiable *F. circinatum* DNA will yield a cycle threshold (Ct) value.

A sample will be considered positive if it produces a Ct value of <40, provided that the amplification curve is exponential and that the negative amplification control and negative extraction control are negative.

A sample will be considered negative if it produces a Ct value of ≥40, provided that the positive nucleic acid control and internal control are positive.

3.4.6 Controls for molecular tests

For the test result obtained to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid isolation and amplification of the target nucleic acid. For PCR a positive nucleic acid control, an internal control and a negative amplification control (no template control) are the minimum controls that should be used.

For the PCR tests described in this diagnostic protocol, it is also recommended that a negative extraction control be included.

Positive nucleic acid control. This control is used to monitor the efficiency of the test method (apart from the extraction). Pre-prepared (stored) genomic DNA from a reference strain of *F. circinatum* or subcloned *F. circinatum* PCR product (CIRC1A/CIRC4A for conventional PCR and SYBR Green real-time PCR; FCIR-F/FCIR-R for real-time PCR with a hydrolysis probe) may be used.

Internal control. For conventional and real-time PCR, internal controls should be incorporated into the protocol to eliminate the possibility of PCR false negatives due to nucleic acid extraction failure or degradation or the presence of PCR inhibitors.

The quality of the DNA extract should be assessed by a relevant method; for example, by spectrophotometry, by using an ad hoc internal amplification control, or by testing the extract in a PCR with universal plant or fungal primers described in the scientific literature.

For conventional PCR and SYBR Green real-time PCR, the ITS1/ITS4 primers targeting the internal transcribed spacers in fungal and plant rDNA (White *et al.*, 1990) may be used in place of the CIRC1A/CIRC4A primers, under the same PCR conditions except for an annealing temperature of 50 °C. The primers are:

ITS1 (forward): 5′-TCC GTA GGT GAA CCT GCG G-3′

ITS4 (reverse): 5′-TCC TCC GCT TAT TGA TAT GC-3′

For real-time PCR using a hydrolysis probe, the 18S uni-F/-R/-P primers and probe targeting plant 18S rDNA (Ioos *et al.*, 2009) may be used in place of the FCIR-F/-R/-P primers and probe, decreasing the annealing-elongation temperature to 65 °C and reading the fluorescence in the appropriate wavelength range for the JOE reporter dye. The primers and probe are:

18S uni-F (forward primer): 5′-GCA AGG CTG AAA CTT AAA GGA A-3′

18S uni-R (reverse primer): 5′-CCA CCA CCC ATA GAA TCA AGA-3′

18S uni-P (probe): 5′-JOE-ACG GAA GGG CAC CAC CAG GAG T-BHQ1-3′

A positive signal with ITS1/ITS4 PCR or a positive Ct value (to be determined by the diagnostic laboratory) with 18S uni-F/-R/-P real-time PCR would indicate that the DNA was successfully extracted, the level of co-extracted inhibiting compounds was sufficiently low, and the DNA was able to be amplified.

Negative amplification control (no template control). This control is necessary for conventional and real-time PCR to rule out false positives due to contamination during preparation of the reaction mixture. PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage.

Negative extraction control. This control is used to monitor contamination during nucleic acid extraction and/or cross-reaction with the host tissue. The control comprises nucleic acid that is extracted from uninfected host tissue and subsequently amplified, or alternatively PCR-grade water. It is recommended that multiple controls be included when large numbers of positive samples are expected.

4. Identification

The requirement for identification of *F. circinatum* is outlined in the flow chart in Figure 1. However, morphological characters of species in the *Fusarium fujikuroi* species complex might be very similar and PCR cross-reaction might occur with phylogenetically close *Fusarium* spp. Therefore, it is recommended that the fungus is isolated and confirmed by sequence analysis.

4.1 Identification of *Fusarium circinatum* by cultural and morphological characteristics

To study colony morphology and pigmentation the isolates are grown on PDA; plates are incubated at 22 °C ± 6 °C under near UV light or in daylight for ten days. On PDA, *F. circinatum* grows relatively rapidly (average 4.7 mm/day at 20 °C) (Nirenberg and O'Donnell, 1998). After ten days, the colony should have an entire margin and white cottony or off-white aerial mycelium with sometimes a salmon-coloured tinge in the middle and/or with a purple to dark violet or yellow pigment in the agar (Figure 5).

To study the formation and type of microconidia, macroconidia and conidiogenous cells the isolates are grown on SNA; plates are incubated at 22 °C ± 6 °C under near UV light or in daylight. Some strains form sterile hyphae only under dark conditions (Aoki *et al.*, 2001); therefore, incubation in the dark may be needed for some strains to form sterile hyphae. Isolates are examined after ten days and confirmed as *F. circinatum* based on the morphological features described by Nirenberg and O'Donnell (1998) and Britz *et al.* (2002). On SNA, microconidia are aggregated in false heads, with branched conidiophores, monophialidic and polyphialidic conidiophores, and obovoid microconidia in aerial mycelium, mostly non-septate or occasionally one-septate (Figure 6(A)). Macroconidia are typically three-septate, with walls that are slightly curved, an apical cell that narrows to an inwardly (i.e. toward the ventral side) curved tip, and a foot-shaped basal cell (Figure 6(B)). Chlamydospores are absent. The above-mentioned characters are typical of several species within the *Fusarium fujikuroi* species complex, particularly *Fusarium subglutinans*. The production of distinctive flexuous/sinuous sterile hyphae, referred to as "coiled" or "circinate" hyphae, distinguishes *F. circinatum* and some other species in the complex, including some recently described species from pine, from *F. subglutinans* (Figure 7). These sinuous hyphae should not be confused with the commonly observed truly coiled hyphae (likely perithecial initials) at the surface of the agar (Figure 8), which may be produced by several species of *Fusarium*, including *Fusarium pseudocircinatum*.

The isolate observed in pure culture can reliably and confidently be assigned to the species *F. circinatum* if all the morphological features described above are observed. Table 4 presents a comparison of *F. circinatum* with other *Fusarium* species that have similar characteristics and that *F. circinatum* may therefore be confused with. In case of doubt, or if at least one characteristic cannot be clearly observed, then a DNA sequence analysis should be conducted (section 4.2).

Table 4. Main cultural and morphological characteristics of commonly encountered *Fusarium* species on pine producing microconidia

Fusarium species	Arrangement of microconidia	Monophialide/polyphialide	Presence of sterile sinuous hyphae
F. circinatum	False head only, on short conidiophores	Monophialides and polyphialides	Yes, more or less clearly sinuous, depending on the isolate
F. subglutinans	False head only, on short conidiophores	Monophialides and polyphialides	No
F. verticillioides	Chains and false head on short conidiophores	Monophialides only	No
F. oxysporum	False head only, on very short (sometimes inconspicuous) conidiophores	Short monophialides only	No
F. solani	False head only, on long conidiophores	Monophialides only, often quite long	No
F. pseudocircinatum	False heads and short chains	Monophialides and occasionally polyphialides	Yes, but distinctively spiral-shaped and unlike those of *F. circinatum*

Source: After Leslie and Summerell (2006).

4.2 Identification of *Fusarium circinatum* by sequence analysis

Regions of the IGS rDNA, such as that amplified by the CIRC1A/CIRC4A primers (Schweigkofler *et al.*, 2004), or the region of the translation elongation factor 1-alpha (*EF-1alpha*) gene amplified by the EF1/EF2 primers (O'Donnell *et al.*, 1998), must be sequenced and used for species identification. The CIRC1A/CIRC4A PCR product may be generated from DNA extracted from a pure fungal culture or from plant tissue or seeds, whereas the EF1/EF2 PCR product may be generated only from DNA extracted from a pure fungal culture.

4.2.1 Identification of *Fusarium circinatum* in pure culture by sequence analysis

Identification of doubtful isolates in pure culture may be ascertained by analysis of the sequence of a barcode or of another relevant phylogenetic marker. In the case of *Fusarium*, several genes may be used for identification with a high level of certainty. The *EF-1alpha* sequence is sufficient to assign the identity of a *Fusarium* strain to *F. circinatum* (O'Donnell *et al.*, 1998; Geiser, 2004) but other markers may also be useful (e.g. largest RNA polymerase II B-subunit (RPB1), second largest RNA polymerase II B-subunit (RPB2), beta-tubulin, IGS) (Steenkamp *et al.*, 2002; O'Donnell *et al.*, 2010). The universal barcode ITS, while very useful for fungi in general, should not be used for the *Fusarium* genus as it is not sufficiently polymorphic for several closely related species, including *F. circinatum*. Moreover, species within the *Fusarium fujikuroi* species complex possess non-orthologous copies of the ITS2 region, which can lead to incorrect phylogenetic inferences (O'Donnell and Cigelnik, 1997). It is recommended that positive results from all PCR tests be verified by sequence analysis.

4.2.1.1 EF-1alpha *sequencing*

The primers are:

EF1 (forward): 5′-ATG GGT AAG GAR GAC AAG AC-3′

EF2 (reverse): 5′-GGA RGT ACC AGT SAT CAT GTT-3′

Using these primers and the PCR detailed in Table 5, a portion of the *EF-1alpha* gene is amplified.

Table 5. EF1/EF2 conventional PCR master mix composition, cycling parameters and amplicons

Reagent	Final concentration
PCR-grade water	–†
PCR buffer	1×
MgCl2	1.5 mM
dNTPs	250 µM
Primer EF1	0.45 µM
Primer EF2	0.45 µM
DNA polymerase	0.5 U
DNA (volume)	2 µl
Cycling parameters	
Initial denaturation‡	94 °C for 5 min
Number of cycles	45
Denaturation	95 °C for 30 s
Annealing	55 °C for 30 s
Elongation	72 °C for 60 s
Final elongation	72 °C for 6 min
Expected amplicons	
Size	Approximately 640 bp

† For a final reaction volume of 20 µl.

‡ According to the DNA polymerase manufacturer's instruction.

bp, base pairs; PCR, polymerase chain reaction.

The *EF-1alpha* PCR product is sequenced; a two-way sequencing with primers EF1 and EF2 as forward and reverse primer, respectively. The consensus sequence, from which the primers' sequences are trimmed, is compared by the Basic Local Alignment Search Tool (BLAST) with those deposited in GenBank (https://www.ncbi.nlm.nih.gov) for numerous phylogenetically close *Fusarium* spp. or with those deposited in the Fusarium-ID database (http://isolate.fusariumdb.org/), selecting the *EF-1alpha* data set. The sequence lying between EF1 and EF2 is sufficiently discriminant to identify *F. circinatum*. The level of identity with the *EF-1alpha* sequence of a reference strain of *F. circinatum* (e.g. GenBank accession number AF160295) should be between 99% and 100%.

4.2.1.2 CIRC1A/CIRC4A sequencing

The CIRC1A/CIRC4A PCR product is sequenced; a two-way sequencing with primers CIRC1A and CIRC4A as forward and reverse primer, respectively. The consensus sequence, from which the primers' sequences are trimmed, is compared by BLAST with those deposited in GenBank (https://www.ncbi.nlm.nih.gov) for numerous phylogenetically close *Fusarium* spp. The sequence lying between CIRC1A and CIRC4A is sufficiently discriminant to identify *F. circinatum*. The level of identity with the IGS sequence of a reference strain of *F. circinatum* (e.g. GenBank accession number AY249397) should be between 99% and 100%.

5. Records

Records and evidence should be retained as described in section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*).

6. Contact Points for Further Information

Further information on this protocol can be obtained from:

Grupo de Investigación en Hongos Fitopatógenos, Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, Camino de Vera s/n, 46022 Valencia, Spain (Mónica Berbegal Martínez; e-mail: mobermar@etsia.upv.es; tel.: +34 963 879 254; fax: +34 963 879 269).

Laboratoire de la Santé des Végétaux – Unité de Mycologie, Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail (ANSES), Domaine de Pixérécourt – Bât. E, CS 40009, F54220 Malzéville, France (Renaud Ioos; e-mail: renaud.ioos@anses.fr; tel.: +33 383 290 080; fax: +33 383 290 022).

Forest Research, Alice Holt Lodge, Farnham, Surrey GU10 4LH, England, United Kingdom (Ana Pérez-Sierra; e-mail: ana.perez-sierra@forestry.gsi.gov.uk; tel.: +44 0300 067 5716; fax: +44 142023653).

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will in turn forward it to the Technical Panel on Diagnostic Protocols (TPDP).

7. Acknowledgements

The first draft of this protocol was written by Mónica Berbegal Martínez (Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, Spain (see preceding section)), Renaud Ioos (Laboratoire de la Santé des Végétaux, ANSES, France (see preceding section)) and Ana Pérez-Sierra (Forest Research, United Kingdom (see preceding section)).

8. References

The present annex may refer to International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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9. Figures

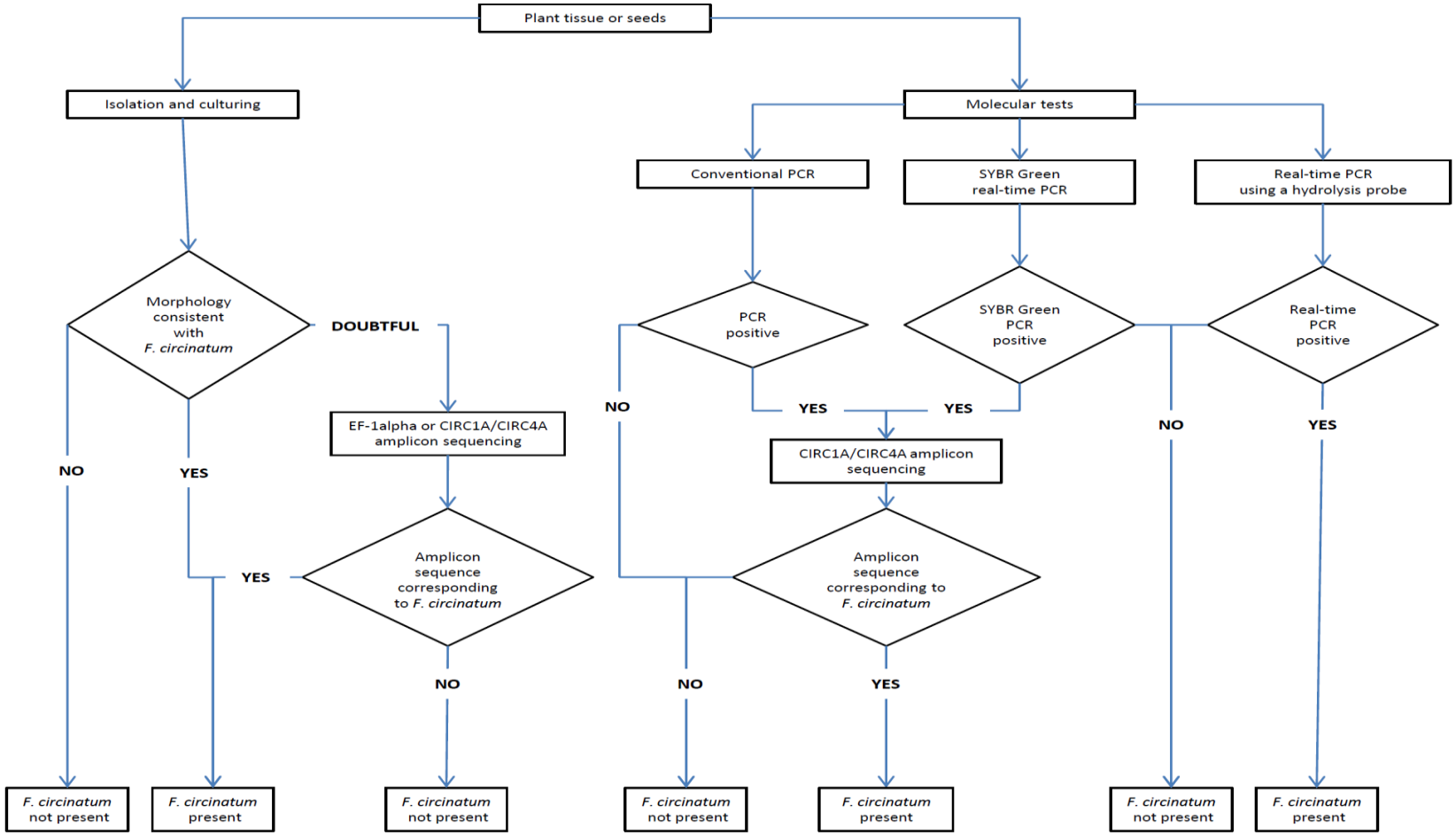

Figure 1. Flow chart for the identification of *Fusarium circinatum* in a sample of plant tissue or seeds.

* It is recommended that the fungus be isolated and confirmed by sequence analysis.

PCR, polymerase chain reaction.

Figure 2. Canker on a *Pinus radiata* trunk caused by *Fusarium circinatum* associated with conspicuous and sometimes resinous exudates.
Photo courtesy A. Pérez-Sierra, Forest Research, United Kingdom.

Figure 3. Removal of the bark on a *P. radiata* trunk shows subcortical lesions with brown and resin-impregnated tissues caused by *F. circinatum*.
Photo courtesy A. Pérez-Sierra, Forest Research, United Kingdom.

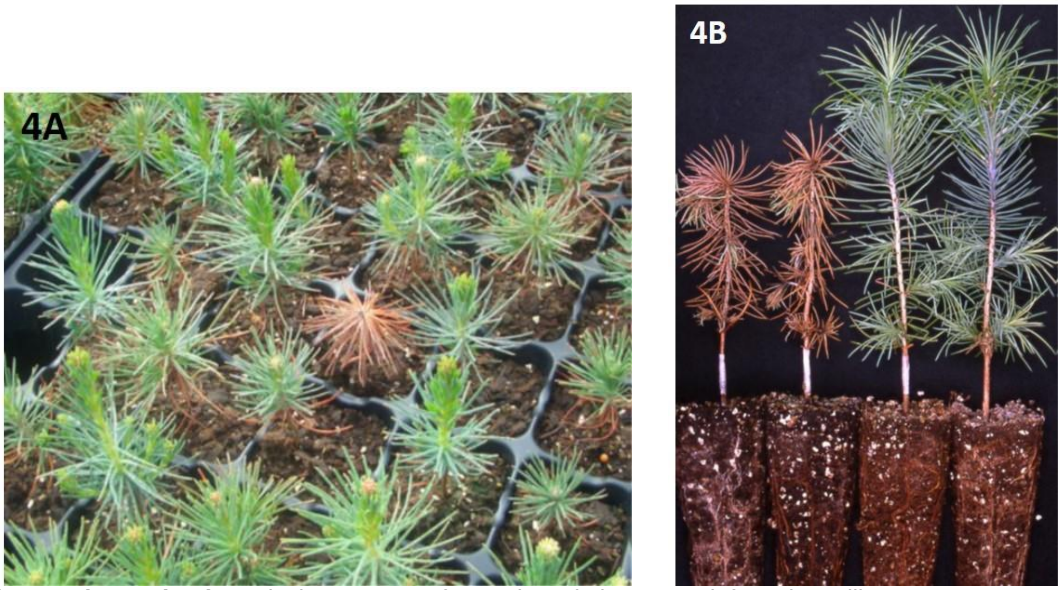

Figure 4 (4A and 4B). Typical symptoms of *Fusarium circinatum* on infected seedlings.
Photos courtesy E. Landeras, Laboratorio de Sanidad Vegetal, Oviedo, Spain.

Figure 5. Colony morphology of *Fusarium circinatum* after 14 days on potato dextrose agar, and the reversed plate. Photos courtesy M. Berbegal Martínez, Universidad Politécnica de Valencia, Spain.

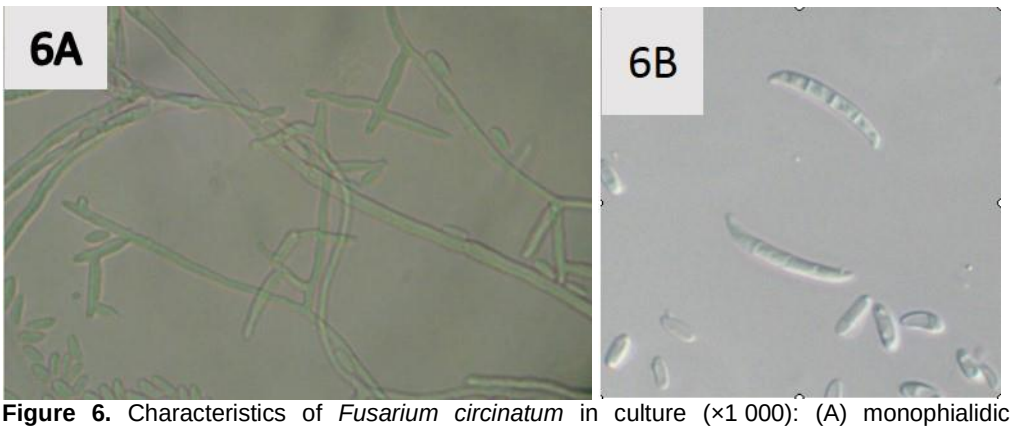

Figure 6. Characteristics of *Fusarium circinatum* in culture (×1 000): (A) monophialidic and polyphialidic conidiophores and microconidia; and (B) macroconidia and microconidia.
Photos courtesy A. Pérez-Sierra, Forest Research, United Kingdom.

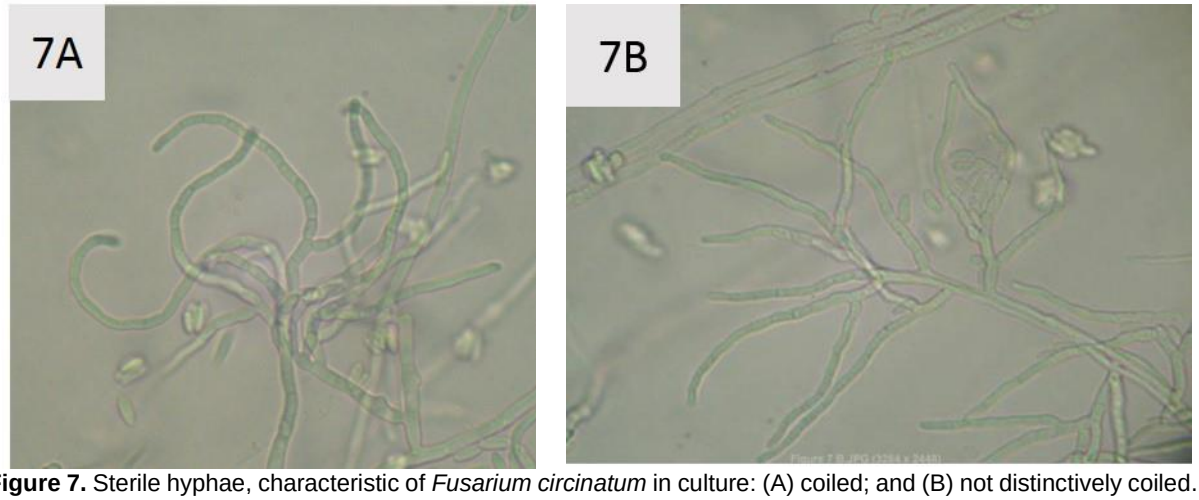

Figure 7. Sterile hyphae, characteristic of *Fusarium circinatum* in culture: (A) coiled; and (B) not distinctively coiled.
Photos courtesy A. Pérez-Sierra, Forest Research, United Kingdom.

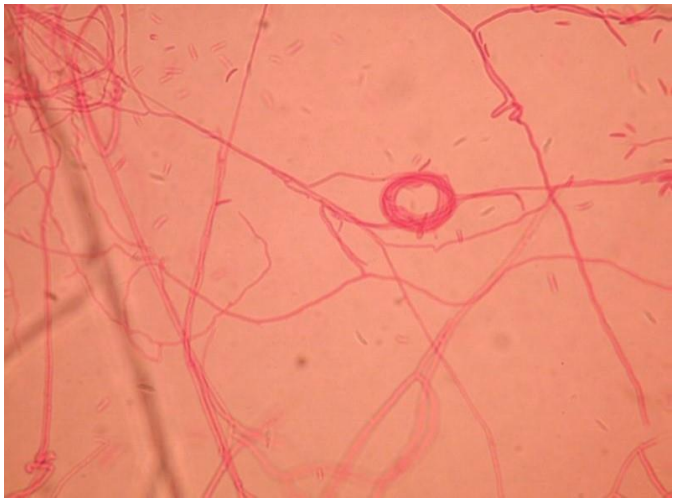

Figure 8. Commonly observed “spiral-wrapped” hyphae at the surface of agar, which may be produced by several species of *Fusarium*.
Photo courtesy R. Ioos, ANSES, Malzéville, France.

Publication history

This is not an official part of the standard

2006-05 SC added original subject: *Gibberella circinata* (syn. of *Fusarium circinatum*) (2006-021)

2015-03 Expert consultation on draft DPs

2015-06 TPDP revised draft

2015-11 SC noted title change from “*Fusarium moniliformis / moniliforme* syn. *F. circinatum*” to “*Fusarium circinatum*”

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2016-07 First consultation

2016-11 TPDP recommended draft to SC for adoption (2016_eTPDP_Nov_02)

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