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| DRAFT ANNEX to ISPM 27: POSPIVIROID SPECIES (EXCEPT POTATO SPINDLE TUBER VIROID (DP 7)) |
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This draft protocol was adapted from EPPO PM 7/138 Pospiviroid 2021 and IPPC DP7 PSTVd 2015 |
| **Main discussion points during development of the diagnostic protocol** (to be updated during development as needed)  |  |
| **Notes**  |  This is a draft document |

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1. Pest Information

Viroids are subviral agents with genomes of 239-401 nucleotides that infect plants. Viroids consist of circular, un-encapsidated single-stranded RNA molecules that do not code for any protein. A viroid replication mechanism uses RNA polymerase, a host-cell enzyme associated with synthesis of messenger RNA from DNA, which catalyses ‘rolling-circle’ synthesis of new RNA using the viroid's RNA as a template (Hammond & Owens, 2006). Viroids are unique among plant pathogens and assigned within two families, the *Avsunviroidae* and the *Pospiviroidae*. Members of the family *Pospiviroidae* replicate in the nucleus and form rod-like secondary structures with conserved structural motifs [*i.e.* the central conserved region (CCR) involved in replication, and the terminal conserved region (TCR) or the terminal conserved hairpin (TCH)] that have taxonomic relevance to assign viroid species within five genera including the genus *Pospiviroid* (Di Serio et al. 2014, Di Serio et al, 2021),

The genus Pospiviroid is comprised of nine viroids species (ICTV online): Potato spindle tuber viroid (PSTVd; type species), Chrysanthemum stunt viroid (CSVd), Citrus exocortis viroid (CEVd), Columnea latent viroid (CLVd), Iresine viroid 1 (IrVd-1), Pepper chat fruit viroid (PCFVd), Tomato apical stunt viroid (TASVd), Tomato chlorotic dwarf viroid (TCDVd), Tomato planta macho viroid (TPMVd, including the former Mexican papita viroid) and one tentative species portulaca latent viroid (PlVd; Verhoeven et al., 2015). Species demarcation is based on sequence similarity level (less than 90% sequence identity of the total viroid genome) and on distinctive biological properties in particular host range and symptoms with respect to the other members of the genus (i.e. differential host range, movement and distribution within the host, differential fitness in competition assays, seed transmission) (Owens et al., 2012, Di Serio et al., 2014). Some pospiviroids represent clusters of very similar genome sequences (>90% sequence identity e.g. PSTVd/TCDVd) but differ in host range and symptom expression (Martinez-Soriano et al., 1996; Singh et al., 1999; Matsushita et al., 2009) and are therefore accepted as distinct species. A recent publication has reported that some CLVd isolates have a sequence similarity of less than 90% within the species taxon, as well as distinct biological characteristics (symptom development and virulence), both of which are important ICTV criteria for viroid classification (Tangkanchanapas et al, 2021). On that basis the authors propose that CLVd should be re-classified into at least three main taxonomic lineages: a “CLVd-tomato Asian lineage” (I), a “CLVd-tomato European lineage” (IV) and a “CLVd-ornamental European lineage” (II), plus two minor lineages (III and V). This document will refer to CLVd as a single homogenous phylogenetic lineage. The latest information on classification of the genus Pospiviroid may be obtained from the International Committee on Taxonomy of Viruses (ICTV) (see <http://ictvonline.org>).

Pospiviroids have been reported worldwide (Faggioli et al 2017). They can cause severe diseases in their hosts, in particular PSTVd in potato and tomato crops. Therefore, pospiviroids are regulated in many countries (see EPPO Global Database <https://gd.eppo.int/taxon/1POSPG>). Pospiviroids can be experimentally transmitted to many plant species. The natural host ranges are less extended and differ between pospiviroid species (Table 1). Pospiviroids are readily transmitted by contact and cutting tools, especially at temperatures above 25°C. In addition, pospiviroids can be spread by vegetative propagation and transmission via seeds. Seed transmission has been shown for several pospiviroids such as: CEVd (Wan Chow Wah and Symons 1999, Singh and Dilworth 2009), PCFVd (Verhoeven et al., 2009), PSTVd (Matsushita and Tsuda, 2016, Fernow et al, 1970, Singh 1970), and TASVd (Antignus et al., 2007). However, lack of seed transmission has also been reported (Faggioli et al., 2015, Verhoeven et al., 2020) and a recent report (Verhoeven et al, 2021) suggests that the role of seed transmission in the spread of pospiviroids in pepper and tomato may have been overestimated. Horizontal transmission through infected pollen has been documented for CSVd, PSTVd and TPMVd (Kryczyński et al., 1988, Singh et al., 1992, Yanagisawa and Matsushita 2018). It has been reported that some pospiviroids can be transmitted by insect vectors under specific ecological conditions (PSTVd Salazar et al., 1995, TPMVd, Galindo et al., 1986, reviewed in Hadidi et al., 2022), however in some cases it cannot be excluded that cross-contamination (such as contact transmission) could have occurred. PSTVd has been reported to be transmitted by aphids when trans-encapsidated in particles of potato leafroll virus (Querci et al., 1997), with the virion acting as a carrier of the viroid RNA (Syller et al., 1997). TASVd and TCDVd have been reported to be transmitted in greenhouses by bumblebees (Bombus ignites), possibly through the transfer of viroid-contaminated pollen (Antignus et al., 2007, Matsuura et al., 2010).

This protocol on detection and identification of pospiviroids is based on EPPO PM7/138 Pospiviroids (EPPO 2021) and includes information from ISPM 27, DP7 Potato spindle tuber viroid (DP7, 2016). PSTVd is included in this pospiviroid protocol because the available diagnostic tests do not allow discrimination between different pospiviroid species. EPPO PM7/138 reports a wide range of tests that detect individual or several pospiviroid species with (where available) information on validation data. Identification of a pospiviroid isolate at species level is only possible by analysis of its nucleotide sequence (preferably full length genome). In comparison to this protocol, the specific IPPC protocol on PSTVd (DP7, 2016) provides more details on the testing of potato and tomato.

2. Taxonomic Information

**Name:** Chrysanthemum stunt viroid (acronym CSVd)

**Synonyms:** Chrysanthemum stunt mottle virus,Chrysanthemum stunt pospiviroid

**Common name:** Measles of chrysanthemum

**Taxonomic position:** *Pospiviroidae***,** *Pospiviroid***.**

**Name:** Citrus exocortis viroid (acronym CEVd)

**Synonyms:**Indian tomato bunchy top viroid, Citrus exocortis pospiviroid

**Common name:** Citrus exocortis

**Taxonomic position:** *Pospiviroidae***,** *Pospiviroid***.**

**Name:** Columnea latent viroid (acronym CLVd)

**Synonyms:** Columnea latent pospiviroid

**Common name:** none

**Taxonomic position:** *Pospiviroidae***,** *Pospiviroid***.**

**Name:** *Iresine viroid 1* (acronym IrVd-1)

**Synonyms:** Iresine pospiviroid, Iresine viroid

**Common name:** none

**Taxonomic position:** *Pospiviroidae***,** *Pospiviroid***.**

**Name** Pepper chat fruit viroid (acronym PCFVd)

**Synonyms:** Pepper chat fruit pospiviroid.

**Common name:** none

**Taxonomic position:** *Pospiviroidae***,** *Pospiviroid***.**

**Name:** Potato spindle tuber viroid (acronym PSTVd)

**Synonyms:** Potato spindle tuber pospiviroid, potato spindle tuber virus, potato gothic virus, tomato bunchy top virus

**Common name:** spindle tuber of potato

**Taxonomic position:** *Pospiviroidae***,** *Pospiviroid***.**

**Name**: Tomato apical stunt viroid (acronym TASVd)

**Synonyms**: Tomato apical stunt pospiviroid

**Common name:** none

**Taxonomic position:** *Pospiviroidae***,** *Pospiviroid***.**

**Name**: Tomato chlorotic dwarf viroid (acronym TCDVd)

**Synonyms**: Tomato chlorotic dwarf pospiviroid

**Common name:** none

**Taxonomic position:** *Pospiviroidae***,** *Pospiviroid***.**

**Name**: Tomato planta macho viroid (acronym TPMVd)

**Synonyms**: Tomato planta macho pospiviroid

**Common name:** Tomato planta macho

**Taxonomic position:** *Pospiviroidae***,** *Pospiviroid*

3. Detection

Symptoms of pospiviroid infections are not specific for the viroid species, *i.e.* variation in symptoms within a species is similar to variation between species and may even be asymptomatic in many hosts. Therefore, tests are required for detection and identification of these viroids. Detection of pospiviroids can be achieved by using the molecular tests shown in Figure 1 and related sections.

Additional information on pospiviroid detection and identification can be found in EPPO PM 7/138 (2021) and for PSTVd detection and identification in DP7 (2016).

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*Note: If a sample is suspected of a viroid infection (i.e. typical symptoms are present) but a test gives a negative result, another test should be carried out for confirmation of the result.*

**Figure 1.** Decision scheme for testing plant samples for pospiviroids. The following sections provide an overview of tests that can either be used for detection, confirmation of detection and/or identification of pospiviroids. Validation data for the recommended tests are presented in Appendix 1 (see EPPO PM 7/138, 2021)

3.1. Host range and symptoms

Pospiviroids are generally distributed over most plant tissue including seed. Their propensity to develop symptoms largely depends on the viroid species, isolates, host species, cultivar and environmental conditions. Infected ornamental species are often symptomless. Although pospiviroids are mainly found in solanaceous species, some have also been reported infecting other plant species (see Table 1) (EPPO PM7/138 2021, EFSA 2011).

**Table 1.** Pospiviroid species natural host range (EPPO PM 7/138, 2021)

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| **Name & Acronym**  | **Host range** |
| *Chrysanthemum stunt viroid* (CSVd) | *Argyranthemum frutescens, Dendranthema x grandiflorum, Gerbera* spp*., Petunia* spp*., Solanum* spp*.,Verbena* spp*.* |
| *Citrus exocortis viroid* (CEVd) | *Cestrum* spp., *Citrus* spp., *Impatiens* spp., *Solanum* spp*., Verbena* spp*.* |
| *Columnea latent viroid* (CLVd) | *Brunfelsia* spp.*, Columnea* spp., *Gloxinia* spp., *Nematanthus wettsteinii*, *Solanum* spp. |
| *Iresine viroid 1* (IrVd-1) | *Alternanthera* spp., *Celosia* spp., *Iresine* spp., *Portulaca* spp.,. *Verbena* spp*., Vinca major* |
| *Pepper chat fruit viroid* (PCFVd) | *Capsicum* spp., *Solanum* spp. |
| *Potato spindle tuber viroid* (PSTVd) | *Capsicum* spp., *Dahlia* spp., *Datura* spp., *Ipomea* spp., *Nicandra* spp.*, Nicotiana* spp., *Persea* spp., *Petunia* spp., *Physalis* spp., *Solanum* spp. |
| *Tomato apical stunt viroid* (TASVd) | *Brugmansia* spp., *Capsicum annuum (seed), Cestrum* spp., *Solanum* spp., *Streptosolen jamesonii* |
| *Tomato chlorotic dwarf viroid* (TCDVd) | *Brugmansia* spp.*, Petunia* spp., *Pittosporum* spp., *Solanum* spp., *Verbena* spp., *Vinca* spp. |
| *Tomato planta macho viroid* (TPMVd) | *Solanum lycopersicum* |

On their main hosts, the following symptoms have been observed (see also EPPO PM 7/138, 2021 for additional information, photos of symptoms and EPPO Global Database <https://gd.eppo.int/photos/>):

*Capsicum annuum.*In pepper natural infections have been recorded for only two pospiviroids i.e., PCFVd and PSTVd. In the case of PCFVd infection, plant growth is slightly reduced, leaves appear pale and fruit size is reduced by up to 50%. In some instances, vein necrosis has been observed (Verhoeven et al., 2009, 2011b). Symptoms of PSTVd in pepper plants were very mild, showing only a wavy margin on the leaves near the top of the plant (Lebas et al., 2005); symptomless infections also occur. In addition to PCFVd and PSTVd, TASVd was detected in an old pepper seed lot (Verhoeven et al., 2017), suggesting that pepper is a natural host of TASVd as well (Verhoeven et al., 2017).

*Chrysanthemum* spp. The main symptom of CSVd in chrysanthemum is stunting (Hollings & Stone, 1973; Diener & Lawson, 1973). Stems might become brittle, readily breaking at the branch point. Other common symptoms are reduced flower size and premature flowering. In certain cultivars, especially red-pigmented ones, symptoms can include flower break or bleaching. Foliar symptoms are less common, and the presence of pale, upright young leaves is often the only indication of infection. Sometimes leaf spots or flecks are observed, that might be associated with leaf distortions (crinkling). However, many chrysanthemum cultivars are symptomless. When symptoms are seen, they are often variable and dependent on environmental conditions, especially temperature and light.

*Citrus spp.* CEVd may cause bark scaling, shelling (exocortis) and splitting of the bark of citrus trees, stunting resulting in significant yield reduction (Semancik & Weathers, 1972a, b; EFSA. 2008, Lin et al, 2015). In Etrog citron, CEVd induces a variety of symptoms ranging from severe stunting, leaf epinasty and rugosity, petiole wrinkle and necrosis, midvein necrosis, and browning of the tip of the leaf blade. that became more pronounced over time. CEVd synergistic effects with other citrus viroids have resulted in enhanced bark scaling or reduced tree growth and yield (reviewed in Zhou et al. 2020)

*Solanum lycopersicum.* In the early stages of pospiviroid infection, a growth reduction and chlorosis in the upper leaves and reduced fruit size are generally observed (Verhoeven et al., 2004). In addition, other types of symptoms such as rugosity and irregular ripening might occur. Growth reduction may develop into stunting and bunchy growth, and the chlorosis may become more severe, turning into reddening, purpling and/or necrosis. At this stage, leaves may become deformed and brittle. As stunting begins, flower and fruit initiation stop. Generally, this stunting is permanent; occasionally, plants may either die or partially recover (EFSA, 2011). Isolates from different tomato-infecting pospiviroids may cause a diversity of symptoms irrespective of the species.

*Solanum tuberosum.* Until recently, PSTVd was the only viroid known to naturally infect cultivated species of potato, however CSVd has been reported in in different potato cultivars and *Solanum nigrum* in different areas; suggesting that CSVd could infect potato naturally (Matsushita et al., 2019, Matsushita et al 2021). PSTVd may cause severe to mild symptoms and also symptomless infections, depending on PSTVd isolate, cultivar and environmental conditions. Severe symptoms might include reduction in plant size, uprightness and clockwise phyllotaxy of the foliage if viewed from above, and dark green and rugose leaves (Pfannenstiel & Slack, 1980). Tubers may be reduced in size, deformed, spindle or dumbbell-shaped, with conspicuous prominent eyes. Under experimental conditions, all pospiviroids (except IrVd-1) could cause tuber symptoms similar to PSTVd (Verhoeven et al., 2004, 2010b).

In relation to *S. tuberosum* it might be relevant to note that PSTVd has been detected in commercial seed lots of *Solanum sisymbriifolium* (Fowkes et al., 2021). *S. sisymbriifolium* is used as a trap crop for the management of potato cyst nematodes (*Globodera pallida* and *Globodera rostochiensis*) in rotation with potato crops. Further studies are needed to investigate the role of *S. sisymbriifolium* as a host of PSTVd and the risk of seed transmission and transmission via roots.

In this diagnostic protocol, tests (including reference to brand names) are described as published, as these defined the original level of analytical sensitivity, analytical specificity (inclusivity as well as exclusivity), selectivity, repeatability and/or reproducibility achieved. Tests can be adapted to local conditions, e.g. using other critical reagents and/or instruments, provided that they are validated for the intended use. Guidelines on test validation for plant pest diagnostics are provided by EPPO PM 7/98 (2021).

3.2 Biological detection

Pospiviroids can be experimentally transmitted to many test (indicator) plants, mostly solanaceous species but also citrus and species from other plant families, depending on the viroid species. Symptom expression has been found to range from very severe (lethal in some cases) to mild and symptomless. In the case of symptomless infections, it is obvious that a bioassay is not suitable for detection. Moreover, the limited host range of some pospiviroids make bioassay a less reliable diagnostic method for pospiviroids. In addition, symptoms induced are not species specific. All pospiviroids (except IrVd-1) could be transmitted to potato and tomato and elicit similar symptoms under controlled conditions (Verhoeven et al., 2004; EFSA, 2011). IrVd-1 is likely not to be detected by bioassay, since no symptoms have been observed in their (limited number of) ornamental hosts, Mechanical inoculation of test plants can be used for propagation and maintenance of isolates and/or production of infected material for further testing and identification. There are no validation data published on the use of bioassay for detection of pospiviroids.

Mechanical inoculation is usually performed using 200–500mg of plant material ground in0.1M phosphate buffer pH7.4 (1:1 w/v)containing carborundum powder (400 mesh). For inoculation of young tomato plants, one or two fully expanded leaves are gently rubbed with the inoculum. Since the viroid concentration in plants is affected by temperature and light intensity, test plants should be grown under controlled conditions, i.e. at least 24°C and a photoperiod of 14 h (Grassmick & Slack; 1985). Lower temperatures and less light may reduce the transmission and multiplication of the viroid, thereby reducing the reliability of the test (Verhoeven et al, 2010). The inoculated plants have to be regularly inspected for symptoms for up to six weeks after inoculation.

Inoculation of *S. lycopersicum* plants (such as cultivars Rutgers, Moneymaker or Sheyenne)will allow the detection of many (but not all) pospiviroids and might provide visual evidence of pathogenicity. For PSTVd mild and severe strains have been described based on symptoms produced by different isolates in cultivar cv. Rutgers (Fernow, 1967), symptoms including stunting, epinasty, rugosity and lateral twisting of new leaflets, leaf chlorosis, reddening, brittleness and necrosis.

In the case of CEVd, typical symptoms of stunting and exocortis can be observed on citrus trees after mechanical inoculation (stem slash inoculation) or grafting onto Etrog citron Arizona 861-S (*Citrus medica*) and *Gynura aurantiaca* grown on rough lemon rootstock (*Citrus jambhiri* Lush. Rutaceae), the bioassay host for citrus viroids (Lin et al, 2015, Dang et al, 2022). Infected leaves showed symptoms such as epinasty, leaf curling, midvein and petiole browning. Stunting can be observed between 3 to 8 months after grafting with CEVd-infected buds (Ito et al, 2002, Lin et al, 2015).

3.3 Sampling

Pospiviroids can infect a wide range of plant species, including both herbaceous and woody species. The viroid concentration in different hosts and tissue types might vary significantly. Therefore, sampling is described for the main hosts and/or matrices. Plant material may be pooled to specific rates depending on the test, tissue, and purpose of testing. In all cases bulking rates must be validated. General guidance on sampling methodologies is described in ISPM 31, 2016 (*Methodologies for sampling of consignments*). Sampling tools should be sanitized with a suitable disinfectant and dried with a paper towel to avoid cross contamination. Samples can be bulked (pooled), provided that the bulk size is adapted to the viroid concentration in the host plant the analytical sensitivity of the test and validated.

3.3.1. Bark and woody tissues

Citrus budwood samples (i.e., stems without leaves and thorns) should be collected from the last mature vegetative flush (approximately 12 to 18 months old), and at multiple locations around the tree canopy to account for any unequal distribution of the viroid in the plant.

Bark tissue from citrus species (e.g. *Citrus medica* L., *Citrus trifoliata*) should be sampled from symptomatic or asymptomatic plants (about 10 months-old plants sampling bark/woody tissues from young flush) and young shoots (Rizza et al., 2009). In the case of trees, which might display scaling symptoms on the rootstock, green bark tissues should be collected during the period of growth, *i.e.* summertime (Ragozzino et al., 2005).

3.3.2. Leaves

In general top and fully expanded young leaves (fully expanded leaf or leaflet), *i.e*. actively growing (non-senescing) tissue, are most suitable for testing. Viroid concentrations might differ considerably dependent on the age/maturity of the plants and environmental conditions (temperature and photoperiod). For leaves of potato and tomato, bulking rates up to 100 have been used for real-time RT-PCR tests; whereas for pepper and ornamentals, such as *Brugmansia* spp., *Chrysanthemum* spp., *Dahlia* spp. and *Solanum jasminoides*, bulking rates of up to 25 were found adequate (E. Meekes, pers. comm.; Verhoeven et al., 2008, 2016). In addition, it should be noted that some plant species contain biochemicals that may inhibit mechanical transmission to test plants (e.g. *Brugmansia* spp.) or amplification in RT-PCR tests (e.g. *Calibrachoa* spp., S. *jasminoides* and *S. jamesonii*).

3.3.3. Microplants

Microplants (essentially solanaceous hosts, such as potato and petunia), should be four to six weeks old with stems of about 5 cm in length and with well-formed leaves. The whole plant can be sampled for testing, or alternatively, the top two-thirds of the plant. In the latter case sampling should be done under aseptic conditions to enable the rest of the plant to continue growing.

3.3.4. Seeds

The likelihood of viroid detection in a seed lot depends on the percentage of contaminated seeds and the viroid concentration in the infected seed(s) (Verhoeven et al., 2015b).This makes it difficult to recommend a sample size and bulking rate (EUPHRESCO, 2010).

For seed lots of pepper and tomato, most common sampling methods rely on weighed samples of approximately 3000 seeds, tested in three subsamples of 1000 seeds. ISTA recommend testing of (sub-) samples of 1000 seeds, that has been validated for real-time RT-PCR (IPPC PSTVd 2015, <https://worldseed.org/wp-content/uploads/2016/05/Tomato_pospiviroids_Jan2015.pdf> ). However, both sample and subsample size might have to be adapted due to technical restrictions or to meet specific import requirements.

3.3.5. Potato tubers

In potato tubers, the highest viroid concentration is found immediately after harvest (Roenhorst et al, 2006). Samples can be taken from tuber eyes, heel-end, peel fragments and flesh cores throughout the whole tuber, since PSTVd has been found to be present in almost equal amounts in different parts of both primarily and secondarily infected tubers (Shamloul et al., 1997; Roenhorst et al., 2006). For testing by real-time RT-PCR, up to 100 cores weighing about 50 mg each may be bulked together (Roenhorst et al, 2006).

3.4 Molecular detection[[1]](#footnote-1)

Different molecular tests are available for the detection of pospiviroids. The sub-sections below describe sample preparation and RNA extraction methods for different host plants and tissue types. The currently most widely used molecular tests i.e. conventional (endpoint) RT-PCR and real-time RT-PCR for testing all tissue types, including seeds, are described. It should be noted that for all molecular tests genomic sequence variability may hamper the detection of specific isolates.

3.4.1 Sample preparation

This section describes sample preparation for RNA extraction for different hosts and tissue types. These initial steps, combined with the RNA extraction, are critical for the outcome of a test and may differ between matrices. Therefore, sample preparation methods should be validated in combination with RNA extraction and PCR test.

To grind (homogenize) material, sampled as described in section 3.3.1 to 3.3.5and 3.1.3, variety of tool can be used to include mortar and pestle, homogenizers e.g. Homex 6 (Bioreba) with extraction bags (Bioreba, Switzerland), bead-beater instrument such as FastPrep, Mixer Mill; TissueLyzer, GenoGrinder and others. For all tissues, freezing the sample e.g. using liquid nitrogen may facilitate grinding and homogenization.

3.4.1.1. Bark (woody tissues) and roots

Bark peel and roots should be chopped into small pieces prior to homogenization using various means. Lyophilization of the tissue prior to processing may help with the homogenization (Dang et al, 2022). Dry grinding (no buffer) is recommended if lyophilized tissue is used for extraction. The following protocol is suggested for testing of bark tissue (Dang et al, 2022).

The phloem-rich bark tissue is peeled using a disposable, single edge razor blade. The peeled bark tissue is chopped into small pieces (4-5 mm) on small disposable chipboards, and 250 mg placed into a 2 mL safe-lock tube (Eppendorf, Hamburg, Germany). All sample tubes are kept on ice during processing and sanitized externally by dipping in an appropriate disinfectant and then in water. Tissue dried tubes are placed in a -80°C freezer for at least two hours prior to lyophilization. Samples are lyophilized for about 24 hours in a FreeZone® Triad™ 74000 freeze-dryer (Labconco®, USA). After lyophilization, a single sterile 4 mm stainless steel grinding ball is added into each sample tube and stored at -80°C until the tissue pulverization and RNA isolation steps. Sample tubes are placed in stainless steel Cryo-Blocks (SPEX SamplePrep, Metuchen, NJ) and chilled with liquid nitrogen using a Cryo-Station (SPEX SamplePrep) for 20 minutes. Samples are ground into a fine powder using a Geno/Grinder® 2010 (SPEX SamplePrep) at 1,680 rpm for 20 seconds, in two cycles. RNA extraction from the pulverized citrus tissue samples is as described in section 3.4.2.1 (Dang et al, 2022). Alternatively, total RNA is extracted from 100mg young bark or leaves (about 10 months-old plants or bark), ground to a fine powder in a mortar with liquid nitrogen, then homogenized in RNA extraction buffer and processed following the manufacturer's instructions (Rizza et al, 2009).

3.4.1.2 Leaves and microplants

Before grinding, water or lysis buffer is added to the plant material, the volume and composition of the buffer depending on the method to be used for nucleic acid extraction. If freezing the sample in liquid nitrogen water or lysis buffer should be added after grinding.

3.4.1.3 Seeds

For seeds, sample preparation and RNA extraction are highly interdependent and are described together in section 3.4.2.3.

3.4.1.4 Tubers

Tuber cores can be ground and homogenized in water or lysis buffer (about 1 g/mL; composition of the buffer depending on the method used for nucleic acid extraction) by using homogenizers (such as Homex 6 with extraction bags, Bioreba). Freezing the cores before adding the water or lysis buffer may facilitate grinding and homogenization.

3.4.2 RNA extraction

In general, a wide range of RNA extraction methods may be used for the different matrices, from commercial kits to methods published in scientific journals. The RNeasy Plant Mini kit (Qiagen), and the Sbeadex® maxi plant kit (LGC genomics) can be used following the manufacturers’ instructions or the instructions described in this Standard where appropriate. For high-throughput RNA extraction, the Sbeadex® maxi plant kit (or MagMax or other) can be used in combination with a KingFisher KF96 system. Other extraction methods, including the CTAB method (Gambino et al, 2008) can also be used. All methods should be validated for the intended use in combination with a specific test (see section 3.3.2.6).

Extracted RNA should be stored refrigerated for short-term storage (<8 hours), at -20°C (<1 month) or at -80°C for longer periods.

For specific applications, the following procedures have been successfully used for the indicated host plants and type of material.

3.4.2.1 Bark and woody tissues

RNA extraction can be performed using 100-500 mg of tissue depending on the RNA extraction method. Validation data are available for methods 3 and 4.

**Method 1***.* RNA extraction is accomplished by combining guanidine lysis buffer with the Qiagen RNeasy Plant Mini Kit (Qiagen) as described by Bernard & Duran-Vila (2006). Approximately 100 mg of tissue is homogenized in RNA extraction buffer (4 M Guanidine isothiocyanate, 100 mM Tris–HCl, 25 mM MgCl2, 25 mM EDTA, pH7.5). The soluble fraction is concentrated by isopropyl alcohol precipitation and resuspended in TE buffer (20 mM Tris–HCl, 1 mM EDTA, pH8.0). Subsequently, the RNA is purified using the Qiagen RNeasy Plant Mini Kit following the manufacturer’s instructions for RNA cleanup and resuspended in 50 µL of water.

**Method 2.** The phenol/guanidine isothiocyanate method (Chomczynski & Sacchi, 1987) or using TRIzol™ reagent (Invitrogen) (Rizza et al, 2009, *Dang et al 2022*). can be used for old bark and other tissues rich in polysaccharides, phenolic compounds or other secondary metabolites. Approximately 100mg to 500mg of bark tissue is homogenized in TRIzol™ (Invitrogen), RNA extraction is undertaken following manufacturer’s instructions.

**Method 3****(Dang *et al,* 202***2*). The pulverized citrus tissues are processed with the MagMAX™ 96 Viral RNA Isolation Kit, utilized with the MagMAX™ Express-96 Deep Well Magnetic Particle Processor (ThermoFisher Scientific, Waltham, MA) following the manufacturer’s recommendations adjusted and optimized for citrus tissue. 750 µL of 4 M guanidine lysis buffer (4 M guanidine thiocyanate, 0.2 M sodium acetate pH 5.0, 2 mM EDTA, 2.5% (w/v) PVP-40 at pH 5.0) is added to each sample. Samples are homogenized using the Geno/Grinder® 2010 at 1,680 rpm for 20 seconds, twice. The crude homogenized extracts are incubated at 4°C for 15 minutes and centrifuged at 4°C for 45 minutes at 17,200 x g. RNA is extracted using the default MagMAX™ program “AM1836\_DW\_50\_V2” of the magnetic particle processor, following recommendation of the manufacturer. Two mL deep well plates are used for the MagMAX™ Express-96 and are prepared as follow: lysis plate (position 1) containing 139 µL of Lysis/Binding Solution Concentrate (premixed with 40 mL of isopropanol), 22 µL of Bead Mix (10 µL of RNA Binding Beads, 10 µL of Lysis/Binding Enhancer, and 2 µL of Carrier RNA), 139 µL of isopropanol, and 150 µL of the processed supernatant; a first set of wash plates (positions 2-3) containing 500 µL of MagMAX™ Wash Solution 1; a second set of wash plates (positions 4-5); containing 500 µL of MagMAX™ Wash Solution 2; the elution plate (position 6) containing 100 µL of elution buffer; and the tip comb plate (position 7) loaded with the MagMAX™ Express-96 Deep Well Tip Comb. Upon completion of the magnetic particle processor run, the elution plate is placed on a magnetic rack for 5 minutes to collect any residual beads. The isolated RNA can be transferred to individual 1.5 µL microcentrifuge tubes and stored at -80°C.

**Method 4 (Dang *et* al, 2022***)*. RNA extraction can also be performed on pulverized tissues with the Qiagen RNeasy® Plant Mini Kit following the manufacturer’s protocol.

3.4.2.2 Leaves and microplants

The following extraction methods were validated in combination with different RT-PCR and real-time RT-PCR tests. For small samples, approximately 100 mg of leaf material is homogenized with lysis buffer from the RNeasy Plant Mini kit (Qiagen) following the instructions of the manufacturer. For large samples, e.g. bulk samples, approximately 1g of tissue is put in an extraction bag and homogenized in 3.5 mL (range 1:2 – 1:5 (w/v)) of GH+ Extraction buffer (6M Guanidine Hydrochloride, 0.2 M sodium acetate pH5; 25 mM EDTA, 2.5% PVP-10), incubated for 10 min at 65°C, and centrifugated for 2 min (~12000 *g*), before nucleic acid extraction using the RNeasy Plant Mini kit or Sbeadex® maxi plant kit according to the instructions of the manufacturer.

3.4.2.3 Seeds

Different equipment and reagents (kits) for sample preparation and RNA extraction in seeds have been compared, resulting in recommendation of the following procedures that performed equally well.

*Homogenization in GH+ buffer*. For both *C. annuum* and *S. lycopersicum* three subsamples of (approximately) 1000 seeds are transferred to a grinding bag (Interscience BagPage 100ml) together with 20 mL of GH+ Extraction buffer\* (see section 3.4.2.2. Leaves). Seeds are soaked at room temperature for 30-60 min before homogenisation with an BagMixer 100 (Interscience) for 90 s (tomato) or 4 min (pepper).

Alternatively, dry seeds can be ground with a Geno/Grinder (SPex SamplePrep P) (Botermans et al, 2020). Six subsamples of (approximately) 500 pepper seeds or three subsamples of (approximately) 1000 tomato seeds are transferred to a 50 mL tube and a steel ball (14 mm) is added. Seeds are ground, tubes upside down, at 1700 rpm for 7 and 4 min for pepper and tomato, respectively. After grinding GH+ buffer[[2]](#footnote-2) is added, 2 times 10 mL for pepper and 20 mL for tomato samples. Tubes are shaken by hand to obtain homogenous solutions. Three times two pepper homogenates are combined and mixed to make three subsamples for further processing.

After homogenization, one mL of the seed homogenate is transferred into a 1.5 mL tube and 30 μL 5M Dithiothreitol added, followed by incubation in a thermomixer at 850 rpm and 65°C for 15 min and centrifugation at 16 000 g for 10 min. For RNA extraction by the RNeasy plant mini kit (Qiagen), 750 μL of the supernatant is loaded on the QIAshredder spin column and centrifuges. Thereafter the manufacturer’s instructions are followed. For high throughput RNA extractions, a Kingfisher KF96 system can be used. In this system 250 μL of the supernatant is transferred to a binding plate containing 600 μL of binding buffer (kit) and 50 μL Sbeadex® maxi plant kit and RNA extracted following the manufacturer’s instructions.

*Homogenisation in phosphate buffer*. For both pepper and tomato, 12 subsamples of 250 seeds can be immersed each in 10 mL of 0.1M phosphate buffer (Na2HPO4/KH2PO4, pH 7.2), incubated at 4°C overnight, and then ground e.g. with a FastPrep homogeniser at 5 m/s for 40s. After centrifugation at 10,000 g at 4°C for 10 min, RNA can be extracted using the RNeasy plant mini kit (Qiagen), following the manufacturer’s instructions with some minor modifications. Briefly, 600 µL of the supernatant is added to 600 µL RLT buffer (without β-mercaptoethanol). Two aliquots of 600 L of this mixture are successively loaded onto the same RNeasy Mini Spin column and centrifuged. RNA is eluted from the RNeasy Mini Spin columns by applying 50 µL of RNase-free warm water (65°C) followed by centrifugation. To maximise RNA recovery, an additional elution step is performed using the same conditions. RNA extracts may be processed separately or three times four RNA extracts may be combined.

In critical cases where the viroid concentration is expected to be low, increasing the pospiviroid RNA concentration might be desirable (Mehle et al., 2017). This can be achieved by transferring 4.5 mL of the supernatant to a 5 mL tube containing 0.5 g Amberlite IRA-900 anion-exchange resin (Polysciences, Warrington, PA, USA). Next, RNA is bound to the resin by continuous stirring (about 27 rpm) at room temperature for 3 h, followed by centrifugation at 5000 g for 1 min and removal of the supernatant. The resin-absorbed RNA is eluted by adding 560 µL AVL buffer (QIAamp Viral RNA minikits; Qiagen, Hilden, Germany) to the pelleted Amberlite beads, followed by incubation and occasional agitation at RT for 10 min. After centrifugation at 5000 g for 1 min, the supernatant (containing the nucleic acids) is transferred to a 1.5 mL tube and applied to the QIAamp column, washed and processed according to the manufacturer’s instructions. Finally, the RNA is eluted from the QIAamp column in 45 µL RNase-free water pre-warmed to 65°C. Note that the QIAamp Viral RNA minikit can be also used for RNA extraction from non-concentrated seed samples.

For samples consisting of small numbers of seeds (<100), a Tissue Lyser (e.g. Qiagen or Retsch) can be used. For larger numbers of seeds, a paddle blender (e.g. MiniMix, Interscience) or homogenizer (e.g. Homex 6) with a suitable quantity of lysis buffer (composition depending on the method used for nucleic acid extraction) can be used. Seeds may also be crushed with a hammer (Bertolini *et al*., 2014b) or by using a mortar and pestle. However, the latter may not be practical for routine use as it may be difficult to prevent cross-contamination.

3.4.2.4 Tubers

The RNeasy Plant Mini kit (Qiagen), CTAB method (Boonham et al 2004), KingFisher total RNA kit (Thermo Scientific) and Sbeadex® Maxi Plant kit (LGC) can be used for RNA extraction from tubers. RNA extraction using the RNeasy Plant Mini kit (Qiagen), CTAB method (Boonham et al 2004) and the KingFisher total RNA kit (Thermo Scientific) was validated in combination with real-time RT-PCR (Boonham et al., 2004, Roenhorst et al, 2005).

3.4.2.6. Other RNA extraction methods

**EDTA method.** Plant tissue may be homogenized (1:4 (w/v)) in a simple lysis buffer (50 mM NaOH, 2.5 mM EDTA) and then incubated (at approximately 25 C for 15 min) and subsequently centrifuged (at 12 000 *g* at 4 C for 15 min). The supernatant can either be used directly for RT-PCR (less sensitive) or spotted onto a nitrocellulose membrane and eluted using sterile distilled water (more sensitive) (Singh *et al*., 2006). The method has been used with RT-PCR and hybridization method (see section 3.4.3.6.2) for *S. lycopersicum,* *S. tuberosum* and a range of ornamental plant species.

**Magnetic bead (Kingfisher) method.** The following automated procedure is based on the use of the KingFisher mL Magnetic Particle Processor (Thermo Scientific).With appropriate adjustment of volumes, other KingFisher models may be used. For each sample, at least 200 mg leaf or tuber tissue or up to 100 seeds are macerated, and then extraction buffer[[3]](#footnote-3) is added immediately at a ratio of 1g leaf or tuber tissue to 10 ml buffer and 1 g seed to 20 ml buffer. Maceration is continued until a clear cell lysate with minimal intact tissue debris is obtained. Approximately 2 ml lysate is decanted into a fresh microcentrifuge tube, which is centrifuged at approximately 5 000 *g* for 1 min. One mL of supernatant is removed and placed in the first tube (A) of the KingFisher mL rack, to which 50 µl vortexed MAP Solution A magnetic beads (Invitek) are added. Tube B has 1 mL GLB added to it; tubes C and D, 1 mL of 70% ethanol each; and tube E, 200 µL water or 1× Tris-EDTA buffer. The tube strip is placed in the KingFisher mL Magnetic Particle Processor and nucleic acid extraction is performed following the Thermo Scientific KingFisher Total RNA kit Instruction Manual.

The magnetic bead extraction method has been used for a wide range of plant species as well as for potato tubers and tomato seeds for real-time RT-PCR assays described in the following sections (see sections 3.3.3.4 and 3.3.4.2) (Roenhorst et al., 2005).

3.4.3 Detection

Reverse transcription – polymerase chain reaction (RT-PCR) using so-called ‘generic’ pospiviroid primers, is an efficient and sensitive method to detect pospiviroids. There are several RT-PCR tests for the ‘generic’ detection of pospiviroids using combinations of different primer sets (Table 3, see Verhoeven et al., 2004, ANSES 2013, Luigi et al., 2014, Olivier et al., 2014). Real-time RT-PCR is the preferred method for high-throughput screening of plant material and seeds. Several real-time RT-PCR tests have been developed to detect a subset of pospiviroids. Some of these tests were evaluated in an inter-laboratory comparison (Olivier et al., 2016). Validation data for the recommended tests are given in Appendix 1 (EPPO PM 7/138, 2021). A list of recommended tests for pospiviroid detection are presented in section 3.4.3 and summarised in Table 2. Other suitable tests for pospiviroid detection are summarised in Table 3.

If appropriate, confirmation of detection should be carried out using an independent test as indicated in the following sections 3.4.3, Tables 2 and 3, or by sequence analysis of the amplicon for identification (see section 4) .

Note that every RT-PCR method used should be validated for the intended use. If other reagents than those recommended are used, the reverse-transcription and/or cycling conditions steps might perform differently and should be validated/adapted accordingly. For all reaction mixes, primers and probes dilutions, molecular grade nuclease-free water should be used. End of PCR cycle temperature storage should range between 4°C to 20°C in compliance with recommended laboratory practice.

3.4.3.1 Conventional RT-PCR

The primer set Pospi1 allows the detection of all known pospiviroids (Verhoeven *et al*. 2004) with the exception of CLVd. Therefore, the Pospi1 primer set is combined with the pCLV4-primer set described by Spieker (1996a) which specifically detects CLVd (Olivier et al., 2014).

**Primers list**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Primer** | **Sequence (5’-3’)** | **Primer location** | **Viroids detected** | **Amplicon****size (bp)** |
| Pospi1-FW | GGGATCCCCGGGGAAAC | 86-1021 | CEVdCSVdIrVd-1PCFVdPSTVdTASVdTCDVdTPMVd | 197 |
| Pospi1-RE | AGCTTCAGTTGT(T/A)TCCACCGGGT | 283-2611 |
|  |  |  |
| pCLVR4 | GGGGCAACTCAGACCGAG C | 102-1202 | CLVd | 370 |
| pCLV4 | GGGGCTCCTGAGACCGCTCTTG-3’ | 101-802 |

1Location in PSTVd NC\_002030; 2Location in CLVd NC\_003538.

**Master mixes composition.** The One-Step RT-PCR Kit (Qiagen) has been shown to be reliable when used for the detection of PSTVd, PCFVd, TPMVd, CEVd, CLVd, CSVd, TASVd and TCDVd in individual samples (EUPHRESCO, 2010).

**Pospi1 primers: CEVd, CSVd, IrVd-1, PCFVd, PSTVd, TASVd, TCDVd, TPMVd**

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagents** | **Working concentration** | **Volume per reaction (µL)**  | **Final concentration** |
| Molecular gradewater | N.A. | 15.0  | N.A. |
| One-step RT-PCR buffer (Qiagen) | 5 x | 5.0 | 1x |
| dNTP mix (Qiagen) | 10 mM | 1.0 | 0.4 mM |
| Pospi1-FW | 10 µM | 1.0 | 0.4 µM |
| Pospi1-RE | 10 µM | 1.0 | 0.4 µM |
| One-Step RT-PCR Enzyme Mix (Qiagen) | - | 1.0 | - |
| RNA template |  | 1.0 |  |
| Total |  | 25.0 |  |

**pCLV4 primers: CLVd**

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagents** | **Working concentration** | **Volume per reaction (µL)**  | **Final concentration** |
| Molecular gradewater  | N.A. | 15.0 | N.A. |
| One-step RT-PCR buffer (Qiagen) | 5 x | 5.0 | 1x |
| dNTP mix (Qiagen) | 10 mM | 1.0 | 0.4 mM |
| pCLVR4 | 10 µM | 0.5 | 0.2 µM |
| pCLV4 | 10 µM | 0.5 | 0.2 µM |
| One-Step RT-PCR Enzyme Mix (Qiagen) | - | 1.0 | - |
| RNA template |  | 2.0 |  |
| Total |  | 25.0 |  |

**Thermocycling conditions**

**Pospi primers.** Reverse transcription at 50°C for 30 min; denaturation at 95°C for 15 min; 14 cycles of denaturation at 94°C for 30 s, annealing at 64°C for 90 s and elongation at 72°C for 45 s, followed by 29 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 90 s and elongation at 72°C for 45 s; terminal elongation at 72°C for 10 min and kept at 20°C.

**pCLV4 primers.** Reverse transcription at 50°C for 30 min; denaturation at 95°C for 15 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 90 s and elongation at 72°C for 45 s; terminal elongation at 72°C for 10 min; and kept at 20°C.

**Expected amplicons.** The PCR products (approximately 197 bp and 370 bp for the Pospi1 and pCLV4 primers, respectively) should be analysed by gel electrophoresis (2% agarose gel).

3.4.3.2 Real-time RT-PCR for the detection of pospiviroid on all tissues except seed

The GenPospi test (Botermans et al., 2013) detects all known pospiviroids in leaves, tubers and fruits. Note that the test is not recommended for testing seeds because of its lack of sensitivity in this matrix. The GenPospi test consists of two reactions running in parallel: the first targets all known pospiviroid except CLVd; the second specifically targets CLVd. In both reactions *nad5* is included as an internal (extraction) control.

**Primers and probes list**

|  |  |  |
| --- | --- | --- |
| **Primers & Probes** | **Sequence (5’-3’)** | **Ref.** |
| *Reaction mix 1* |
| TCR-F 1-1 | TTCCTGTGGTTCACACCTGACC | 1 |
| TCR-F 1-3 | CCTGTGGTGCTCACCTGACC | 1 |
| TCR-F 1-4 | CCTGTGGTGCACTCCTGACC | 1 |
| TCR-F PCFVd | TGGTGCCTCCCCCGAA | 1 |
| TCR-F IrVd | AATGGTTGCACCCCTGACC | 1 |
| TR-R1 | GGAAGGGTGAAAACCCTGTTT | 1 |
| TR-R CEVd | AGGAAGGAGACGAGCTCCTGTT | 1 |
| TR-R6 | GAAAGGAAGGATGAAAATCCTGTTTC | 1 |
| pUCCR | *FAM*-CCGGGGAAACCTGGA-*MGB* | 1 |
| *Reaction mix 2* |
| CLVd-F | GGTTCACACCTGACCCTGCAG | 2 |
| CLVd-F2 | AAACTCGTGGTTCCTGTGGTT | 2 |
| CLVd-R | CGCTCGGTCTGAGTTGCC | 2 |
| CLVd-P | *FAM*-AGCGGTCTCAGGAGCCCCGG-*BHQ1* | 2 |
| *Internal Control* |
| nad5-F | GATGCTTCTTGGGGCTTCTTGTT | 3 |
| nad5-R | CTCCAGTCACCAACATTGGCATAA | 3 |
| nad5-P | *VIC*-AGGATCCGCATAGCCCTCGATTTATGTG-*BHQ1* | 1 |

References: 1Botermans *et al.*, 2013; 2Monger *et al.*, 2010; 3Menzel *et al.*, 2002

**Master mixes composition**

**GenPospi primer mix**

|  |  |  |  |
| --- | --- | --- | --- |
| **GenPospi-primer mix** | **Stock concentration (µM)** | **Volume (µL)** | **Final concentration (µM)** |
| Molecular grade water | **-** | 720 | **-** |
| TCR-F 1-1 | 100 | 10 | 1.25 |
| TCR-F 1-3 | 100  | 10 | 1.25 |
| TCR-F 1-4 | 100  | 10 | 1.25 |
| TCR-F IrVd | 100  | 10 | 1.25 |
| TCR-F PCFVd | 100  | 10 | 1.25 |
| TR-R1 | 100  | 10 | 1.25 |
| TR-R CEVd | 100  | 10 | 1.25 |
| TR-R6 | 100  | 10 | 1.25 |
| Total  |  | 800 |  |

**GenPospi Reaction Mix: CEVd, IrVd, PCFVd, PSTVd, TASVd, TCDVd, TPMVd, including nad5**

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagent** | **Working concentration (µM)** | **Volume per reaction (µL)**  | **Final concentration (µM)** |
| Molecular gradewater | N.A. | 1.65 | N.A. |
| 2× TaqMan RNA-to-CT 1-Step Kit mix | 2x | 12.5 | 1x |
| TaqMan® RT enzyme mix\* | 40x | 0.6 | ~1x |
| GenPospi- primer mix | 1,25 each | 6.0 | 0,3 each |
| Primer nad5-F  | 10 | 0.75 | 0.3 |
| Primer nad5-R | 10 | 0.75 | 0.3 |
| TaqMan® probe pUCCR | 10  | 0.25 | 0.1 |
| TaqMan® probe nad5-P | 10  | 0.5 | 0.2 |
| RNA  |  | 2.0 |  |
| Total |  | 25.0 |  |

\* The use of reagent TaqMan RNA-to-CT 1-Step Kit (Applied Biosystems) is critical as Ct values have been found to increase by 8-10 when using other kits (Botermans *et al.*, 2013).

**CLVd Reaction Mix: CLVd including nad5**

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagents** | **Working concentration (µM)** | **Volume per reaction (µL)**  | **Final concentration (µM)** |
| Molecular gradewater | N.A. | 5.4 | N.A. |
| 2× TaqMan® RT-PCR mix\* | 2x | 12.5 | 1x |
| TaqMan® RT enzyme mix\* | 40x | 0.6 | ~1x |
| Primer CLVd-F | 10 | 0.75 | 0.3 |
| Primer CLVd-F2 | 10 | 0.75 | 0.3 |
| Primer nad5-F | 10 | 0.75 | 0.3 |
| Primer CLVd-R | 10 | 0.75 | 0.3 |
| Primer nad5-R | 10 | 0.75 | 0.3 |
| TaqMan® probe CLVd-P | 10  | 0.25 | 0.1 |
| TaqMan® probe nad5-P | 10  | 0.5 | 0.2 |
| RNA |  | 2.0 |  |
| Total |  | 25.0 |  |

\*TaqMan RNA-to-CT 1 Step Kit, ABI. Note that the use of this reagent can be critical as Ct values have been found to increase by 8-10 when using another kit (Botermans *et al.*, 2013).

**Thermocycling conditions.** Thermocycling conditions are 50 °C for 10 min, 95 °C for 2 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min for the three mixes above.

3.4.3.3 Real-time RT-PCR for the detection of pospiviroids in seeds: Pospisense test (Botermans et al, 2020)

The PospiSense test (Botermans et al., 2020) allows sensitive detection in seeds of all pospiviroids known to infect pepper and tomato naturally. The test makes use of a single fluorophore, which implies that it does not discriminate between species. The test is described for samples of app. 3000 seeds, tested in 3 subsamples of 1000 seeds. The test consists of two reactions running in parallel: PospiSense 1 and PospiSense 2, together targeting CEVd, CLVd, PCFVd, PSTVd, TASVd, TCDVd and TPMVd. In both reactions DLVd is used as an internal (extraction) control. At high concentrations individual pospiviroid might produce a signal in both reactions.

**Primers and probes list**

|  |  |  |
| --- | --- | --- |
| **Primers & Probes** | **Sequence (5’-3’)** | **Ref.** |
| *PospiSense 1* |
| PospiFW1 | TGCGCTGTCGCTTCG  | 1 |
| PospiFW5a | CCTTCCTTTCTTCGGGTTTC | 1 |
| PospiRV1 | AGAAAAAGCGGCGCTTG | 1 |
| PospiRV2 | TAGAGAAAAAGCGGTTCTCGG | 1 |
| PospiRV5a | GAAAAAGCACCTCTGTCAGTTGTA | 1 |
| CLVd-F | GGTTCACACCTGACCCTGCAG | 2 |
| CLVd-F2 | AAACTCGTGGTTCCTGTGGTT | 2 |
| CLVd-R | CGCTCGGTCTGAGTTGCC | 2 |
| PospiP1a | FAM-CGGTGGAAACAACTG-MGB | 1 |
| PospiP3a | FAM-CGGCCTTCTCGCGCA-MGB | 1 |
| CLVd-P | FAM-AGCGGTCTCAGGAGCCCCGG-BHQ1 | 2 |
| *PospiSense 2* |
| PospiFW6a | GGATCTTTCTTGAGGTTCCTGT | 1 |
| PospiFW6b | GGAACTTTCTTGAGGTTCCTGT | 1 |
| PospiFW6c | TCTTTCCTTGTGGTTCCTGTG | 1 |
| PospiRV6a | CGACTTCCTCCAGGTTTCC | 1 |
| PopspiP5 | FAM-CTGCAGGGTCAGGTG-MGB | 1 |
| *Internal Control* |
| DaVd1-FT | GCTCCGCTCCTTGTAGCTTT | 3 |
| DaVd1-RT | AGGAGGTGGAGACCTCTTGG | 3 |
| DaVd1-P | Texas Red-CTGACTCGAGGACGCGACCG-BHQ2 | 3 |

References:1Botermans *et al.* (2020), 2Monger *et al.* (2010);3Naktuinbouw (unpublished).

**PospiSense 1 primers mix**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Stock concentration (µM)** | **Volume (µL)** | **Final concentration (µM)** |
| Molecular grade water | **-** | 20 | **-** |
| PospiFW1 | 100 | 10 | 10 |
| PospiFW5a | 100  | 10 | 10 |
| PospiRV1 | 100  | 10 | 10 |
| PospiRV2 | 100  | 10 | 10 |
| PospiRV5a | 100  | 10 | 10 |
| CLVd-F | 100  | 10 | 10 |
| CLVd-F2 | 100  | 10 | 10 |
| CLVd-R | 100 | 10 | 10 |
| Total  |  | 100 |  |

**PospiSense 1 probes mix**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Stock concentration (µM)** | **Volume (µL)** | **Final concentration (µM)** |
| Molecular grade water | **-** | 70 | **-** |
| PospiP1a | 100 | 10 | 10 |
| PospiP3a | 100  | 10 | 10 |
| CLVd-P | 100  | 10 | 10 |
| Total  |  | 100 |  |

**PospiSense 2 primers mix**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Stock concentration (µM)** | **Volume (µL)** | **Final concentration (µM)** |
| Molecular grade water  | **-** | 60 | **-** |
| PospiFW6a | 100 | 10 | 10 |
| PospiFW6b | 100  | 10 | 10 |
| PospiFW6c | 100  | 10 | 10 |
| PospiRV6a | 100 | 10 | 10 |
| Total  |  | 100 |  |

**DLVd primers mix (internal control)**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Stock concentration (µM)** | **Volume (µL)** | **Final concentration (µM)** |
| Molecular grade water | **-** | 80 | **-** |
| DaVd1-FT | 100 | 10 | 10 |
| DaVd1-RT | 100  | 10 | 10 |
| Total  |  | 100 |  |

**Master mixes composition.** The test has been successfully performed on different real-time PCR systems including CFX96 (Bio-Rad Laboratories) and QuantStudio™ 6 Flex Real-Time PCR System (ThermoFisher Scientific).

**PospiSense Reaction mix 1: CLVd, PCFVd, PSTVd, TCDVd, TPMVd + DLVd**

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagents** | **Working concentration** | **Volume per reaction (µL)**  | **Final concentration** |
| Molecular gradewater  | N.A. | 11.2 | N.A. |
| RT-PCR mix (Quanta Biosciences) | 4x | 5.0 µL | 1x |
| PospiSense 1 primer mix  | 10 µM each | 0.6 | 0.24 µM  |
| PospiSense 1 probe mix | 10 µM each | 0.2 | 0.08 µM |
| DLVd primer mix | 10 µM each | 0.6 | 0.24 µM |
| DLVd probe | 10 µM  | 0.4 | 0.16 µM |
| Subtotal |  | 18 |  |
| RNA |  | 2.0 |  |
| Total |  | 20 |  |

**PospiSense Reaction mix 2: CEVd, TASVd + DLVd**

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagent** | **Working concentration** | **Volume per reaction (µL)**  | **Final concentration** |
| Molecular gradewater  | N.A. | 11.2 | N.A. |
| RT-PCR mix (Quanta Biosciences) | 4x | 5.0 | 1x |
| PospiSense 2- primer mix | 10 µM each | 0.6 | 0.24 µM  |
| PospiP5 - probe | 10 µM | 0.2 | 0.08 µM |
| DLVd - primer mix | 10 µM each | 0.6 | 0.24 µM |
| DLVd - probe  | 10 µM  | 0.4 | 0.16 µM |
| Subtotal |  | 18 |  |
| RNA |  | 2.0 |  |
| Total |  | 20 |  |

**Thermocycling conditions.** Thermocycling conditions are 50 °C for 10 min, 95 °C for 2 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

3.4.3.4. Real-time RT-PCR for the detection of pospiviroids in seeds: Naktuinbouw (2017).

The Naktuinbouw test (2017) allows sensitive detection in seeds of all pospiviroids known to infect pepper and tomato naturally. The test is described for samples of app. 3000 seeds, tested in 3 subsamples of 1000 seeds. The test consists of four reactions running in parallel: A (PCFVd, PSTVd, TCDVd, and TPMVd (not all isolates), B (CEVd and CLVd), C (TPMVd (genotype not detected by Mix A; GenBank acc. no. NC\_001558) and D (TASVd). In reactions A and B, DLVd is used as an internal (extraction) control. In reaction C, Nad5 is used as internal control. In reaction D no internal control is used

**Primers and probes list**

|  |  |  |
| --- | --- | --- |
| **Primers & Probes** | **Sequence (5’-3’)** | **Ref.** |
| **Mix A** |  |  |
| PSTV-231F | GCCCCCTTTGCGCTGT | 1 |
| PSTV-296R | AAGCGGTTCTCGGGAGCTT | 1 |
| PSTV-251T | *FAM*-CAGTTGTTTCCACCGGGTAGTAGCCGA-*BHQ1*  | 1 |
| PCFVd-F | TCTTCTAAGGGTGCCTGTGG | 2 |
| PCFVd-R | GCTTGCTTCCCCTTTCTTTT | 2 |
| PCFVd-P | *VIC*-CTCCCCCGAAGCCCGCTTAG-*BHQ1* | 2 |
| **Mix B** |  |  |
| CLVd-F | GGTTCACACCTGACCCTGCAG | 3 |
| CLVd-F2 | AAACTCGTGGTTCCTGTGGTT | 3 |
| CLVd-R | CGCTCGGTCTGAGTTGCC | 3 |
| CLVd-P | *FAM*-AGCGGTCTCAGGAGCCCCGG-*BHQ1* | 3 |
| CEVd-F2-304 | CTCCACATCCGRTCGTCGCTGA | 3 |
| CEVd-R2-399 | TGGGGTTGAAGCTTCAGTTGT | 3 |
| CEVd-P2-337 | *FAM*-CCCTCGCCCGGAGCTTCTCTCTG-*BHQ1*  | 3 |
| **Mix C** |  |  |
| TPMVd-F1 | AAAAAAGAATTGCGGCCAAA | 2 |
| TPMVd-R | GCGACTCCTTCGCCAGTTC | 2 |
| pUCCR | *FAM*-CCGGGGAAACCTGGA-*MGB* | 4 |
| **Mix D** |  |  |
| TASVd-F2-200 | CKGGTTTCCWTCCTCTCGC | 3 |
| TASVd-R2-269 | CGGGTAGTCTCCAGAGAGAAG | 3 |
| TASVd-P2-228 | *FAM*-TCTTCGGCCCTCGCCCGR-*BHQ1* | 3 |
| **Internal Controls** |  |  |
| DaVd1-FT | GCTCCGCTCCTTGTAGCTTT | 2 |
| DaVd1-RT | AGGAGGTGGAGACCTCTTGG | 2 |
| DaVd1-P | *Texas red*-CTGACTCGAGGACGCGACCG-*BHQ2* | 2 |
| nad5-F | GATGCTTCTTGGGGCTTCTTGTT | 5 |
| nad5-R | CTCCAGTCACCAACATTGGCATAA | 5 |
| nad5-P | *VIC*-AGGATCCGCATAGCCCTCGATTTATGTG-*BHQ1* | 4 |

References: 1Boonham *et al.* (2004); 2Naktuinbouw (2017); 3Monger *et al.* (2010); 4Botermans *et al.* (2013); 5Menzel *et al.* (2002).The test has been successfully performed on different real-time PCR systems including CFX96 (Bio-Rad Laboratories). and QuantStudio™ 5 & 7 Flex Real-Time PCR System (ThermoFisher Scientific).).Further details can be found at the website of Naktuinbouw (Naktuinbouw, 2017)

**Reaction mix A primers**

|  |  |  |  |
| --- | --- | --- | --- |
| **Primers RMA** | **Stock concentration (µM)** | **Volume (µL)** | **Final concentration (µM)** |
| Molecular grade water\* | - | 400 | - |
| PSTV-231F | 100 | 100 | 10 |
| PSTV-296R | 100 | 100 | 10 |
| PCFVd-F | 100 | 100 | 10 |
| PCFVd-R | 100 | 100 | 10 |
| DaVd1-FT | 100 | 100 | 10 |
| DaVd1-RT | 100 | 100 | 10 |
| Total |  | 1000 |  |

**Reaction mix A probes**

|  |  |  |  |
| --- | --- | --- | --- |
| **Probes RMA** | **Stock concentration (µM)** | **Volume (µL)** | **Final concentration (µM)** |
| Molecular grade water\* | - | 350 | - |
| PSTV-251T | 100 | 50 | 10 |
| PCFVd-P | 100 | 50 | 10 |
| DaVd1-P | 100 | 50 | 10 |
| Total |  | 500 |  |

**Reaction mix B primers**

|  |  |  |  |
| --- | --- | --- | --- |
| **Primers RMB** | **Stock concentration (µM)** | **Volume (µL)** | **Final concentration (µM)** |
| Molecular grade water\* | - | 300 | - |
| CLVd-F | 100 | 100 | 10 |
| CLVd-F2 | 100 | 100 | 10 |
| CLVd-R | 100 | 100 | 10 |
| CEVd-F2-304 | 100 | 100 | 10 |
| CEVd-R2-399 | 100 | 100 | 10 |
| DaVd1-FT | 100 | 100 | 10 |
| DaVd1-RT | 100 | 100 | 10 |
| Total |  | 1000 |  |

.

**Reaction mix B probes**

|  |  |  |  |
| --- | --- | --- | --- |
| **Probes RMB** | **Stock concentration (µM)** | **Volume (µL)** | **Final concentration (µM)** |
| Molecular grade water\* | - | 350 | - |
| CLVd-P | 100 | 50 | 10 |
| CEVd-P2 | 100 | 50 | 10 |
| DaVd1-P | 100 | 50 | 10 |
| Total |  | 500 |  |

**Reaction mix C primers**

|  |  |  |  |
| --- | --- | --- | --- |
| **Primers RMC** | **Stock concentration (µM)** | **Volume (µL)** | **Final concentration (µM)** |
| Molecular grade water\* | - | 600 | - |
| TPMVd-F1 | 100 | 100 | 10 |
| TPMVd-R | 100 | 100 | 10 |
| nad5-F | 100 | 100 | 10 |
| nad5-R | 100 | 100 | 10 |
| Total |  | 1000 |  |

**Reaction mix C probes**

|  |  |  |  |
| --- | --- | --- | --- |
| **Probes RMC** | **Stock concentration (µM)** | **Volume (µL)** | **Final concentration (µM)** |
| Molecular grade water\* | - | 400 | - |
| pUCCR | 100 | 50 | 10 |
| nad5-P | 100 | 50 | 10 |
| Total |  | 500 |  |

**Master mixes composition.** All reactions can be performed with the UltraPlex 1-Step ToughMix 4x (Quanta Biosciences) and AgPath-IDTM One-step RT-PCR mix (Ambion; P/N: 4387424). These mixes have been shown to improve the performance in comparison with the qScript XLT Multiplex One-step RT qPCR Tough Mix 2x (Quanta Biosciences), which has been used for validation of the original protocol (TESTA, 2015). Pipetting schemes are provided for the UltraPlex 1-Step ToughMix 4x (Quanta Biosciences).

**Reaction mix A: PCFVd, PSTVd, TCDVd, TPMVd + DLVd**

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagent** | **Working concentration (µM)** | **Volume per reaction (µL)**  | **Final concentration (µM)** |
| Molecular gradewater\* | N.A. | 11.5 | N.A. |
| 4× RT-PCR mix (Quanta Biosciences) | 4x | 6.25 | 1x |
| RMA primers mix | 10 (each) | 0.75 | 0.3 (each) |
| RMA probes mix | 10 (each) | 0.5 | 0.2 (each) |
| Subtotal |  | 19.0 |  |
| RNA |  | 6.0 |  |
| Total  |  | 25.0 |  |

**Reaction mix B: CEVd, CLVd + DLVd**

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagent** | **Working concentration (µM)** | **Volume per reaction (µL)**  | **Final concentration (µM)** |
| Molecular gradewater\*  | N.A. | 11.5 | N.A. |
| 4× RT-PCR mix (Quanta Biosciences) | 4x | 6.25 | 1x |
| RMB primers mix | 10 (each) | 0.75 | 0.3 (each) |
| RM2 Probes mix | 10 each | 0.5 | 0.2 (each) |
| Subtotal |  | 19.0 |  |
| RNA |  | 6.0 |  |
| Total |  | 25.0 |  |

**Reaction mix C: TPMVd + nad5**

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagent** | **Working concentration (µM)** | **Volume per reaction (µL)**  | **Final concentration (µM)** |
| Molecular gradewater\* | N.A. | 11.5 | N.A. |
| 4× RT-PCR mix (Quanta Biosciences) | 4x | 6.25 | 1x |
| RMC primers mix | 10 (each) | 0.75 | 0.3 (each) |
| RMC Probes mix | 10 (each) | 0.5 | 0.2 (each) |
| Subtotal |  | 19.0 |  |
| RNA |  | 6.0 |  |
| Total |  | 25.0 |  |

**Reaction mix D: TASVd**

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagent** | **Working concentration (µM)** | **Volume per reaction (µL)**  | **Final concentration (µM)** |
| Molecular gradewater\*  | N.A. | 10.75 | N.A. |
| 4× RT-PCR mix (Quanta Biosciences) | 4x | 6.25 | 1x |
| TASVd-F2-200 | 10  | 0.75 | 0.3  |
| TASVd-R2-269 | 10  | 0.75 | 0.3  |
| TASVd-P2-228 | 10  | 0.5 | 0.2  |
| Subtotal |  | 19.0 |  |
| RNA |  | 6.0 |  |
| Total |  | 25.0 |  |

**Thermocycling conditions.** Thermocycling conditions are 50 °C for 10 min, 95 °C for 2 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

* + - 1. ***Real-time RT-PCR for the detection of CSVd in all matrices***

The described test uses two independent real-time RT-PCR reactions for CSVd and COX (Fera, UK). The primers and probe described by Mumford et al. (2000) are used to detect CSVd and the COX primers and probes are used as internal amplification control (IAC). Consequently, using COX as IAC this test does not allow monitoring of the reverse-transcription step as opposed to using NAD5 primers and probes as described in other tests.

|  |  |  |  |
| --- | --- | --- | --- |
| **Primers & Probes** | **Sequence (5’-3’)** | **Primer location1** | **Viroids detected** |
| CSVd 220F | CTGCCCTAGCCCGGTCTT | 222-239 | CSVd  |
| CSVd 297R | GGAAAAAAAGGCGTTGAAGCTT | 278-289 |
| CSVd 249T | CAGTTGTTTCCACCGGGTAGTAGCCAA | 251-278 |
| COX-F | CGTGCGATTCCAGATTATCCA |  |  |
| COX-R | CAACTACGGATATATAAGRRCCRRACCTG |  |  |
| COXsol-1511T probe | FAM-AGGGCATTCCATCCAGCGTAAGCA-BHQ1 |  |  |

1 Location in CSVd NC\_002015

**Master mixes composition.** This test has been successfully used on a wide range of plant species and matrices. The test has been successfully performed on different real-time PCR systems including ABI7900HT (Applied Biosystems).

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagents** | **Working concentration (µM)** | **Volume per reaction (µL)**  | **Final concentration (µM)** |
| *CSVd Primers/Probe mix* |
| CSVd 220F | 7.5 | 1 | 0.375 |
| CSVd 297R | 7.5 | 1 | 0.375 |
| CSVd 249T | 5 | 0.5 | 0.125 |
| Total |  | 2.5 |  |
| *CSVd reaction mix* |
| Molecular gradewater | N.A. | 6.45 | N.A. |
| iTaq Master Mix (BioRad) | 2x | 10 | 1X |
| iScript RNase H+ Reverse Transcriptase |  | 0.05 | 1X |
| CSVd Primers/Probe mix |  | 2.5 |  |
| RNA | 5 | 1 |  |
| Total |  | 20 |  |
| *COX Primers/Probe mix* |
| COX F | 7.5 | 1 | 0.375 |
| COX R | 7.5 | 1 | 0.375 |
| COXsol-1511T probe | 5 | 0.5 | 0.125 |
| Total |  | 2.5 |  |
| *COX Reaction mix*  |
| Molecular gradewater | N.A. | 6.45 | N.A. |
|  iTaq Master Mix (BioRad) | 2x | 10 | 1X |
| iScript RNase H+ Reverse Transcriptase |  | 0.05 | 1X |
| COX Primers/Probe mix |  | 2.5 |  |
| RNA |  | 1 |  |
| Total |  | 20 |  |

**Thermocycling conditions.** Thermocycling conditions are 50 °C for 10 min, 95 °C for 2 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

3.4.3.6 Other detection methods

3.4.3.6.1 High Throughput Sequencing

High throughput sequencing (HTS), also known as next-generation sequencing (NGS) or deep sequencing, enables the simultaneous detection of plant viruses and viroids without a priori knowledge of what may be present. These technologies are broad-spectrum and generic in nature and can potentially be used to replace a wide range of conventional methods (Lebas et al, 2022). HTS could be used to partially or fully sequence pospiviroid isolates and successfully detect and/or identify previously described and/or unknown pospiviroid species. Note that the limit of detection by HTS can be ‘higher’ than that of real-time RT-PCR tests.

Further information and recommendations on the use of HTS as a diagnostic tool for phytosanitary purposes have been published (Lebas et al, 2022, IPPC CPM Recommendation R-08, 2019 Recommendation on: Preparing to use high-throughput sequencing (HTS) technologies as a diagnostic tool for phytosanitary purposes).

3.4.3.6.2 Hybridization with a DIG-labelled RNA probe

Nucleic-acid hybridisation using probes that cross-hybridize with other pospiviroids at low stringency conditions has been used for viroid detection (Owens and Diener, 1981, Singh, 1999). Nucleic acid-hybridisation using a Digoxigenin (DIG)-labelled PSTVd cRNA probe (Agdia Inc. USA) is a sensitive detection method but less amenable to high-throughput screening in comparison to real-time PCR tests. A full-length monomer PSTVd DIG-labelled cRNA probe will detect all known pospiviroids from a range of hosts including *Petunia* spp., *S. jasminoides*, *S. lycopersicum* and *S. tuberosum* (Monger & Jeffries, 2015; Torchetti et al., 2012). Sensitivity of detection was at least 17 pg PSTVd (Jeffries and James, 2005). Probe preparation, sample and test membrane preparation, and hybridisation conditions are as described in EPPO PM 7/138 (2021) and IPPC DP7 (2016).

3.4.3.6.3. Other methods and kits.

A list of additional tests detecting several or individual pospiviroid species have been listed in Table 3 (EPPO PM7/138, 2021) and peer-reviewed journals (Hammond & Zhang, 2016, Kovalskaya & Hammond, 2022). A range of commercial kits are available for the detection of specific pospiviroid species using either real-time PCR, isothermal amplification or hybridisation methods. Testing laboratories should follow recommendations for users and validate these tests for the intended use.

# 3.5 Controls for molecular tests

For the test result obtained to be considered reliable, appropriate controls -depending on the type of test used and the level of certainty required – should be included for each series of nucleic acid extraction and amplification of the target. For RT-PCR, a positive amplification control and a negative amplification control (no template control) are the minimum controls that should be used.

**Positive amplification control (PAC).** This control is used to ensure that amplification of a known target happens as expected (apart from the extraction). Pospiviroid infected RNA extract, viroid RNA or a synthetic control (e.g. cloned PCR product) can be used. Furthermore, gBlocks gene fragments for specific pospiviroid species such as PSTVd (Adkar-Purushothama *et al*, 2017, EPPO PM 7/139, 2016) can be used as PAC. An additional control at the limit of detection (not mandatory) may also be used.

**Internal control (IC).** An internal control should be included in the RT-PCR test to reduce the possibility of false negatives due to nucleic acid extraction failure or degradation, or the presence of PCR inhibitors. For conventional and real-time RT-PCR, a plant housekeeping gene such as COX and NAD5 can be used. Although COX has been used as an internal control in this protocol, COX primers will amplify RNA and DNA and, therefore, does not control the RT step. The *nad5* mitochondrial *NADH dehydrogenase 5* gene has been shown to bea reliable indicator of the performance of the RNA extraction and RT step for conventional RT-PCR (Menzel *et al*., 2002) and real-time RT-PCR (Botermans et al., 2013). It has been tested against many plant speciesincluding *S. bonariensis*, *S. dulcamara*, *S. jasminoides, S. nigrum*, *S. pseudocapsicum*, *S. rantonnetii* and *S. sisymbrifolium*, *Acnistus arborescens*, *Atropa belladonna*, *Brugmansia* spp., *Capsicum* spp., *Cestrum* spp., *Lochroma cyanea*, *Nicotiana* spp. and *Physalis* spp. (Seigner *et al*., 2008). The *nad5* primers span an exon- intron junction and will therefore not amplify DNA. As an alternative an external (unrelated) target such as DLVd can be used as an internal extraction control. The internal control primers can be used in a duplex reaction with the pospiviroid primers or as a two separate (simplex) reaction should the analytical sensitivity of the test be reduced in a duplex reaction.

**Negative amplification control (NAC or no template control - NTC).** This control rules out false positives due to contamination of reagents and during preparation of the reaction mixture. The PCR-grade water that was used to prepare the reaction mixture is added instead of RNA at the amplification stage.

**Positive extraction control (PEC).** This control is used to ensure that the target viroid nucleic acid extracted is of sufficient quantity and quality for PCR-based detection. Viroid nucleic acids are extracted from infected host tissue or healthy plant tissue that has been spiked with the viroid. Care needs to be taken to avoid cross-contamination due to aerosols from the positive control. The sequence of the positive control used in the laboratory should be known so that this sequence can be readily compared with the sequence obtained for the samples in order to trace potential cross-contamination by the positive control. Alternatively, synthetic positive controls can be used.

**Negative extraction control (NEC).** 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. Multiple negative extraction controls are recommended to be included when large numbers of positive samples are expected (whenever a non-infected matrix is available in sufficient quantity).

3.6 Interpretation of results from conventional and real-time RT-PCR

3.6.1 Conventional RT-PCR

The viroid-specific RT-PCR will be considered valid only if the controls produce the expected results:

* NEC and NAC should produce no amplicons
* PEC, PAC, as well as IC if applicable should produce amplicons of the correct size (note that in the case of a positive sample, the amplicon of the IC might not be produced or in a lower amount)

When these conditions are met:

* A test will be considered positive if amplicons are produced of the correct size
* A test will be considered negative if it produces no band or a band of a different size

Tests should be repeated if any contradictory or unclear results are obtained.

For identification of the viroid species the RT-PCR product must be sequenced.

3.6.2 Real-time RT-PCR

The real-time RT-PCR will be considered valid only if the controls produce the expected results:

* NEC and NAC should give no amplification
* PEC and PAC, as well as IC if applicable, should give exponential amplification curves (note that in the case of a positive sample, the amplification curve of the IC might not be produced) or in a lower amount)

When these conditions are met:

* A test will be considered positive if it produces an exponential amplification curve
* A test will be considered negative if it does not produce an amplification curve or a curve which is not exponential.

Tests should be repeated if any contradictory or unclear results are obtained.

A Ct cut-off value could be applied according to laboratory validation data.

4. Identification

Pospiviroid species can be identified by sequence analysis of the amplicon obtained by the conventional RT-PCR test (3.4.3.1) or high throughput sequencing (3.4.3.6.1), followed by comparison of the sequence with public genetic sequence databases. If the amount of amplicon is low or if a mixed infection is suspected, cloning and sequencing the PCR products may be effective for identification. In critical cases, e.g. the first finding in a country or in a new host, the laboratory may have the result confirmed by another test or laboratory.

EPPO PM7/129 DNA barcoding as identification tool for a number of regulated pests provides general guidance on sequencing and sequence analysis. For the identification of pospiviroids, preferably the sequence of the complete genome should be used for further analysis. According to the International Committee on Taxonomy of Viruses (ICTV) the main criterion for species identification is more than 90% sequence identity (Owens et al., 2012). However, if the sequence obtained shows identity close to 90%, additional parameters should be included, such as biological properties. The ICTV Viroid Study Group is currently discussing the viroid classification and the criteria for species demarcation.

The test using the Pospi1 primers (Verhoeven et al., 2004) has been found to be the most sensitive conventional RT-PCR test, in some cases comparable to real-time RT-PCR. However, this test is not the first choice for identification, as the amplicon only covers about half of the pospiviroid genome. Nevertheless, thus far this partial sequence appeared suitable for a correct identification of isolates (NPPO-NL, 2013a).

The Pospi2 primers (Verhoeven et al., 2017) which have the opposite orientation can be used to sequence the complete genome, although this test is less sensitive than the Pospi1 test. Therefore, it should be noted that in some cases where it is not feasible to obtain the complete genome sequence. Table 3 gives an overview of primer sets that can be used for identification of the different posiviroids.

A positive sample detected by real-time RT-PCR, should, if required for confirmation or identification, be retested using either a different real-time PCR test (no identification) or a conventional RT-PCR to enable the amplicon to be sequenced and identified. However, because of the higher analytical sensitivity of the real-time RT-PCR, an amplicon may not be obtained with conventional RT-PCR. Especially in the case of seed testing, where viroid concentrations might be low, conventional RT-PCR tests might lack the analytical sensitivity to produce an amplicon. Choices for further testing have to be adjusted depending on the initial test. Examples of test combinations suitable to substantiate a result are described in Roenhorst et al. (2018) and in Tables 2 and 3.

To obtain the complete genome(s) sequence(s), both RT- PCR primers are used for Sanger sequencing. The edited consensus sequence (determined by combining the two sequences to a consensus sequence) can then be compared with pospiviroid sequences in a relevant public database (such as GenBank non-redundant nucleotide database) using a local alignment tool (such as BLASTN). Further sequence analysis should be performed by multiple sequence alignment and phylogenetic analysis using appropriate software (such as MEGA or CLUSTALW). For identification, it is advisable to use the consensus sequence starting at position 1 of the viroid genome for comparison with public nucleotide databases.

When 100% sequence accuracy is required, for example when a sequence is to be submitted to a database or when a new viroid species is supposed, it is recommended to perform a second “RT-PCR to cover the region of the primer sequences used for the first RT-PCR as well as any ambiguous bases. Design of a new set of primers from the initial sequence may be required for this purpose.

5. Records

Records and evidence should be retained as described in section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*).

* In instances where other contracting parties may be affected by the results of the test results, in particular in cases of non-compliance or when a (regulated) pospiviroid is found in an area for the first time or in a new host, the following materials should be kept in a manner that ensures complete traceability.
* If relevant and still available, material of the original sample, stored at −80oC or freeze-dried.
* RNA extractions stored at −80oC
* RT-PCR amplicons stored at −20oC to −80oC
* DNA sequence trace files used to generate the consensus sequence

6. Contact Points for Further Information

Further information on this protocol can be obtained from:

SASA, The Scottish Government, Roddinglaw Road, Edinburgh EH12 9FJ, Scotland, UK (Dr C. Lacomme, e-mail: christophe.lacomme@sasa.gov.scot).

Netherlands Institute for Vectors, Invasive Plants and Plant health (NIVIP), National Plant Protection Organization (NPPO) of the Netherlands, PO Box 9102, 6700 HC Wageningen, the Netherlands (Dr J.W. Roenhorst, e-mail: j.w.roenhorst@nvwa.nl; C. Oplaat, e-mail: a.g.oplaat@nvwa.nl; MSc, and M. Botermans, e-mail: m.botermans@nvwa.nl; MSc.)

Department of Environment and Primary Industries, Biosciences Research Division, AgriBio, 5 Ring Road, La Trobe University, Bundoora, Victoria 3083, Australia (Dr B. Rodoni, e-mail: brendan.rodoni@depi.vic.gov.au).

*To be completed…*

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Thanks are due to…*to complete*

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Appendix 1: Validation data for the tests presented.

Note that performance criteria might be different when using another nucleic acid/RNA extraction method and PCR reagents, which implies that each laboratory needs to verify the performance of the test. If not specified, PCR reagents were as specified in the protocols.

**1. Conventional RT-PCR - Performance criteria available (Section 3.4.3.1).**

- Nucleic acid extraction method: RNeasy Plant Mini Kit (Qiagen)

- Information on validation: Validation data were generated according to PM 7/98 Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity at NIVIP The Netherlands (Botermans et al., 2013; EPPO PM 7/138, 2021). Validation data are available here <https://dc.eppo.int/validation_data/dwvalidation?id=204> *Validation of a conventional RT-PCR assay for detection and preliminary identification of pospiviroids (expect CLVd) by Pospi1-FW/Pospi1-RE.*

The Pospi1 test was validated with the SuperScript® One-Step RT-PCR (Qiagen) kit at NIVIP, The Netherlands (Botermans et al., 2013; EPPO PM 7/138, 2021). Validation data are available at <https://dc.eppo.int/validation_data/dwvalidation?id=204> *Validation of a conventional RT-PCR assay for detection and preliminary identification of pospiviroids (except CLVd) by Pospi1-FW/Pospi1-RE.*

The pCLV4 test has been validated with the SuperScript® One-Step RT-PCR kit with Platinum® Taq DNA polymerase (ThermoFisher Scientific™) at LSV Anses, France.

Both tests have been compared for detection of pospiviroids in tomato leaves and seeds by inter-laboratory comparison (Olivier et al., 2016).

***Pospi1 primers***

***Analytical sensitivity***: Pospi1 primers detected all pospiviroids (except CLVd) up to a dilution between 102-105, depending on the viroid species and concentration in the original plant material. [Note that this performance criterium has been expressed as relative infection rate in the EPPO standard, but that both values are based on the same data.] Amplicons could be successfully sequenced up to a dilution of 102.

***Analytical specificity***.: Pospi1 primers have been found to detect all pospiviroid isolates (except CLVd) encountered at the NPPO-NL thus far. No reactions were obtained for isolates of the following viroid species in the family Avsunviroidae, i.e. Avocado sunblotch viroid (*Avsunviroid*), Chrysanthemum chlorotic mottle viroid (*Pelamoviroid*), Eggplant latent viroid (*Elaviroid*), and family *Pospiviroidae*, i.e. Apple scar skin viroid (*Apscaviroid*), Coleus blumei viroid 1 (Coleviroid), Hop stunt viroid (*Hostuviroid*). *In silico* analysis did not reveal cross reactions with other tomato-infecting viruses and host plant sequences. Only a cross reaction was observed for an isolate of *Hop latent viroid* (*Cocadviroid*).

***Selectivity:*** No apparent matrix effects have been observed in a broad variety of host plants, in particular in the families *Apocynaceae*, *Gesneriaceae* and *Solanaceae*.

***Repeatability & reproducibility*:** 100%.

***pCLV4 primers***

***Analytical sensitivit*y:** pCLV4 primers detected all tested CLVd isolates up to at least a relative infection rate of 1% for dilution of infected tomato leaves in healthy tomato leaves (10-2; 6 replicates for each sample).

***Analytical specificity*:** So far, pCLV4 primers have been found to detect all CLVd isolates encountered at LSV-ANSES. No cross reactions were obtained for isolates of other viroid species in the genus *Pospiviroid*. In silico analysis did not reveal cross reactions with other tomato-infecting viruses and host plant sequences (6 replicates for each sample).

***Selectivity*:** No apparent matrix effects have been observed in a broad variety of host plants, in particular in the families *Asteraceae*, *Chenopodiaceae* and *Solanaceae* (6 replicates for each sample).

***Repeatability & reproducibility*:** The test was validated in both an intra- and inter-laboratory comparison. Repeatability and reproducibility were shown to be 100% (6 replicates for each sample).

2. Real-time RT-PCR for the detection of pospiviroids on all tissues except seed (Section 3.4.3.2).

- *Nucleic acid extraction method:* RNeasy Plant Mini Kit (Qiagen)

- *Information on validation:* Validation data were generated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* at NIVIP The Netherlands (Botermans et al., 2013; EPPO PM 7/138, 2021). Validation data are provided in the publication and at <https://dc.eppo.int/validation_data/dwvalidation?id=204>

***Analytical sensitivity***: The GenPospi test was found to detect isolates from all the known pospiviroid species up to a relative infection rate of 0.13% in tomato leaf material (which equals a 770-fold dilution).

***Analytical specificity***: The GenPospi test was found to detect all 33 tested isolates of the targeted pospiviroids, i.e. CEVd (3), CLVd (3), CSVd (4), IrVd-1 (2), PCFVd (1), PSTVd (10), TASVd (3), TCDVd (5) and TPMVd (2). No reactions were obtained for isolates of the following viroid species in the family *Avsunviridae*, *i.e.* Avocado sunblotch viroid (*Avsunviroid*), Chrysanthemum chlorotic mottle viroid (*Pelamoviroid*), Eggplant latent viroid (*Elaviroid*), and family *Pospiviroidae, i.e.* Apple scar skin viroid (*Apscaviroid*), Coleus blumei viroid 1 (*Coleviroid*), Hop latent viroid (*Cocadviroid*), Hop stunt viroid (*Hostuviroid*) and (tomato)viruses: Alfalfa mosaic virus (AMV), Cucumber mosaic virus (CMV), Pepino mosaic virus (PepMV), Potato virus Y (PVY), Tomato mosaic virus (ToMV), Tobacco mosaic virus (TMV), Tomato chlorosis virus (ToCV) and Tomato yellow leaf curl virus (TYLCV).

***Selectivity***: No apparent matrix effects were observed in a wide range of host plants, including a range of tomato cultivars.

***Repeatability & reproducibility***: The test was validated in both an intra- and inter-laboratory comparison for IrVd-1, PSTVd, TASVd and TCDVd and repeatability and reproducibility were shown to be 100%.

3. Real-time RT-PCR for the detection of pospiviroids on seed (Section 3.4.3.3).

Validation data were generated according to PM 7/98 Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity (NIVIP, The Netherlands) (Botermans et al., 2020).

**Analytical sensitivity:** For both tomato and pepper seeds one contaminated seed in a sample of 1000 seeds could be detected for CEVd, CLVd, PCFVd, PSTVd, TASVd, TCDVd and TPMVd.

**Analytical specificity:** The PospiSense test was found to detect all 40 tested isolates of the seven target pospiviroid species, i.e. CEVd (5), CLVd (5), PCFVd (3), PSTVd (12), TASVd (6), TCDVd (6) and TPMVd (3).

Cross reactions have been found to occur with CSVd, Eggplant latent viroid (Elaviroid) and IrVd-1, when present in high concentrations. Of these viroid species, however, no natural infections in pepper and tomato have been reported. Also one out of two isolates of Tomato infectious chlorosis virus produced a cross reaction when present at a high concentration.

No cross reactions were observed for the hostuviroid Hop stunt viroid, and the following pepper and tomato viruses: Alfalfa mosaic virus, Cucumber mosaic virus, Pepper mild mottle virus, Pepino mosaic virus, Potato virus Y, Tobacco mosaic virus, Tomato chlorosis virus, Tomato mosaic virus, Tomato spotted wilt virus and Tomato yellow leaf curl virus. Furthermore, no cross reactions have been observed for Clavibacter michiganensis subsp. michiganensis.

**Selectivity**: No apparent matrix effects were observed for pepper and tomato seeds.

**Repeatability & reproducibility**: The test was validated in both an intra- and inter-laboratory comparison. For pepper seeds contaminated with PSTVd, TASVd and both PCFVd and CLVd, as well as tomato seeds contaminated with TASVd, TCDVd and TPMVd, both repeatability and reproducibility were 100%, respectively.

**Diagnostic sensitivity and diagnostic specificity**: Comparison of the PospiSense with the Real-time RT-PCR method for seed testing of Naktuinbouw (Section 3.4.3.4) by testing 40 pospiviroid-infected samples and 4 healthy samples showed 100% concordance. It should be noted, however, that the PospiSense (Botermans et al, 2020) test appeared less sensitive for CEVd and TASVd than the Naktuibouw test,

**4. Real-time RT-PCR for the detection of pospiviroids in seeds (Naktuinbouw (2017) (section 3.4.3.4**).

- *Nucleic acid extraction method:* Sbeadex® maxi plant kit (LGC)

- *Information on validation:* Validation data (EPPO database) were generated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic* activity (Naktuinbouw, The Netherlands). Validation data are available at <http://dc.eppo.int/validationlist.php>. *Pospiviroids: validation sheets for ‘Detection of pospiviroids by real-time RT-PCR on tomato and pepper seeds* and *Detection of Potato spindle tuber viroid (PSTVd) and/or Tomato chlorotic dwarf viroid (TCDVd) in tomato seed with real-time RT-PCR (TaqMan RT-PCR) (Boonham et al, 2004)*.

*Analytical sensitivity:* For all seven viroids at least the 1000x dilution was. Only for TPMVd not all 1000x dilutions were detected below the Ct threshold of 32.

*Analytical specificity*: No cross reactions with 29 isolates of other viruses and viroids tested. No false negatives were observed for all primer sets and none of the non-target viroids and viruses reacted with the Real-time RT-PCRs. Some acceptable cross-reactivity of TASVd isolates with the CEVd/CLVd primer mix (B) was observed.

*Selectivity*: No apparent matrix effects were observed for pepper and tomato seeds.

*Repeatability & reproducibility:* 100% repeatability and reproducibility for all target species.

5. Real-time RT-PCR for the detection of CSVd in all matrices (Section 3.4.3.5).

- *Nucleic extraction method*: KingFisher mL Magnetic Particle Processor (Thermo Scientific).

- *Information on validation*: Validation data were generated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity*. Fera, UK (as of 04/2019, Fera pers. comm.).

***Analytical sensitivity***: CSVd was detected in chrysanthemum up to a dilution of 1:105. There was no difference when using different RNA extraction methods (CTAB, Promega SV and Thermo Scientific KingFisher Total RNA kit).

***Analytical specificity***: By testing variants of CSVd and synthetic oligonucleotides it has been shown that this test detects all known sequence variants.

***Selectivity***: CSVd was detected in a wide range of chrysanthemum samples representing more than 750 samples from more than 20 chrysanthemum varieties. No apparent matrix effects were observed.

***Repeatability & reproducibility***: The test was validated in both an intra- and inter-laboratory comparison. The test displayed high repeatability and reproducibility (Ct values deviation between 0.2 to 2 with an average of 1 for 20 independent samples tested).

**Table 2**: Recommended test for the detection of all listed pospiviroid species. Position and size of amplicon are given for RT-PCR tests only.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Section** | **Primers & Probes** | **CSVd** | **CEVd** | **CLVd** | **IrVd-1** | **PCFVd** | **PSTVd** | **TASVd** | **TCDVd** | **TPMVd** | **Position of amplicona** | **Size** | **References on validation** | **Reference** |
| **3.4.3.1** | Pospi1b | + | + | - | + | + | + | + | + | + | 86-283 PSTVd NC\_002030 | 198 nt | EPPO validation data NPPO-NL (2013a) | Verhoeven *et al.*, 2004 |
| pCLV | nt | - | + | - | nt | nt | - | nt | nt | 102-101 CLVd NC\_003538 | 370 nt | Olivier *et al.*, 2016; NPPO-NL (unp.) | Spieker, 1996 |
| **3.4.3.2** |  GenPospi | + | + | - | + | + | + | + | + | + | N.A. | - | EPPO validation data NPPO-NL (2013c) | Botermans *et al.*, 2013 |
| CLVd | - | - | + | - | - | - | - | - | - | N.A. | - | EPPO validation data NPPO-NL (2013c) | Monger *et al.*, 2010 |
| **3.4.3.3** | PospiSense1 | nt | - | + | nt | + | + | - | + | + | N.A. | - | Botermans *et al*; 2020; | Botermans *et al*; 2020; Monger *et al.*, 2010 |
| PospiSense2 | nt | + | - | nt | - | - | + | - | - | N.A. |  | Botermans *et al*; 2020; | Botermans *et al 2020* |
| **3.4.3.4** | Mix A | nt | - | - | - | + | + | - | + | +e | N.A. | - | Testa (2015); EPPO validation data (Naktuinbouw,2017) | Boonham *et al.*, 2004; Naktuinbouw, 2017 |
| Mix B | - | + | + | nt | - | - | +f | - | - | N.A. | - | Testa (2015); EPPO validation data (Naktuinbouw, 2017) | Monger *et al.*, 2010; Naktuinbouw, 2017 |
| Mix C | nt | - | - | nt | - | - | - | - | + | N.A. | - | Testa (2015); EPPO validation data (Naktuinbouw, 2017) | Botermans *et al.*, 2013; Naktuinbouw, 2017 |
| Mix D | nt | - | - | nt | - | - | + | - | - | N.A. | - | Testa (2015); EPPO validation data (Naktuinbouw, 2017) | Monger *et al.*, 2010 |
| **3.4.3.5** | CSVd  | + | nt | nt | nt | nt | - | nt | nt | nt | N.A. | - | c.f. Section 4 (Fera, UK) | Mumford *et al.* 2000 |

+ detected; - not detected; nt not tested; a Position of amplicon in reference sequence of indicated species in NCBI GenBank; b Sequence of PCR product can be used for identification; c It is known that not all isolates of CLVd (GenBank acc. no. FM995506.1) will be detected (Steyer et al., 2010); d Complete sequence includes primer sequences (because of the circular genome it is advisable to include these sequences in BLAST searches); e It is known that at least one isolate of TPMVd (GenBank acc. no. K00817.1) will not or only be poorly detected (Testa, 2015; EPPO validation data (Naktuinbouw, 2017); f May cross react with some isolates (Monger et al., 2010).

**Table 3**: Overview of additional PCR-based tests suitable for pospiviroid detection and/or identification. Position and size of amplicon are given for RT-PCR tests only.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Reference** | **Primers & probes** | **CSVd** | **CEVd** | **CLVd** | **IrVd-1** | **PCFVd** | **PSTVd** | **TASVd** | **TCDVd** | **TPMVd** | **Position of amplicon\*** | **Size** | **References on validation** |
| Boonham *et al.*, 2004 | PSTV | - | - | nt | - | - | + | - | + | +a | N.A. | - | Naktuinbouw (2012a), NAK (2015) |
| Hooftman *et al.*, 1996 | CSVdh/c | + | nt | nt | nt | nt | nt | nt | nt | nt | 62-112 CSVd NC\_002015 | complete genomeb | c.f. ref. |
| Mumford *et al.*, 2000c | Vir2/1 | + | + | nt | nt | nt | + | nt | nt | nt | 17-280 CSVd NC\_002015 | 264 nt | c.f. section 4 (Fera, UK) |
| CSVd | + | nt | nt | nt | nt | - | nt | nt | nt | N.A. | - |
| Önelge, 1997 | CEVd | nt | + | nt | nt | nt | nt | +d | nt | nt | 80-117 CEVd NC\_002015 | complete genomeb | c.f. ref. |
| Shamloul *et al.*, 1997 | 3H1/2H1e | nt | nt | nt | nt | nt | + | nt | + | +a | 69-113 PSTVd NC\_002030 | complete genomeb | EPPO validation data EPPO validation data NPPO-NL (2013d) |
| Spieker *et al.*, 1996a | pCLVR4/pCLV4 | nt | - | + | - | nt | nt | - | nt | nt | 102-101 CLVd NC\_003538 | complete genomeb | c.f. ref., NPPO-NL (unp.) |
| Verhoeven *et al.*, 2009 | AP-FW1/RE2 | nt | nt | nt | nt | + | nt | nt | nt | nt | 178-164 PCFVd NC\_011590 | *ca.* complete genome (-13nt) | c.f. ref. |
| Verhoeven *et al.,* 2004 | Vid-FW/RW | - | - | + | - | - | + | - | + | - | 355-354 PSTVd NC\_002030 | complete genome | EPPO validation data NPPO-NL (2013b) |
| Verhoeven *et al.*, 2010b | IrVd-1 | nt | nt | nt | + | nt | nt | nt | nt | nt | 168-167 IrVd-1 NC\_003613 | complete genomeb | c.f. ref. |
| Verhoeven *et al.*, 2017 | Pospi2f | + | + | - | + | + | + | + | + | + | 261-103 PSTVd NC\_002030 | *ca*. half genome | c.f. ref. |
| Monger *et al.*, 2010; Naktuinbouw (unp.) | Generic | + | + | + | nt | nt | + | + | + | nt | N.A. | - | c.f. ref.Testa (2015) |
| CEVd | - | + | - | nt | - | - | +g | - | - | N.A. | - |
| CLVd | - | - | + | nt | - | - | - | - | - | N.A. | - |
| TASVd | - | - | - | nt | - | - | + | - | - | N.A. | - |
| Naktuinbouw (unp.) | PCFVd | nt | - | - | nt | + | - | - | - | - | N.A. | - | Testa, (2015) |

+ detected; - not detected; nt not tested; \*Position of amplicon in reference sequence of indicated species in NCBI GenBank. Amplicon size is given where relevant for cloning and sequencing purposes; nt: not tested. Unp.: unpublished. a It is known that at least one isolate of TPMVd (GenBank acc. no. K00817.1) will not or only be poorly detected (Testa, 2015; EPPO validation data; Naktuinbouw, 2017). b Complete sequence includes primer sequences (because of the circular genome it might be advisable to include these sequences in BLAST searches); c Test described in EPPO DP PM 7/6(1); if not described refer to EPPO (2002) EPPO Bulletin 32, 245-253; d All tested TASVd isolates tested at NPPO-NL were detected so far; e Primer names used in IPPC protocol DP-07 PSTVd; f Primers complementary to pospi1; g CEVd primers and probe cross react with TASVd isolates.

1. 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 validated. [↑](#footnote-ref-1)
2. At this stage an external positive control such as Dahlia latent viroid (DLVd) for real-time RT-PCR, could be added to the homogenization buffer. [↑](#footnote-ref-2)
3. Extraction buffer consists of 200 μL of 8% (w/v) tetrasodium pyrophosphate (TNaPP) solution (pH10) and 100 μL Antifoam B Emulsion (Sigma) added to 9.8 ml Guanidine Lysis Buffer (8M Guanidine Hydrochloride, 20mM Na2EDTA, 3% (w/v) PVP-10, 25mM citric acid monohydrate, 1mM tri-sodium citrate, 0.5% Triton X-100, 25% ethanol). [↑](#footnote-ref-3)