2024 FIRST CONSULTATION

30 January – 03 May 2024

Compiled comments for 2024 First Consultation: 2018-031_DP_Pospiviroid_species

Summary

Participants

Colombia	Completed	Reviewer	Referencias Bibliográficas: Fernow KH, Peterson LC & Plaisted RL (1970) Spindle tuber virus in seeds and pollen of infected plants. American Potato Journal 47, 75–80. Singh RP (1970) Seed transmission of potato spindle tuber virus in tomato and potato. American Potato Journal 47, 225–227. Merriam D & Bonde R (1954) Dissemination of spindle tuber by contaminated tractor wheels and by foliage contact with diseased plants. Phytopathology 44, 111. Manzer FE & Merriam D (1961) Field transmission of potato spindle tuber virus and virus X by cultivating and hilling equipment. American Potato Journal 38, 346–352. Morris TJ & Smith EM (1977) Potato spindle tuber disease: procedures for the detection of viroid RNA and certification of disease-free potato tubers. Phytopathology 67, 145–150 Gos RW (1926) Transmission of potato spindle tuber disease by cutting knives and seed piece contact. Phytopathology 16, 299–304.

T (Type) - B = Bullet, C = Comment, P = Proposed Change, R = Rating
S (Status) - A = Accepted, C = Closed, O = Open, W = Withdrawn, M = Merged

Para	Text	T	Comment
G	(General Comment)	C	*Category : SUBSTANTIVE* **(456) Barbados (3 May 2024 9:02 PM)** Barbados has no objections to this diagnostic protocol being added as an annex to ISPM 27. It is well researched and provides the necessary info for both large and small lots that have to be assayed.
G	(General Comment)	C	*Category : EDITORIAL* **(438) Nepal (1 May 2024 1:36 PM)** Nepal does not have any comment on the document
G	(General Comment)	C	*Category : SUBSTANTIVE* **(437) Philippines (1 May 2024 11:48 AM)**

			PH has no further comments on the Draft annexes to ISPM 27 Genus Pospiviroid (2018-031)
G	(General Comment)	C	*Category : SUBSTANTIVE* **(243) Guyana (3 Apr 2024 9:31 PM)** Guyana commends the efforts of the IPPC and TP in providing such a relevant and informative protocol. There are no objections at this time.
G	(General Comment)	C	*Category : TECHNICAL* **(1) Congo (1 Feb 2024 5:51 AM)** i approve the draft annexe to ISPM 27
29	Johanna ROENHORST and Carla OPLAAT (Netherlands Institute for Vectors, Invasive Plants and Plant health, ~~National Plant Protection Organization~~Netherlands Food and Consumer Product Safety Authority, Wageningen, NL)	P	*Category : EDITORIAL* **(278) EPPO (30 Apr 2024 7:53 PM)**
33	Ellis ~~Meeks Naktuinbouw~~Meekes (Naktuinbouw, ~~NL~~NL)	P	*Category : EDITORIAL* **(279) EPPO (30 Apr 2024 7:53 PM)**
34	Harrie ~~Koenraadt~~Koenraadt (Naktuinbouw, ~~Naktuinbouw, NL~~NL)	P	*Category : EDITORIAL* **(280) EPPO (30 Apr 2024 7:53 PM)**
52	Viroids are subviral agents that infect plants. A viroid consists of a circular, un-encapsidated single-stranded RNA molecule, with a genome of ~~239–401~~239–434 bp nucleotides, that does 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 and Owens, 2006). Viroids are unique among plant pathogens and are assigned to 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 the assignment of viroid species within five genera including the genus *Pospiviroid* (Di Serio *et al.*, 2014; Di Serio *et al.*, 2021a).	P	*Category : TECHNICAL* **(451) New Zealand (3 May 2024 5:23 AM)** Genome size of AHVd is 434 bp. Suggest changing the genome size of viroid to 239 – 434 bp nucleotides.
52	Viroids are subviral agents that infect plants. A viroid consists of a circular, un-encapsidated single-stranded RNA molecule, with a genome of ~~239–401~~250-430 nucleotides, that does 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	P	*Category : SUBSTANTIVE* **(272) China (24 Apr 2024 8:06 AM)** According to ICTV, members of family Avsunviroidae are composed of circular single-stranded RNAs ranging in size from 246 to 434 nt, and members of the family Pospiviroidae are 246–375. If the authors decided to give an

	viroid's RNA as a template (Hammond and Owens, 2006). Viroids are unique among plant pathogens and are assigned to 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 the assignment of viroid species within five genera including the genus *Pospiviroid* (Di Serio *et al.*, 2014; Di Serio *et al.*, 2021a).		exact range, the length of viroids' genome should recheck. Considering the new isolates are being report very now and then, China recommends to use the description of ICTV, which was about 250–430 nt.
52	Viroids are subviral agents that infect plants. A viroid consists of a circular, un-encapsidated single-stranded RNA molecule, with a genome of 239–401 nucleotides, that does 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 and Owens, 2006). Viroids are unique among plant pathogens and are assigned to 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 the assignment of viroid species within five genera including the genus *Pospiviroid* (Di Serio *et al.*, 2014; Di Serio *et al.*, 2021a).	C	*Category : TECHNICAL* **(244) Canada (19 Apr 2024 2:52 PM)** Some viroids are reported to have larger genomes e.g. Citrus exocortis viroid isolate D-104 (AY671952.1) has 475 bp genome
52	Viroids are subviral agents that infect plants. A viroid consists of a closed circular, un-encapsidated single-stranded RNA molecule, with a genome of 239–401 nucleotides, that does 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 and Owens, 2006). Viroids are unique among plant pathogens and are assigned to 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	P	*Category : TECHNICAL* **(231) United States of America (1 Apr 2024 7:38 PM)** for technical precision

	the assignment of viroid species within five genera including the genus *Pospiviroid* (Di Serio *et al.*, 2014; Di Serio *et al.*, 2021a).		
53	The genus *Pospiviroid* consists of ten viroid species (ICTV, n.d.). The corresponding viroids and the species to which they belong are as follows: chrysanthemum stunt viroid (CSVd; species *Chrysanthemum stunt viroid*), citrus exocortis viroid (CEVd; species *Citrus exocortis viroid*), Columnea latent viroid (CLVd; species *Columnea latent viroid*), iresine viroid 1 (IrVd-1; species *Iresine viroid 1*), pepper chat fruit viroid (PCFVd; species *Pepper chat fruit viroid*), portulaca latent viroid (PLVd; species *Pospiviroid plvd*; Verhoeven *et al*., 2015; Di Serio *et al*., 2021a), potato spindle tuber viroid (PSTVd; species *Potato spindle tuber viroid*; type species), tomato apical stunt viroid (TASVd; species *Tomato apical stunt viroid*), tomato chlorotic dwarf viroid (TCDVd; species *Tomato chlorotic dwarf viroid*) and tomato planta macho viroid (TPMVd; species *Tomato planta macho viroid*, including the former *Mexican papita viroid*). Species demarcation is based on sequence similarity level (less than 90% sequence identity of the total viroid genome) and on distinctive biological properties, particularly 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, differential 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 and TCDVd) but differ in host range and symptom expression (Martinez-Soriano *et al*., 1996; Singh, Nie and Singh, 1999; Matsushita, Usugi and Tsuda, 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 criteria used by the International Committee on Taxonomy of Viruses (ICTV) for viroid classification (Tangkanchanapas *et al.*, 2021). On that basis, the authors of the publication propose that CLVd should be reclassified 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 diagnostic protocol will refer to CLVd as a single homogenous phylogenetic lineage. The	C	*Category : TECHNICAL* **(255) Canada (22 Apr 2024 4:36 PM)** Not sure, if this is the best way to state the date. I suggest using the date when you last time checked the ICTV website.

	latest information on classification of the genus *Pospiviroid* may be obtained from the ICTV (n.d.).		
54	Pospiviroids have been reported worldwide (Faggioli *et al*., 2017). They can cause severe diseases in their hosts, particularly *Solanum tuberosum* (potato) and *Solanum lycopersicum* (tomato) crops in the case of PSTVd. Therefore, pospiviroids are regulated in many countries (see EPPO, n.d.(a)). Pospiviroids can be experimentally transmitted to many plant species, but their natural host ranges differ between different pospiviroids (Table 1). Pospiviroids are readily transmitted by contact and cutting tools, especially at temperatures above 25 °C. In addition, pospiviroids can 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 (Fernow, Peterson and Plaisted, 1970; Singh 1970; Matsushita and Tsuda, 2016) and TASVd (Antignus, Lachman and Pearlsman, 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 *Capsicum annuum* (pepper) and *S. lycopersicum* may have been overestimated. Horizontal transmission through infected pollen has been documented for CSVd, PSTVd and TPMVd (Kryczyński, Paduch-Cichal and Skrzeczkowski, 1988; Singh, Boucher and Somerville, 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, Lopez and Aguilar, 1986; reviewed in Hadidi, Sun and Randles, 2022); however, in some cases it cannot be excluded that cross-contamination (such as contact transmission) could have occurred. Potato spindle tuber viroid 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, Marczewski and Pawłowicz, 1997). Transmission of TASVd and TCDVd by *Bombus ignitus* (bumblebee) in greenhouses has also been reported, with the transmission possibly being through the transfer of viroid-contaminated pollen (Antignus *et al.*, 2007; Matsuura *et al.*, 2010).	C	*Category : EDITORIAL* **(285) EPPO (30 Apr 2024 7:53 PM)** It is suggested to add natural infection of tomato by CEVd, CLVd, PSTVd, TCDVd (Verhoeven et al, 2004)

54	Pospiviroids have been reported worldwide (Faggioli *et al*., 2017). They can cause severe diseases in their hosts, particularly *Solanum tuberosum* (potato) and *Solanum lycopersicum* (tomato) crops in the case of PSTVd. Therefore, pospiviroids are regulated in many countries (see EPPO, n.d.(a)). Pospiviroids can be experimentally transmitted to many plant species, but their natural host ranges differ between different pospiviroids (Table 1). Pospiviroids are readily transmitted by contact and cutting tools, especially at temperatures above 25 °C. In addition, pospiviroids can 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 (Fernow, Peterson and Plaisted, 1970; Singh 1970; Matsushita and Tsuda, 2016) and TASVd (Antignus, Lachman and Pearlsman, 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 *Capsicum annuum* (pepper) and *S. lycopersicum* may have been overestimated. Horizontal transmission through infected pollen has been documented for CSVd, PSTVd and TPMVd (Kryczyński, Paduch-Cichal and Skrzeczkowski, 1988; Singh, Boucher and Somerville, 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, Lopez and Aguilar, 1986; reviewed in Hadidi, Sun and Randles, 2022); however, in some cases it cannot be excluded that cross-contamination (such as contact transmission) could have occurred. Potato spindle tuber viroid 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, Marczewski and Pawłowicz, 1997). Transmission of TASVd and TCDVd by *Bombus ignitus* (bumblebee) in greenhouses has also been reported, with the transmission possibly being through the transfer of viroid-contaminated pollen (Antignus *et al.*, 2007; Matsuura *et al.*, 2010).	C	*Category : EDITORIAL* **(284) EPPO (30 Apr 2024 7:53 PM)** also refer to Van Bogaert et al 2015 who conducted controlled transmission experiments with B terrestis, M. persicae and Macrolophus pygmaeus. DOI 10.1007/s10658-015-0766-9
54	Pospiviroids have been reported worldwide (Faggioli *et al*., 2017). They can cause severe diseases in their hosts, particularly *Solanum tuberosum* (potato) and *Solanum lycopersicum* (tomato) crops in the case of PSTVd. Therefore,	C	*Category : EDITORIAL* **(283) EPPO (30 Apr 2024 7:53 PM)** Suggestion to rephrase since authors stated that it was not possible to discrimate whehter transmission

	pospiviroids are regulated in many countries (see EPPO, n.d.(a)). Pospiviroids can be experimentally transmitted to many plant species, but their natural host ranges differ between different pospiviroids (Table 1). Pospiviroids are readily transmitted by contact and cutting tools, especially at temperatures above 25 °C. In addition, pospiviroids can 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 (Fernow, Peterson and Plaisted, 1970; Singh 1970; Matsushita and Tsuda, 2016) and TASVd (Antignus, Lachman and Pearlsman, 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 *Capsicum annuum* (pepper) and *S. lycopersicum* may have been overestimated. Horizontal transmission through infected pollen has been documented for CSVd, PSTVd and TPMVd (Kryczyński, Paduch-Cichal and Skrzeczkowski, 1988; Singh, Boucher and Somerville, 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, Lopez and Aguilar, 1986; reviewed in Hadidi, Sun and Randles, 2022); however, in some cases it cannot be excluded that cross-contamination (such as contact transmission) could have occurred. Potato spindle tuber viroid 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, Marczewski and Pawłowicz, 1997). Transmission of TASVd and TCDVd by *Bombus ignitus* (bumblebee) in greenhouses has also been reported, with the transmission possibly being through the transfer of viroid-contaminated pollen (Antignus, Lachman and Pearlsman, 2007; Matsuura *et al.*, 2010).		was by wounding of flowers (mechanical transfer) or by introduction of infected pollen to the stigma, and this information is lacking.
54	Pospiviroids have been reported worldwide (Faggioli *et al*., 2017). They can cause severe diseases in their hosts, particularly *Solanum tuberosum* (potato) and *Solanum lycopersicum* (tomato) crops in the case of PSTVd. Therefore, pospiviroids are regulated in many countries (see EPPO, n.d.(a)). Pospiviroids can be experimentally transmitted to many plant species, but their natural host ranges	P	*Category : EDITORIAL* **(282) EPPO (30 Apr 2024 7:53 PM)**

	differ between different pospiviroids (Table 1). Pospiviroids are readily transmitted by contact and cutting tools, especially at temperatures above 25 °C. In addition, pospiviroids can 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 (Fernow, Peterson and Plaisted, 1970; Singh 1970; Matsushita and Tsuda, 2016) and TASVd (Antignus, Lachman and Pearlsman, 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 *Capsicum annuum* (pepper) and *S. lycopersicum* may have been overestimated. Horizontal transmission through infected pollen has been documented for CSVd, PSTVd and TPMVd (Kryczyński, Paduch-Cichal and Skrzeczkowski, 1988; Singh, Boucher and Somerville, 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, Lopez and Aguilar, 1986; reviewed in Hadidi, Sun and Randles, 2022); however, in some cases it cannot be excluded that cross-contamination (such as contact transmission) could have occurred. Potato spindle tuber viroid 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, Marczewski and Pawłowicz, 1997). Transmission of TASVd and TCDVd by *Bombus ignitus* (bumblebee) in greenhouses has also been reported, with the transmission possibly being through the transfer of viroid-contaminated pollen ~~(Antignus~~(Antignus *et al.*, ~~Lachman and Pearlsman,~~ 2007; Matsuura *et al.*, 2010).		
54	Pospiviroids have been reported worldwide (Faggioli *et al*., 2017). They can cause severe diseases in their hosts, particularly *Solanum tuberosum* (potato) and *Solanum lycopersicum* (tomato) crops in the case of PSTVd. Therefore, pospiviroids are regulated in many countries (see EPPO, n.d.(a)). Pospiviroids can be experimentally transmitted to many plant species, but their natural host ranges differ between different pospiviroids (Table 1). Pospiviroids are readily transmitted by contact and cutting tools, especially at temperatures above 25 °C. In addition, pospiviroids can spread by vegetative propagation and transmission via seeds. Seed	C	*Category : EDITORIAL* **(281) EPPO (30 Apr 2024 7:53 PM)** For other pospiviroids causing diseases in tomato, see Verhoeven et al., 2004. Add information and reference? This explains why several pospiviroids are regulated, as is stated in the next sentence.

	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 (Fernow, Peterson and Plaisted, 1970; Singh 1970; Matsushita and Tsuda, 2016) and TASVd (Antignus, Lachman and Pearlsman, 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 *Capsicum annuum* (pepper) and *S. lycopersicum* may have been overestimated. Horizontal transmission through infected pollen has been documented for CSVd, PSTVd and TPMVd (Kryczyński, Paduch-Cichal and Skrzeczkowski, 1988; Singh, Boucher and Somerville, 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, Lopez and Aguilar, 1986; reviewed in Hadidi, Sun and Randles, 2022); however, in some cases it cannot be excluded that cross-contamination (such as contact transmission) could have occurred. Potato spindle tuber viroid 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, Marczewski and Pawłowicz, 1997). Transmission of TASVd and TCDVd by *Bombus ignitus* (bumblebee) in greenhouses has also been reported, with the transmission possibly being through the transfer of viroid-contaminated pollen (Antignus, Lachman and Pearlsman, 2007; Matsuura *et al.*, 2010).		
56	**Viroids name:** chrysanthemum stunt viroid (acronym CSVd)	C	*Category : EDITORIAL* **(286) EPPO (30 Apr 2024 7:53 PM)** Please check (consistency of) capital use for virus names
57	**Species name:** **Pospiviroid impedichysanthemi**~~*Chrysanthemum stunt viroid*~~	P	*Category : TECHNICAL* **(450) New Zealand (3 May 2024 5:21 AM)** Species names of viroids need to be updated according to the current ICTV naming. Chrysanthemum stunt viroid to Pospiviroid impedichysanthemi
61	**Viroid name:** citrus exocortis viroid (acronym CEVd)	C	*Category : EDITORIAL* **(287) EPPO (30 Apr 2024 7:53 PM)** Space when new species is mentioned?

62	**Species name:** - not assigned~~*Citrus exocortis viroid*~~	P	*Category : TECHNICAL* **(232) United States of America (1 Apr 2024 7:39 PM)** corrected taxonomy
88	**Species name:** *Pospiviroid plvd*	C	*Category : EDITORIAL* **(288) EPPO (30 Apr 2024 7:53 PM)** A comment may be needed to indicate that the viroid name is used instead of the species name, although there is not a lot of info regarding this viroid in the protocol
108	~~Symptoms of pospiviroid infections are not specific to each viroid: variation in symptoms within each viroid is similar to variation between viroids and an infection may be asymptomatic in many hosts. Detection and identification of pospiviroids can be achieved by using the molecular methods shown in Figure 1 and the corresponding sections of this protocol. Very few molecular methods are available that are specific to one viroid, with the majority of methods detecting a few viroids simultaneously because of a lack of primer specificity~~Symptoms of pospiviroid infections are not specific to each viroid: variation in symptoms within each viroid is similar to variation between viroids and an infection may be asymptomatic in many hosts. Detection and identification of pospiviroids can be achieved by using the molecular methods shown in Figure 1 and the corresponding sections of this protocol. There are very few molecular methods that are specific to one viroid; most tests are non-specific and can also detect non-target viroids . Additional information on pospiviroid detection and identification can be found in EPPO (2021a) and on PSTVd detection and identification in diagnostic protocol (DP) No. 7 (ISPM 27 (*Diagnostic protocols for regulated* pests), Annex 7).	P	*Category : TECHNICAL* **(290) EPPO (30 Apr 2024 7:53 PM)**
108	Symptoms of pospiviroid infections are not specific to each viroid: variation in symptoms within each viroid is similar to variation between viroids and an infection may be asymptomatic in many hosts. Detection and identification of pospiviroids can be achieved by using the molecular methods shown in Figure 1 and the corresponding sections of this protocol. Very few molecular methods are available that are specific to one viroid, with the majority of methods detecting a few viroids simultaneously because of a lack of primer specificity. Additional information on pospiviroid detection and identification can be found in EPPO (2021a) and on PSTVd detection and identification in diagnostic protocol (DP)	C	*Category : EDITORIAL* **(289) EPPO (30 Apr 2024 7:53 PM)** Throughout the whole document, "method" is used where "test" should be the correct term, according to EPPO definition. Please verify throughout the whole document.

	No. 7 (ISPM 27 (*Diagnostic protocols for regulated* pests), Annex 7).		
109		C	*Category : EDITORIAL* **(291) EPPO (30 Apr 2024 7:53 PM)** The lowest box: delete if applicaable (as the text in the box already starts with "if approprate")
115	Pospiviroids are generally distributed within most tissues of the plant, including seed. Their propensity to stimulate the development of symptoms largely depends on the viroid and isolate, the host species and cultivar, and the environmental conditions. Infected ornamental species are often symptomless. Although pospiviroids are often found in solanaceous species, some have also been reported infecting other plant species (see Table 1) (EFSA Panel on Plant Health, 2011; EPPO, 2021a).	C	*Category : EDITORIAL* **(294) EPPO (30 Apr 2024 7:53 PM)** No change of text is requested
115	Pospiviroids are generally distributed within most tissues of the plant, including seed. Their propensity to stimulate the development of symptoms largely depends on the viroid and isolate, the host species and cultivar, and the environmental conditions. Infected ornamental species are often symptomless. Although pospiviroids are often found in solanaceous species, some have also been reported infecting other plant species (see Table 1) (EFSA Panel on Plant Health, 2011; EPPO, 2021a).	C	*Category : TECHNICAL* **(293) EPPO (30 Apr 2024 7:53 PM)** This is one of the reasons why we would not recommend the sample preparation procedure by Sang et al., 2006 (EDTA method). Further explanations are given later in the corresponding Chapter (3.4.2.2.).
115	Pospiviroids are generally distributed within most tissues of the plant, including seed. Their propensity to stimulate the development of symptoms largely depends on the viroid and isolate, the host species and cultivar, and the environmental conditions. Infected ornamental species are often symptomless. Although pospiviroids are often found in solanaceous species, some have also been reported infecting other plant species (see Table 1) (EFSA Panel on Plant Health, 2011; EPPO, 2021a).	C	*Category : EDITORIAL* **(292) EPPO (30 Apr 2024 7:53 PM)** This suggests that there are tissues in which the viroid is not present. Is there a reference? If not, I woould suggest to rephrase.
116	**Table 1.** Natural host range of members of the *Pospiviroid* genus	C	*Category : EDITORIAL* **(295) EPPO (30 Apr 2024 7:53 PM)** Please verify font of the header of tables. These seem to be different from the font of the main text. Is this on purpose?
116	**Table 1.** Natural host range of members of the *Pospiviroid* genus	C	*Category : TECHNICAL* **(265) Japan (23 Apr 2024 5:01 AM)** Japan proposes the host range of Table 1 be reviewed as this table includes plants reported as

			experimental host (PCFVd, TPMVd : Petunia spp., Yanagisawa and Matsushita (2017, EJPP, 149, 211–217)). If it is difficult to describe all natural hosts in case of listing only natural hosts in the Table 1, the title of the Table 1 could be changed to "Representative natural hosts of members of the Pospiviroid genus".
120	~~*Ageratum*~~*Ageratum* spp., *Argyranthemum frutescens*, *Chrysanthemum ×morifolium*, *Dahlia* spp., *Gerbera* spp., *Pericallis* spp., *Petunia* spp., *Physalis alkekengi*, *Solanum* spp., *Verbena* spp., *Vinca* spp.	P	*Category : EDITORIAL* **(445) Brazil (2 May 2024 6:11 PM)**
121	Citrus exocortis viroid (CEVd)	C	*Category : TECHNICAL* **(227) CA (19 Mar 2024 6:12 PM)** In order to complete the transmition message, it is proposed to adition those paragraphs: Citrus spp. In citrus trees, CEVd may cause scaling, shelling (exocortis) and splitting of the bark and stunted growth, resulting in significant yield reduction (Semancik and Weathers, 1972a, 1972b; EFSA, 2008; Lin et al., 2015). In Citrus medica (Etrog citron), CEVd may induce a variety of symptoms including severe stunting, leaf epinasty and rugosity, petiole wrinkle and necrosis, midvein necrosis, and browning of the tip of the leaf blade that become more pronounced over time. Synergistic effects of CEVd with other citrus viroids have resulted in enhanced bark scaling or reduced tree growth and yield (reviewed in Zhou et al., 2020) CEVd can be transmitted from one tree to another by budding or grafting, also by pruning activities, however, it is not transmitted by seed and no vectors have been known to spread the disease. P Category : SUBSTANTIVE (4) Colombia (28 feb. 2024 20:45) In order to complete the transmition message, it is proposed to adition those paragraphs O
122	*Cestrum* spp., *Citrus* spp., *Glandularia pulchella*, *Impatiens* spp., *Lycianthes rantonnetii, Petunia* spp., *Petunia* × *Calibrachoa*, *Solanum* spp., *Verbena* spp., *Vitis* spp.	P	*Category : TECHNICAL* **(446) Brazil (2 May 2024 6:14 PM)** There is a report of Citrus exocortis viroid (CEVd) on Vitis spp. in Brazil. Reference: https://doi.org/10.1590/S0100-

			41582006000500002
128	*Capsicum* spp., *Petunia* spp., *Solanum* spp.	C	*Category : EDITORIAL* **(297) EPPO (30 Apr 2024 7:53 PM)** it is suggested to keep natural hosts only as title of the table
128	*Capsicum* spp., *Petunia* spp., *Solanum* spp.	C	*Category : SUBSTANTIVE* **(296) EPPO (30 Apr 2024 7:53 PM)** Was this a natural infection? If not, please indicate or delete
136	*Brugmansia* spp., *Calibrachoa* spp., *Petunia* spp., *Pittosporum* spp., *Solanum* spp., *Verbena* spp., *Vinca* spp~~.~~*. Dahlia pinnata Cav.*	P	*Category : SUBSTANTIVE* **(273) China (24 Apr 2024 9:07 AM)** https://doi.org/10.1094/PDIS-02-23-0389-PDN
138	*Petunia* spp., *Solanum lycopersicum*	C	*Category : SUBSTANTIVE* **(298) EPPO (30 Apr 2024 7:53 PM)** Was this a natural infection? If not, please indicate or delete
139	*Source:* Based and updated from ~~EPPO~~EPPO Global Database, n.d.(a) and EPPO, 2021a.	P	*Category : EDITORIAL* **(299) EPPO (30 Apr 2024 7:53 PM)**
139	*Source:* Based and updated from EPPO, n.d.(a) and EPPO, 2021a.	C	*Category : TECHNICAL* **(266) Japan (23 Apr 2024 6:52 AM)** Japan proposes inclusion of all references in "Source". For example, host plant of Portulaca latent viroid (PLVd) is not mentioned in EPPO, n.d.(a) and EPPO, 2021a..We propose all reference materials included in Source after checking whether it is natural host or not. Otherwise, it could add "mainly" as "Source: Based and updated from mainly EPPO, n.d.(a) and EPPO, 2021a."
140	On their main hosts, the following symptoms have been observed (see also EPPO (2021a) for additional information and photos of symptoms in EPPO Global Database (n.d.(b))).	P	*Category : EDITORIAL* **(300) EPPO (30 Apr 2024 7:53 PM)**
142	***Chrysanthemum ~~×morifolium~~× morifolium* (chrysanthemum).** The main symptom of CSVd in *C. ~~×morifolium~~× morifolium* is stunting (Diener and Lawson, 1973; Hollings and Stone, 1973). Stems may 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	P	*Category : EDITORIAL* **(301) EPPO (30 Apr 2024 7:53 PM)** Note that a blanc space is missing after × used in plant names. Please check throughout the document

	presence of pale, upright young leaves is often the only indication of infection. Sometimes, leaf spots or flecks are observed, which may be associated with leaf distortions (crinkling). However, many *C.* ~~×~~× *morifolium* cultivars are symptomless when infected. ~~When symptoms~~ Symptoms are ~~seen, they are~~ often variable and dependent on environmental conditions, especially temperature and light.		
143	***Citrus* spp.** In citrus trees, CEVd may cause scaling, shelling (exocortis) and splitting of the bark and stunted growth, resulting in significant yield reduction (Semancik and Weathers, 1972a, 1972b; EFSA, 2008; Lin *et al.*, 2015). In *Citrus medica* (Etrog citron), CEVd may induce a variety of symptoms including severe stunting, leaf epinasty and rugosity, petiole wrinkle and necrosis, midvein necrosis, and browning of the tip of the leaf blade that become more pronounced over time. Synergistic effects of CEVd with other citrus viroids have resulted in enhanced bark scaling or reduced tree growth and yield (reviewed in Zhou *et al.*, 2020).	P	*Category : EDITORIAL* **(302) EPPO (30 Apr 2024 7:53 PM)**
143	***Citrus* spp.** In citrus trees, CEVd may cause scaling, shelling (exocortis) and splitting of the bark and stunted growth, resulting in significant yield reduction (Semancik and Weathers, 1972a, 1972b; EFSA, 2008; Lin *et al.*, 2015). In *Citrus medica* (Etrog citron), CEVd may induce a variety of symptoms including severe stunting, leaf epinasty and rugosity, petiole wrinkle and necrosis, midvein necrosis, and browning of the tip of the leaf blade that become more pronounced over time. Synergistic effects of CEVd with other citrus viroids have resulted in enhanced bark scaling or reduced tree growth and yield (reviewed in Zhou *et al.*, 2020) CEVd can be transmitted from one tree to another by budding or grafting, also by pruning activities, however, it is not transmitted by seed and no vectors have been known to spread the disease.	P	*Category : TECHNICAL* **(228) CA (20 Mar 2024 2:47 PM)** n order to complete the transmition message, it is proposed to adition those paragraphs
143	***Citrus* spp.** In citrus trees, CEVd may cause scaling, shelling (exocortis) and splitting of the bark and stunted growth, resulting in significant yield reduction (Semancik and Weathers, 1972a, 1972b; EFSA, 2008; Lin *et al.*, 2015). In *Citrus medica* (Etrog citron), CEVd may induce a variety of symptoms including severe stunting, leaf epinasty and rugosity, petiole wrinkle and necrosis, midvein necrosis, and browning of the tip of the leaf blade that become more pronounced over time. Synergistic effects of CEVd with other citrus viroids have resulted in enhanced bark scaling or reduced tree growth and yield (reviewed in Zhou *et al.*, 2020)	P	*Category : SUBSTANTIVE* **(4) Colombia (28 Feb 2024 8:45 PM)** In order to complete the transmition message, it is proposed to adition those paragraphs

	CEVd can be transmitted from one tree to another by budding or grafting, also by pruning activities, however, it is not transmitted by seed and no vectors have been known to spread the disease.		
145	***Solanum tuberosum* (potato).** Until recently, PSTVd was the only viroid known to naturally infect cultivated species of *S. tuberosum*. However, CSVd has been reported in different *S. tuberosum* cultivars, suggesting that CSVd could infect *S. tuberosum* naturally (Matsushita *et al.*, 2019; Matsushita *et al*., 2021). Potato spindle tuber viroid may cause severe to mild symptoms as well as symptomless infections, depending on the PSTVd isolate, *S. tuberosum* cultivar and environmental conditions. Severe symptoms may include reduction in plant size, uprightness and clockwise phyllotaxy of the foliage if viewed from above, and dark green and rugose leaves (Pfannenstiel and Slack, 1980). Tubers may be reduced in size, deformed, spindle- or dumbbell-shaped, with prominent eyes. Under experimental conditions, all pospiviroids (except IrVd-1) can cause tuber symptoms similar to PSTVd (Verhoeven *et al.*, 2004, 2010). In potato, PSTVd is mainly transmitted through the use of infected planting material, produced vegetatively or as botanical seed (Fernow et al., 1970; Singh, 1970). The viroid can also be transmitted mechanically. It survives in dried plant sap and can contaminate tools and storage facilities. Transmission can occur in the growing crop by plant-to-plant contact and passage of machinery (Merriam and Bonde, 1954; Manzer and Merriam, 1961). The extent of mechanical transmission depends on a number of factors, including the host plant species or cultivar serving as the inoculum source, viroid concentration, frequency and severity of damage, and temperature. The viroid may be present in a much lower concentration in plants grown at 25° than at 30°C (Morris & Smith, 1977). Tuber-to-tuber transmission can also occur at the contact of the cutting blades and the seed if the seed tubers are cut into pieces (Gos, 1926).	P	*Category : TECHNICAL* **(229) CA (20 Mar 2024 2:48 PM)** In order to complete the transmition message, it is proposed to adition those paragraphs
145	***Solanum tuberosum* (potato).** Until recently, PSTVd was the only viroid known to naturally infect cultivated species of *S. tuberosum*. However, CSVd has been reported in different *S. tuberosum* cultivars, suggesting that CSVd could infect *S. tuberosum* naturally (Matsushita *et al.*, 2019; Matsushita *et al*., 2021). Potato spindle tuber viroid may cause severe to mild symptoms as well as symptomless	P	*Category : SUBSTANTIVE* **(2) Colombia (28 Feb 2024 8:39 PM)** In order to complete the transmition message, it is proposed to adition those paragraphs

	infections, depending on the PSTVd isolate, *S. tuberosum* cultivar and environmental conditions. Severe symptoms may include reduction in plant size, uprightness and clockwise phyllotaxy of the foliage if viewed from above, and dark green and rugose leaves (Pfannenstiel and Slack, 1980). Tubers may be reduced in size, deformed, spindle- or dumbbell-shaped, with prominent eyes. Under experimental conditions, all pospiviroids (except IrVd-1) can cause tuber symptoms similar to PSTVd (Verhoeven *et al.*, 2004, 2010). In potato, PSTVd is mainly transmitted through the use of infected planting material, produced vegetatively or as botanical seed (Fernow et al., 1970; Singh, 1970). The viroid can also be transmitted mechanically. It survives in dried plant sap and can contaminate tools and storage facilities. Transmission can occur in the growing crop by plant-to-plant contact and passage of machinery (Merriam and Bonde, 1954; Manzer and Merriam, 1961). The extent of mechanical transmission depends on a number of factors, including the host plant species or cultivar serving as the inoculum source, viroid concentration, frequency and severity of damage, and temperature. The viroid may be present in a much lower concentration in plants grown at 25° than at 30°C (Morris & Smith, 1977). Tuber-to-tuber transmission can also occur at the contact of the cutting blades and the seed if the seed tubers are cut into pieces (Gos, 1926).		
147	**3.2 ~~Biological detection~~Bioassay**	P	*Category : EDITORIAL* **(303) EPPO (30 Apr 2024 7:53 PM)**
148	Pospiviroids can be experimentally transmitted to many indicator plants – mostly solanaceous species but also citrus and species from other plant families, depending on the viroid. Symptom expression has been found to range from severe (lethal in some cases) to mild and symptomless. However, the restricted host range of some pospiviroids limits the reliability of such biological detection as a diagnostic method. In addition, the symptoms induced are not viroid specific and may indicate the presence of other viroids or viruses. All pospiviroids (except IrVd-1) could be transmitted to ~~Pospiviroids can be experimentally transmitted to many indicator plants – mostly solanaceous species but also citrus and species from other plant families, depending on the viroid. Symptom expression has been found to range from severe (lethal in some cases) to mild and symptomless. However, the restricted host range of some pospiviroids limits the reliability of such biological~~	P	*Category : EDITORIAL* **(304) EPPO (30 Apr 2024 7:53 PM)** Created by merging other changes together

	~~detection as a diagnostic method for pospiviroids. In addition, the symptoms induced are not viroid specific and may indicate the presence of other viroids and viruses. For example, all pospiviroids (except IrVd-1) could be transmitted to~~ *S. tuberosum* and *S. lycopersicum* and elicit similar symptoms under controlled conditions (Verhoeven *et al*., 2004; EFSA Panel on Plant Health, 2011)~~; IrVd-1 is not likely to be detected by biological methods since no symptoms have been observed in its ornamental hosts~~. Furthermore, there are no validation data published on the use of biological methods for the detection of pospiviroids. However, despite these drawbacks as a detection method, mechanical inoculation of indicator plants can be used for propagation and maintenance of isolates or production of infected material for further testing and identification.		
151	Inoculation of *S. lycopersicum* plants (such as cultivars 'Rutgers', 'Moneymaker' or 'Sheyenne') will allow the detection of many (but not all) pospiviroids and may provide visual evidence of pathogenicity. For PSTVd, mild and severe strains have been described based on symptoms produced by different isolates in cultivar 'Rutgers' (Fernow, 1967), with symptoms including stunting, epinasty, rugosity and lateral twisting of new leaflets, leaf chlorosis, reddening, brittleness and necrosis. Similar symptoms can, however, be caused by other viruses or viroids.	C	*Category : TECHNICAL* **(447) Brazil (2 May 2024 6:59 PM)** It seems to be contradictory to the first paragraph, where is said "the symptoms induced are not viroid specific and may indicate de presence of other viroids and viruses".
151	Inoculation of *S. lycopersicum* plants (such as cultivars 'Rutgers', 'Moneymaker' or 'Sheyenne') will allow the detection of many (but not all) pospiviroids and may provide visual evidence of pathogenicity. For PSTVd, mild and severe strains have been described based on symptoms produced by different isolates in cultivar 'Rutgers' (Fernow, 1967), with symptoms including stunting, epinasty, rugosity and lateral twisting of new leaflets, leaf chlorosis, reddening, brittleness and necrosis. Similar symptoms can, however, be caused by other viruses or viroids.	C	*Category : EDITORIAL* **(306) EPPO (30 Apr 2024 7:53 PM)** also for eg CLVd mild and severe strains have been described on Solanaceaous plants, also on tomato , incl Rutgers, however also host plant associated (tomato vs pepper vs eggplant) (Tangkanchanapas et al. 2021) https://doi.org/10.3390/ microorganisms9061117
151	Inoculation of *S. lycopersicum* plants (such as cultivars 'Rutgers', 'Moneymaker' or 'Sheyenne') will allow the detection of many (but not all) pospiviroids and may provide visual evidence of pathogenicity. For PSTVd, mild and severe strains have been described based on symptoms produced by different isolates in cultivar 'Rutgers' (Fernow, 1967), with symptoms including stunting, epinasty, rugosity and lateral twisting of new leaflets, leaf chlorosis, reddening, brittleness and necrosis. Similar symptoms can, however, be caused by other ~~viruses~~ viroids or	P	*Category : EDITORIAL* **(305) EPPO (30 Apr 2024 7:53 PM)**

	~~viroids~~viruses.		
152	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 *C. medica* 'Arizona 861-S' grown on *Citrus jambhiri* (rough lemon) rootstock, the biological-detection host for citrus viroids, and onto *Gynura aurantiaca* (purple velvet) (Lin *et al.*, 2015; Dang *et al.*, 2022). Infected leaves show symptoms such as epinasty, leaf curling, and midvein and petiole browning. Stunting can be observed between three to eight months after grafting with CEVd-infected buds (Lin *et al*., 2015).	C	*Category : EDITORIAL* **(307) EPPO (30 Apr 2024 7:53 PM)** Sentence too long. Please split.
153	Some plant species (e.g. *Brugmansia* spp.) are unsuitable as indicator plants as they contain biochemicals that may inhibit the transmission of viroids.	C	*Category : SUBSTANTIVE* **(311) EPPO (30 Apr 2024 7:53 PM)** It is suggested to give aanother example than Brugmansia spp
153	Some plant species (e.g. *Brugmansia* spp.) are unsuitable as indicator plants ~~as they contain biochemicals that may inhibit the transmission of viroids~~due to poor mechanical infection rate.	P	*Category : TECHNICAL* **(310) EPPO (30 Apr 2024 7:53 PM)**
153	Some plant species (e.g. *Brugmansia* spp.) are unsuitable as indicator plants as they contain biochemicals that may inhibit the transmission of viroids.	C	*Category : TECHNICAL* **(309) EPPO (30 Apr 2024 7:53 PM)** Is there a reference for this statement?
153	Some plant species (e.g. *Brugmansia* spp.) are unsuitable as indicator plants as they contain biochemicals that may inhibit the transmission of viroids.	C	*Category : SUBSTANTIVE* **(308) EPPO (30 Apr 2024 7:53 PM)** Is this relevant? I wonder wether Brugmansia is used as an indicator plant at all. We incoulated this host in the frame of an experiment, but did not use it as indicator plant
155	Pospiviroids can infect a wide range of plant species, including both herbaceous and woody species. The viroid concentration in different hosts and tissue types can vary significantly. Sampling methods for the main hosts or matrices are described in this section. The number of individual samples in one pooled sample (the pool rate) depends on the detection method, the tissue being tested and the purpose of testing. The pool rate should also be adapted to the viroid concentration in the host plant and the analytical sensitivity of the detection method, and should be validated. General guidance on sampling methodologies is described in ISPM 31 (*Methodologies for sampling of consignments*). Sampling tools should be sanitized with a suitable disinfectant and dried with a paper towel to avoid cross-contamination.	C	*Category : EDITORIAL* **(314) EPPO (30 Apr 2024 7:53 PM)** Rephrase by deleting "adapted to viroid concentration"
155	Pospiviroids can infect a wide range of plant species, including both herbaceous	C	*Category : SUBSTANTIVE*

	and woody species. The viroid concentration in different hosts and tissue types can vary significantly. Sampling methods for the main hosts or matrices are described in this section. The number of individual samples in one pooled sample (the pool rate) depends on the detection method, the tissue being tested and the purpose of testing. The pool rate should also be adapted to the viroid concentration in the host plant and the analytical sensitivity of the detection method, and should be validated. General guidance on sampling methodologies is described in ISPM 31 (*Methodologies for sampling of consignments*). Sampling tools should be sanitized with a suitable disinfectant and dried with a paper towel to avoid cross-contamination.		**(313) EPPO (30 Apr 2024 7:53 PM)** How to validate? Viroid concentrations may differ considerably depending on the age of the plants and the environmental conditions. Maybe one could add… if feasible/possible. This could be a hurdle for accreditation for many laboratories. The host range can be huge and we have different matrixes as well!
155	Pospiviroids can infect a wide range of plant species, including both herbaceous and woody species. The viroid concentration in different hosts and tissue types can vary significantly. Sampling methods for the main hosts or matrices are described in this section. The number of individual samples in one pooled sample (the pool rate) depends on the detection method, the tissue being tested and the purpose of testing. The pool rate should also be adapted to the viroid concentration in the host plant and the analytical sensitivity of the detection method, and should be validated. General guidance on sampling methodologies is described in ISPM 31 (*Methodologies for sampling of consignments*). ~~Sampling tools should be sanitized with a suitable disinfectant and dried with a paper towel to avoid cross-contamination.~~	P	*Category : EDITORIAL* **(312) EPPO (30 Apr 2024 7:53 PM)** Delete? I think it is common knowledge that you should work with clean tools. Moreover, what is a suitable disinfectant in this case?
155	Pospiviroids can infect a wide range of plant species, including both herbaceous and woody species. The viroid concentration in different hosts and tissue types can vary significantly. Sampling methods for the main hosts or matrices are described in this section. The number of individual samples in one pooled sample (the pool rate) depends on the detection method, the tissue being tested and the purpose of testing. The pool rate should also be adapted to the viroid concentration in the host plant and the analytical sensitivity of the detection method, and should be validated. General guidance on sampling methodologies is described in ISPM 31 (*Methodologies for sampling of consignments*). Sampling tools should be sanitized with a suitable disinfectant and dried with a paper towel to avoid cross-contamination.	P	*Category : TECHNICAL* **(230) CA (20 Mar 2024 2:49 PM)** In order to complete the sampling message, it is proposed to adition these paragraph

	Sampling should be aimed at capturing plants or parts of plants that present symptoms associated with the viroid if they present symptoms, or sampling young plant tissue in the case of asymptomatic plants.		
155	Pospiviroids can infect a wide range of plant species, including both herbaceous and woody species. The viroid concentration in different hosts and tissue types can vary significantly. Sampling methods for the main hosts or matrices are described in this section. The number of individual samples in one pooled sample (the pool rate) depends on the detection method, the tissue being tested and the purpose of testing. The pool rate should also be adapted to the viroid concentration in the host plant and the analytical sensitivity of the detection method, and should be validated. General guidance on sampling methodologies is described in ISPM 31 (*Methodologies for sampling of consignments*). Sampling tools should be sanitized with a suitable disinfectant and dried with a paper towel to avoid cross-contamination. Sampling should be aimed at capturing plants or parts of plants that present symptoms associated with the viroid if they present symptoms, or sampling young plant tissue in the case of asymptomatic plants.	P	*Category : SUBSTANTIVE* **(3) Colombia (28 Feb 2024 8:42 PM)** In order to complete the sampling message, it is proposed to adition these paragraph
158	Bark or woody tissue from citrus species should be sampled from the young flush of symptomatic or asymptomatic plants (when the plant is approximately ten months old) and from young shoots (Rizza *et al*., 2009). In the case of trees, which may display scaling symptoms on the rootstock, green bark tissue should be collected during the period of growth (i.e. summertime) (Ragozzino, Faggioli and Barba, 2005).	C	*Category : EDITORIAL* **(315) EPPO (30 Apr 2024 7:53 PM)** How does this relate to the previous sentence? Does this sentence apply to shrubs? Please rephrase
160	In general, fully expanded young leaves, consisting of non-senescent tissue, are the most suitable for testing. Viroid concentrations may differ considerably depending on the age of the plants and the environmental conditions (temperature and photoperiod). For leaves of *S. tuberosum* and *S. lycopersicum*, pooling rates up to 100 have been used for real-time, reverse transcription–polymerase chain reaction (RT-PCR) tests; whereas for *C. annuum* and ornamentals, such as *Brugmansia* spp., *C.* ~~x~~x *morifolium*, *Dahlia* spp. and *Solanum jasminoides*, pooling rates of up to 25 have been found to be adequate (Verhoeven *et al*., 2008, 2016; van Brunschot	P	*Category : TECHNICAL* **(316) EPPO (30 Apr 2024 7:53 PM)**

	et al., 2014). S~~However, some~~ ome plant species (e.g. *Calibrachoa* spp., *Solanum laxum* and *Solanum ~~jamesii~~jamesonii*)) contain biochemicals that may inhibit amplification in RT-PCR tests. Dilution of ~~samples~~ RNA extract has been shown to alleviate inhibition, although this may have an impact on the ~~sensitivity of detection~~analytical sensitivity.		
160	In general, fully expanded young leaves, consisting of non-senescent tissue, are the most suitable for testing. Viroid concentrations may differ considerably depending on the age of the plants and the environmental conditions (temperature and photoperiod). For leaves of *S. tuberosum* and *S. lycopersicum*, pooling rates up to 100 have been used for real-time, reverse transcription–polymerase chain reaction (RT-PCR) tests; whereas for *C. annuum* and ornamentals, such as *Brugmansia* spp., *C.* ×*morifolium*, *Dahlia* spp. and *Solanum jasminoides*, pooling rates of up to 25 have been found to be adequate (Verhoeven *et al*., 2008, 2016; van Brunschot *et al*., 2014). However, some plant species (e.g. *Calibrachoa* spp., *Solanum laxum* and *Solanum jamesii*)) contain biochemicals that may inhibit amplification in RT-PCR tests. Dilution of samples has been shown to alleviate inhibition, although this may have an impact on the sensitivity of detection.	C	*Category : TECHNICAL* **(233) United States of America (1 Apr 2024 7:41 PM)** Any citations for the last two sentences?
162	Microplants of solanaceous hosts, such as *S. tuberosum* and *Petunia* ×*atkinsiana* (petunia), should be four to six weeks old with stems approximately 5 cm long and with well-formed leaves. Either the whole plant can be sampled for testing or just the top two-thirds of the plant. In the latter case, sampling should be carried out under aseptic conditions to enable the rest of the plant to continue growing.	C	*Category : TECHNICAL* **(317) EPPO (30 Apr 2024 7:53 PM)** Plants can be older than 4-6 weeks, this is a minimum. Suggested change in the text.
164	The likelihood of viroid detection in a seed lot depends on the percentage of infected seeds and the viroid concentration in the infected seed or seeds. This makes it difficult to recommend a sample size and bulking rate (Euphresco, 2010).	C	*Category : SUBSTANTIVE* **(319) EPPO (30 Apr 2024 7:53 PM)** Very good to tell that it is difficult. I appreciate that a recommendation is given anyway. This greatly helps in decision making without consultation of other standards and/or publications.
164	The likelihood of viroid detection in a seed lot depends on the percentage of infected seeds and the viroid concentration in or on the infected seed or seeds. This makes it difficult to recommend a sample size and ~~bulking~~ pooling rate (Euphresco, 2010).	P	*Category : EDITORIAL* **(318) EPPO (30 Apr 2024 7:53 PM)**
164	The likelihood of viroid detection in a seed lot depends on the percentage of infected seeds and the viroid concentration in the infected seed or seeds. This	C	*Category : TECHNICAL* **(271) Japan (23 Apr 2024 7:53 AM)**

	makes it difficult to recommend a sample size and bulking rate (Euphresco, 2010).		Japan proposes adding information that viroid may infect internal tissues such as seed embryo, not only surface of seeds. As Matsushita and Tsuda (2016, EJPP, 145;1007–1011) reported, "the seed transmission of PSTVd in tomato could also be attributable to the indirect invasion of the embryo".
165	For seed lots of *C. annuum* and *S. lycopersicum*, most common sampling methods rely on weighed samples of approximately 3 000 seeds, tested in three subsamples of 1 000 seeds. The International Seed Federation recommends testing of subsamples of 1 000 seeds in a method that has been validated for real-time RT-PCR (ISF, 2015). However, sample size may be adapted to address technical restrictions or to meet specific phytosanitary import requirements.	C	*Category : TECHNICAL* **(455) Brazil (3 May 2024 3:11 PM)** This is suitable to the seed industry and their big lots. What about small consignments in international trade, any % suggestion?
165	For seed lots of *C. annuum* and *S. lycopersicum*, most common sampling methods rely on weighed samples of approximately 3 000 seeds, tested in three subsamples of 1 000 seeds. The International Seed Federation recommends testing of subsamples of 1 000 seeds in a method that has been validated for real-time RT-PCR (ISF, 2015). However, both subsample and sample size may be adapted to address technical restrictions or to meet specific phytosanitary import requirements.	P	*Category : EDITORIAL* **(320) EPPO (30 Apr 2024 7:53 PM)**
165	For seed lots of *C. annuum* and *S. lycopersicum*, most common sampling methods rely on weighed samples of approximately 3 000 seeds, tested in three subsamples of 1 000 seeds. The International Seed Federation recommends testing of subsamples of 1 000 seeds in a method that has been validated for real-time RT-PCR (ISF, 2015). However, sample size may be adapted to address technical restrictions or to meet specific phytosanitary import requirements.	C	*Category : EDITORIAL* **(256) Canada (22 Apr 2024 4:38 PM)** Is this the style of writing for IPPC? I mean space between 1 and 000? Please check.
165	For seed lots of *C. annuum* and *S. lycopersicum*, most common sampling methods rely on weighed samples of approximately ~~3~~ 3,000 seeds, tested in three subsamples of 1 000 seeds. The International Seed Federation recommends testing of subsamples of 1 000 seeds in a method that has been validated for real-time RT-PCR (ISF, 2015). However, sample size may be adapted to address technical restrictions or to meet specific phytosanitary import requirements.	P	*Category : EDITORIAL* **(245) Canada (19 Apr 2024 2:54 PM)**
167	In *S. tuberosum* tubers, the highest viroid concentration is found immediately after harvest (Roenhorst *et al.*, 2006). Potato spindle tuber viroid has been found to be	P	*Category : EDITORIAL* **(321) EPPO (30 Apr 2024 7:53 PM)**

	present in almost equal amounts in different parts of infected tubers, regardless of whether the infection is a primary or secondary infection (Shamloul *et al*., 1997; Roenhorst *et al*., 2006). Therefore, samples can be taken from the heel end or from tuber eyes, peel fragments and flesh cores throughout the whole tuber. For testing by real-time RT-PCR, up to 100 cores weighing approximately 50 mg each may be ~~bulked~~ pooled (Roenhorst *et al.*, 2006).		
171	**3.4.1 Sample preparation**	C	*Category : EDITORIAL* **(322) EPPO (30 Apr 2024 7:53 PM)** Consider to combine this paragraph with 3.4.2 not only for seeds but also for the other matrices, seeing the references to the addition of buffers in relation to the RNA extraction method. The seperation appears artiicial and not logic.
175	Bark peel and roots should be chopped into small pieces before homogenization. Lyophilization of the tissue before 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 the testing of citrus bark tissue (Dang *et al*., 2022)~~.~~:	P	*Category : EDITORIAL* **(439) Brazil (2 May 2024 3:51 PM)** To improve the flow
176	The phloem-rich bark tissue is peeled using a disposable, single-edged 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. 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 freezer at approximately −80 °C for at least 2 h before lyophilization. For RNA extraction, liquid nitrogen is added to the lyophilized tissue and samples are ground into a fine powder. Section 3.4.2.1 describes the next steps of the extraction (Dang *et al*., 2022). Alternatively, total RNA is extracted from 100 mg young bark or leaves (plants or bark of plants approximately 10 months old), ground to a fine powder in a mortar with liquid nitrogen, then homogenized in TRIzol Reagent (Invitrogen)[1] buffer and processed following the manufacturer's instructions (Rizza *et al.*, 2009).	P	*Category : EDITORIAL* **(440) Brazil (2 May 2024 3:54 PM)** A separate paragraph is necessary once, despite being for citrus bark tissue, this is not in Dang et al, 2022 but in Rizza et al., 2009.
176	The phloem-rich bark tissue is peeled using a disposable, single-edged razor blade. The peeled bark tissue is chopped into small pieces (4–5 mm) on small disposable	C	*Category : EDITORIAL* **(325) EPPO (30 Apr 2024 7:53 PM)** Right section? Seems part of RNA extraction.

	chipboards, and 250 mg placed into a 2 mL safe-lock tube. 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 freezer at approximately −80 °C for at least 2 h before lyophilization. For RNA extraction, liquid nitrogen is added to the lyophilized tissue and samples are ground into a fine powder. Section 3.4.2.1 describes the next steps of the extraction (Dang *et al*., 2022). Alternatively, total RNA is extracted from 100 mg young bark or leaves (plants or bark of plants approximately 10 months old), ground to a fine powder in a mortar with liquid nitrogen, then homogenized in TRIzol Reagent (Invitrogen)[1] buffer and processed following the manufacturer's instructions (Rizza *et al.*, 2009).		
176	The phloem-rich bark tissue is peeled using a disposable, single-edged 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. 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 freezer at approximately −80 °C for at least 2 h before lyophilization. For RNA extraction, liquid nitrogen is added to the lyophilized tissue and samples are ground into a fine powder. Section 3.4.2.1 describes the next steps of the extraction (Dang *et al*., 2022). Alternatively, total RNA is extracted from 100 mg young bark or leaves (plants or bark of plants approximately 10 months old), ground to a fine powder in a mortar with liquid nitrogen, then homogenized in TRIzol Reagent (Invitrogen)[1] buffer and processed following the manufacturer's instructions (Rizza *et al.*, 2009).	C	*Category : EDITORIAL* **(324) EPPO (30 Apr 2024 7:53 PM)** This section is on bark, woody tissues and roots, not on leaves, please omit
176	The phloem-rich bark tissue is peeled using a disposable, single-edged 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. 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 freezer at approximately −80 °C for at least 2 h before lyophilization. For RNA extraction, liquid nitrogen is added to the lyophilized tissue and samples are ground into a fine powder. Section 3.4.2.1 describes the next steps of the extraction (Dang *et al*., 2022). Alternatively, total RNA is extracted from 100 mg young bark or leaves	C	*Category : SUBSTANTIVE* **(323) EPPO (30 Apr 2024 7:53 PM)** Too much detail. Any knife, can be used, provided that it is sterile. Level of detail applies to the whole section

	(plants or bark of plants approximately 10 months old), ground to a fine powder in a mortar with liquid nitrogen, then homogenized in TRIzol Reagent (Invitrogen)[1] buffer and processed following the manufacturer's instructions (Rizza *et al.*, 2009).		
178	Before grinding, water or buffer is added to the plant material; the volume and composition of the buffer depends on the method to be used for RNA extraction. If freezing the sample in liquid nitrogen, water or lysis buffer should be added after grinding.	C	*Category : EDITORIAL* **(326) EPPO (30 Apr 2024 7:53 PM)** Add reference to 3.4.2 sections
184	A wide range of RNA extraction methods may be used, from commercial kits to methods published in scientific journals, with particular methods being appropriate for particular matrices (see sections 3.4.2.1–3.4.2.4). The RNeasy Plant Mini Kit (QIAGEN)[1] and the sbeadex Maxi Plant Kit (LGC Biosearch Technologies)[1] can be used where appropriate following the manufacturers' instructions or the instructions described in this diagnostic protocol. For high-throughput RNA extraction, the sbeadex Maxi Plant Kit (or MagMAX (Applied Biosystems)[1] or other) can be used in combination with a KingFisher KF96 system (Thermo Scientific).[1] Other extraction methods, including the cetyltrimethylammonium bromide (CTAB) method (Gambino, Perrone and Gribaudo, 2008), can also be used.	C	*Category : TECHNICAL* **(449) New Zealand (3 May 2024 5:19 AM)** CTAB method using Kingfisher mL system is only mentioned for RNA extraction from tubers. Has this method tried with other type of samples? QIAGEN kit is mostly used for extraction? Is InviMag Plant DNA Mini Kit tried?
184	A wide range of RNA extraction methods may be used, from commercial kits to methods published in scientific journals, with particular methods being appropriate for particular matrices (see sections 3.4.2.1–3.4.2.4). The RNeasy Plant Mini Kit (QIAGEN)[1] and the sbeadex Maxi Plant Kit (LGC Biosearch Technologies)[1] can be used where appropriate following the manufacturers' instructions or the instructions described in this diagnostic protocol. For high-throughput RNA extraction, the sbeadex Maxi Plant Kit (or MagMAX (Applied Biosystems)[1] or other) can be used in combination with a KingFisher KF96 system (Thermo Scientific).[1] Other extraction methods, including the cetyltrimethylammonium bromide (CTAB) method (Gambino, Perrone and Gribaudo, 2008), can also be used.	C	*Category : EDITORIAL* **(330) EPPO (30 Apr 2024 7:53 PM)** Rephrase to be less prescriptive for the methods used, for example add 'such as' or 'e.g.'
184	A wide range of RNA extraction methods may be used, from commercial kits to methods published in scientific journals, with particular methods being appropriate for particular matrices (see sections 3.4.2.1–3.4.2.4). The RNeasy Plant Mini Kit	C	*Category : TECHNICAL* **(329) EPPO (30 Apr 2024 7:53 PM)** In GEVES they use and they have validated the Promega Maxwell HT96 Simply RNA, to see if you

	(QIAGEN)[1] and the sbeadex Maxi Plant Kit (LGC Biosearch Technologies)[1] can be used where appropriate following the manufacturers' instructions or the instructions described in this diagnostic protocol. For high-throughput RNA extraction, the sbeadex Maxi Plant Kit (or MagMAX (Applied Biosystems)[1] or other) can be used in combination with a KingFisher KF96 system (Thermo Scientific).[1] Other extraction methods, including the cetyltrimethylammonium bromide (CTAB) method (Gambino, Perrone and Gribaudo, 2008), can also be used.		want to add it as an additional example.
184	A wide range of RNA extraction methods may be used, from commercial kits to methods published in scientific journals, with particular methods being appropriate for particular matrices (see sections 3.4.2.1–3.4.2.4). The RNeasy Plant Mini Kit (QIAGEN)[1] and the sbeadex Maxi Plant Kit (LGC Biosearch Technologies)[1] can be used where appropriate following the manufacturers' instructions or the instructions described in this diagnostic protocol. For high-throughput RNA extraction, the sbeadex Maxi Plant Kit (or MagMAX (Applied Biosystems)[1] or other) can be used in combination with a KingFisher KF96 system (Thermo Scientific).[1] Maxwell® RSC Plant RNA Kit (Cat.# AS1500, PROMEGA) can be used in combination with a Maxwell® RSC Instrument (Cat.# AS4500, PROMEGA). Other extraction methods, including the cetyltrimethylammonium bromide (CTAB) method (Gambino, Perrone and Gribaudo, 2008), can also be used.	P	*Category : TECHNICAL* **(328) EPPO (30 Apr 2024 7:53 PM)**
184	A wide range of RNA extraction methods may be used, from commercial kits to methods published in scientific journals, with particular methods being appropriate for particular matrices (see sections 3.4.2.1–3.4.2.4). The RNeasy Plant Mini Kit (QIAGEN)[1] and the sbeadex Maxi Plant Kit (LGC Biosearch Technologies)[1] can be used where appropriate following the manufacturers' instructions or the instructions described in this diagnostic protocol. For high-throughput RNA extraction, the sbeadex Maxi Plant Kit (or MagMAX (Applied Biosystems)[1] or other) can be used in combination with a KingFisher KF96 system (Thermo Scientific).[1] Other extraction methods, including the cetyltrimethylammonium bromide (CTAB) method (Gambino, Perrone and Gribaudo, 2008), can also be used.	C	*Category : EDITORIAL* **(327) EPPO (30 Apr 2024 7:53 PM)** Redundant ??
187	**Method 1.** Extraction of RNA is accomplished by combining guanidine lysis	C	*Category : TECHNICAL*

	buffer with the RNeasy Plant Mini Kit (QIAGEN)[1] as described by Bernard and Duran-Vila (2006). Approximately 100 mg of tissue is homogenized in RNA extraction buffer (4 M guanidine isothiocyanate, 100 mM Tris-HCl, 25 mM $MgCl_2$, 25 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5). The RNA in the soluble fraction is concentrated by isopropyl alcohol precipitation and resuspended in TE buffer (20 mM Tris-HCl, 1 mM EDTA, pH 8.0). Subsequently, the RNA is purified using the RNeasy Plant Mini Kit (QIAGEN)[1] following the manufacturer's instructions for RNA cleanup and resuspended in 50 µL water.		**(331) EPPO (30 Apr 2024 7:53 PM)** In which volume? The w/v ratio of sample/extraction-buffer is most probably critical. The w/v ratio should be recommended or at least a range should be given.
188	**Method 2.** Approximately 100–500 mg ~~bark~~ of tissue is homogenized in TRIzol Reagent (Invitrogen)[1] and RNA extraction is undertaken following the manufacturer's instructions (Chomczynski and Sacchi, 1987; Rizza *et al.*, 2009; Dang *et al.*, 2022).	P	*Category : EDITORIAL* **(332) EPPO (30 Apr 2024 7:53 PM)**
189	**Method 3.** The pulverized bark or woody tissue from citrus trees is processed with the MagMAX-96 Viral RNA Isolation Kit (Applied Biosystems),[1] using the MagMAX Express-96 Deep Well Magnetic Particle Processor (Applied Biosystems),[1] following the manufacturer's recommendations and as described in Dang *et al.* (2022).[1]	C	*Category : EDITORIAL* **(333) EPPO (30 Apr 2024 7:53 PM)** How much? For both method 3 and 4 provide the same information as for method 1 and 2.
192	**Commercial kits.** For small samples, approximately ~~100~~ 100 mg leaf material is homogenized with lysis buffer from the RNeasy Plant Mini Kit (QIAGEN)[1] following the manufacturer's instructions. For larger ~~samples~~sample sizes, such as pooled samples, approximately ~~1~~ 1 g plant tissue is put in an extraction bag and homogenized in 3.~~5~~ 5 mL (between 1:2 and 1:5 (w/v)) GH+ extraction buffer ~~(6~~ (6 M guanidine hydrochloride, 0.~~2~~ 2 M sodium acetate ~~pH~~ pH 5, ~~25~~ 25 mM EDTA, 2.5% polyvinylpyrrolidone 10% (PVP-10)), incubated for ~~10~~ 10 min at ~~65~~ 65 °C and centrifugated for ~~2~~ 2 min (approximately ~~12 000~~ 12 000 *g*), before nucleic acid extraction using the RNeasy Plant Mini Kit (QIAGEN)[1] or the sbeadex Maxi Plant Kit (LGC Biosearch Technologies)[1] according to the manufacturer's instructions.	P	*Category : EDITORIAL* **(336) EPPO (30 Apr 2024 7:53 PM)** Created by merging other changes together
192	**Commercial kits.** For small samples, approximately 100 mg leaf material is homogenized with lysis buffer from the RNeasy Plant Mini Kit (QIAGEN)[1] following the manufacturer's instructions. For larger samples, such as pooled samples, approximately 1 g plant tissue is put in an extraction bag and	C	*Category : TECHNICAL* **(335) EPPO (30 Apr 2024 7:53 PM)** Recommendation from the EPPO protocol for sbeadex extraction : 250 ul lysate are transferred to a binding plate containing 450 ul of binding buffer

	homogenized in 3.5 mL (between 1:2 and 1:5 (w/v)) GH+ extraction buffer (6 M guanidine hydrochloride, 0.2 M sodium acetate pH 5, 25 mM EDTA, 2.5% polyvinylpyrrolidone 10% (PVP-10)), incubated for 10 min at 65 °C and centrifugated for 2 min (approximately 12 000 *g*), before nucleic acid extraction using the RNeasy Plant Mini Kit (QIAGEN)[1] or the sbeadex Maxi Plant Kit (LGC Biosearch Technologies)[1] according to the manufacturer's instructions.		and 50 ul of particle suspension. This information is also given for seeds in this protocol.
192	**Commercial kits.** For small samples, approximately 100 mg leaf material is homogenized with lysis buffer from the RNeasy Plant Mini Kit (QIAGEN)[1] following the manufacturer's instructions. For larger samples, such as pooled samples, approximately 1 g plant tissue is put in an extraction bag and homogenized in 3.5 mL (between 1:2 and 1:5 (w/v)) GH+ extraction buffer (6 M guanidine hydrochloride, 0.2 M sodium acetate pH 5, 25 mM EDTA, 2.5% polyvinylpyrrolidone 10% (PVP-10)), incubated for 10 min at 65 °C and centrifugated for 2 min (approximately 12 000 *g*), before nucleic acid extraction using the RNeasy Plant Mini Kit (QIAGEN)[1] or the sbeadex Maxi Plant Kit (LGC Biosearch Technologies)[1] according to the manufacturer's instructions.	C	*Category : EDITORIAL* **(334) EPPO (30 Apr 2024 7:53 PM)** This should be mentioned for bark samples as well. What about CTAB extraction?
193	**EDTA method.** Plant tissue is homogenized (1:4 (w/v)) in a simple lysis buffer (50 mM NaOH, 2.5 mM EDTA) and then incubated (at approximately 25 ºC for 15 min) and subsequently centrifuged (at 12 000 *g* at 4 °C for 15 min). The supernatant is either used directly for RT-PCR or spotted onto a nitrocellulose membrane and eluted using sterile distilled water (Singh *et al*., 2006). The method has been used with RT-PCR and hybridization methods (see section 3.4.3.5 below) for *S. lycopersicum*, *S. tuberosum* and a range of ornamental plant species.	C	*Category : SUBSTANTIVE* **(338) EPPO (30 Apr 2024 7:53 PM)** This is not a purification! The publication by Singh et al., 2006 focuses on ornamental samples. It is also shown that the sensitivity is drastically reduced compared to phenol extraction. It should be specified that this method is not very sensitive and should not be used for asymptomatic samples. It should definitely not be used for pooling of samples in screenings. In the publication they mention that "usually" viroid concentration is high. This statement is in contradiction with other publications where in general it is noted that pospiviroid concentration can show great variability depending on host, tissue and environmental conditions. This statement is even in contradiction with this document. If this method is mentioned the scope and limitations should be clearly stated.
193	**EDTA method.** Plant tissue is homogenized (1:4 (w/v)) in a ~~simple~~ sample lysis buffer (50 mM NaOH, 2.5 mM EDTA) and then incubated (at approximately 25 ºC	P	*Category : EDITORIAL* **(337) EPPO (30 Apr 2024 7:53 PM)**

	for 15 min) and subsequently centrifuged (at 12 000 *g* at 4 °C for 15 min). The supernatant is either used directly for RT-PCR or spotted onto a nitrocellulose membrane and eluted using sterile distilled water (Singh *et al*., 2006). The method has been used with RT-PCR and hybridization methods (see section 3.4.3.5 below) for *S. lycopersicum*, *S. tuberosum* and a range of ornamental plant species.		
195	**Homogenization in GH+ extraction buffer.** For both *C. annuum* and *S. lycopersicum*, three subsamples of (approximately) 1 000 seeds are transferred to a grinding bag of suitable size together with 20 mL (*S. lycopersicum*) or 40 mL (*C. annuum*) GH+ extraction buffer (see section 3.4.2.2). Seeds are soaked at room temperature for 30–60 min before homogenization with a BagMixer MiniMix 100 P CC (Interscience)[1] for 90 s (*S. lycopersicum*) or at least 4 min (*C. annuum*). Other equipment maybe used and the time of homogenization maybe adjusted accordingly.	C	*Category : TECHNICAL* **(340) EPPO (30 Apr 2024 7:53 PM)** At GEVES they have deleted this step and this was validated internally. Could you propose this step as an option?
195	**Homogenization in GH+ extraction buffer.** For both *C. annuum* and *S. lycopersicum*, three subsamples of (approximately) 1 000 seeds are transferred to a grinding bag of suitable size together with 40 mL (C. annuum) or 20 mL (*S. lycopersicum*) ~~or 40 mL (*C. annuum*)~~ GH+ extraction buffer (see section 3.4.2.2). Seeds are soaked at room temperature for 30–60 min before homogenization with a BagMixer MiniMix 100 P CC (Interscience)[1] for 90 s (*S. lycopersicum*) or at least 4 min (*C. annuum*). Other equipment maybe used and the time of homogenization ~~maybe~~ can be adjusted accordingly.	P	*Category : EDITORIAL* **(339) EPPO (30 Apr 2024 7:53 PM)**
195	**Homogenization in GH+ extraction buffer.** For both *C. annuum* and *S. lycopersicum*, three subsamples of (approximately) 1 000 seeds are transferred to a grinding bag of suitable size together with 20 mL (*S. lycopersicum*) or 40 mL (*C. annuum*) GH+ extraction buffer (see section 3.4.2.2). Seeds are soaked at room temperature for 30–60 min before homogenization with a BagMixer MiniMix 100 P CC (Interscience)[1] for 90 s (*S. lycopersicum*) or at least 4 min (*C. annuum*). Other equipment maybe used and the time of homogenization maybe adjusted accordingly.	C	*Category : TECHNICAL* **(269) Japan (23 Apr 2024 7:50 AM)** Japan proposes adding a footnote on notes on use of BagMixer MiniMix 100 PCC (Interscience), for example; This model is suitable for crushing relatively soft samples. As described in section 3.3.4, viroids (or Pospiviroids) can also infect internal tissues such as seed embryos. When crushing hard samples such as seeds, it is appropriate to use other models that can expose the internal tissues of the seeds to avoid false negatives.
195	**Homogenization in GH+ extraction buffer.** For both *C. annuum* and *S. lycopersicum*, three subsamples of (approximately) 1 000 seeds are transferred to	C	*Category : TECHNICAL* **(234) United States of America (1 Apr 2024**

	a grinding bag of suitable size together with 20 mL (*S. lycopersicum*) or 40 mL (*C. annuum*) GH+ extraction buffer (see section 3.4.2.2). Seeds are soaked at room temperature for 30–60 min before homogenization with a BagMixer MiniMix 100 P CC (Interscience)[1] for 90 s (*S. lycopersicum*) or at least 4 min (*C. annuum*). Other equipment maybe used and the time of homogenization maybe adjusted accordingly.		**7:43 PM)** Is the subsample size for C. annuum (pepper) not 500 seeds and a total of six subsamples?
196	Alternatively, dry seeds can be ground with a Geno/Grinder (SPEX SamplePrep)[1] (Botermans *et al*., 2020). Six subsamples of approximately 500 *C. annuum* seeds or three subsamples of approximately 1 000 *S. lycopersicum* seeds are transferred to a 50 mL tube (one subsample per tube) and a steel ball (14 mm) is added. Seeds are ground, with the tubes upside down, at 1700 rpm for 7 min and 4 min for *C. annuum* and *S. lycopersicum*, respectively. After grinding, GH+ buffer is added: 20 mL for *C. annuum* and 20 mL for *S. lycopersicum* samples. At this stage, a positive extraction control such as dahlia latent viroid (DLVd) for real-time RT-PCR, can be added to the homogenization buffer. Tubes are shaken by hand to obtain homogenous solutions. Each pair of *C. annuum* homogenates (out of six) are combined and mixed to make three subsamples for further processing.	C	*Category : TECHNICAL* **(345) EPPO (30 Apr 2024 7:53 PM)** For example they sample in GEVES 100µL of each to combine.
196	Alternatively, dry seeds can be ground with a Geno/Grinder (SPEX SamplePrep)[1] (Botermans *et al*., 2020). Six subsamples of approximately 500 *C. annuum* seeds or three subsamples of approximately 1 000 *S. lycopersicum* seeds are transferred to a 50 mL tube (one subsample per tube) and a steel ball (14 mm) is added. Seeds are ground, with the tubes upside down, at 1700 rpm for 7 min and 4 min for *C. annuum* and *S. lycopersicum*, respectively. After grinding, GH+ buffer is added: 20 mL for *C. annuum* and 20 mL for *S. lycopersicum* samples. At this stage, a positive extraction control such as dahlia latent viroid (DLVd) for real-time RT-PCR, can be added to the homogenization buffer. Tubes are shaken by hand to obtain homogenous solutions. Each pair of *C. annuum* homogenates (out of six) are combined and mixed to make three subsamples for further processing.	C	*Category : TECHNICAL* **(344) EPPO (30 Apr 2024 7:53 PM)** To clarify that the DLVd can be added in the homogeneization buffer previously to the distribution to each sample to gain time.
196	Alternatively, dry seeds can be ground with a Geno/Grinder (SPEX SamplePrep)[1] (Botermans *et al*., 2020). Six subsamples of approximately 500 *C. annuum* seeds or three subsamples of approximately	C	*Category : TECHNICAL* **(343) EPPO (30 Apr 2024 7:53 PM)** Not 10 mL for C. annuum? The Botermans et al., 2020 protocol says "For

	1 000 *S. lycopersicum* seeds are transferred to a 50 mL tube (one subsample per tube) and a steel ball (14 mm) is added. Seeds are ground, with the tubes upside down, at 1700 rpm for 7 min and 4 min for *C. annuum* and *S. lycopersicum*, respectively. After grinding, GH+ buffer is added: 20 mL for *C. annuum* and 20 mL for *S. lycopersicum* samples. At this stage, a positive extraction control such as dahlia latent viroid (DLVd) for real-time RT-PCR, can be added to the homogenization buffer. Tubes are shaken by hand to obtain homogenous solutions. Each pair of *C. annuum* homogenates (out of six) are combined and mixed to make three subsamples for further processing.		pepper seeds the same procedure was followed except that the subsamples of c.1000 seeds were subdivided into 2x500 seeds before grinding with a Geno/Grinder for 7 min. After grinding 10 ml DLVd-spiked GH+ extraction buffer was added to each of the six tubes, followed by combining and mixing of the contents of the two tubes of a subsample before further processing."
196	Alternatively, dry seeds can be ground with a Geno/Grinder (SPEX SamplePrep)[1] (Botermans *et al*., 2020). Six subsamples of approximately 500 *C. annuum* seeds or three subsamples of approximately 1 000 *S. lycopersicum* seeds are transferred to a 50 mL tube (one subsample per tube) and a steel ball (14 mm) is added. Seeds are ground, with the tubes upside down, at 1700 rpm for 7 min and 4 min for *C. annuum* and *S. lycopersicum*, respectively. After grinding, GH+ buffer is added: 20 mL for *C. annuum* and 20 mL for *S. lycopersicum* samples. At this stage, a positive extraction control such as dahlia latent viroid (DLVd) for real-time RT-PCR, can be added to the homogenization buffer. Tubes are shaken by hand to obtain homogenous solutions. Each pair of *C. annuum* homogenates (out of six) are combined and mixed to make three subsamples for further processing.	C	*Category : EDITORIAL* **(342) EPPO (30 Apr 2024 7:53 PM)** Delete? I wonder if the extraction control needs an example, as each lab can make its own choice.
196	Alternatively, dry seeds can be ground with a Geno/Grinder (SPEX SamplePrep)[1] (Botermans *et al*., 2020). Six subsamples of approximately 500 *C. annuum* seeds or three subsamples of approximately 1 000 *S. lycopersicum* seeds are transferred to a 50 mL tube (one subsample per tube) and a steel ball (14 mm) is added. Seeds are ground, with the tubes upside down, at 1700 rpm for 7 min for *C. annuum* and 4 min for ~~*CS*~~. ~~*annuum*~~*lycopersicum* ~~and~~ seeds~~*S. lycopersicum*, respectively~~. After grinding, GH+ buffer is added: 20 mL for *C. annuum* and 20 mL for *S. lycopersicum* samples. At this stage, a positive extraction control such as dahlia latent viroid (DLVd) for real-time RT-PCR, can be added to the homogenization buffer. Tubes are shaken by hand to obtain homogenous solutions. ~~Each pair of~~ Two *C. annuum* homogenates (out of six) are combined and mixed to make three	P	*Category : EDITORIAL* **(341) EPPO (30 Apr 2024 7:53 PM)**

	subsamples for further processing.		
196	Alternatively, dry seeds can be ground with a Geno/Grinder (SPEX SamplePrep)[1] (Botermans *et al*., 2020). Six subsamples of approximately 500 *C. annuum* seeds or three subsamples of approximately 1 000 *S. lycopersicum* seeds are transferred to a 50 mL tube (one subsample per tube) and a steel ball (14 mm) is added. Seeds are ground, with the tubes upside down, at 1700 rpm for 7 min and 4 min for *C. annuum* and *S. lycopersicum*, respectively. After grinding, GH+ buffer is added: 20 mL for *C. annuum* and 20 mL for *S. lycopersicum* samples. At this stage, a positive extraction control such as ~~dahlia~~ Dahlia latent viroid (DLVd) for real-time RT-PCR, can be added to the homogenization buffer. Tubes are shaken by hand to obtain homogenous solutions. Each pair of *C. annuum* homogenates (out of six) are combined and mixed to make three subsamples for further processing.	P	*Category : TECHNICAL* **(257) Canada (22 Apr 2024 4:40 PM)** Shouldn't Dahlia start with capital letter as Columnea?
196	Alternatively, dry seeds can be ground with a Geno/Grinder (SPEX SamplePrep)[1] (Botermans *et al*., 2020). Six subsamples of approximately 500 *C. annuum* seeds or three subsamples of approximately 1 000 *S. lycopersicum* seeds are transferred to a 50 mL tube (one subsample per tube) and a steel ball (14 mm) is added. Seeds are ground, with the tubes upside down, at 1700 rpm for 7 min and 4 min for *C. annuum* and *S. lycopersicum*, respectively. After grinding, GH+ buffer is added: 20 mL for *C. annuum* and 20 mL for *S. lycopersicum* samples. At this stage, a positive extraction control such as dahlia latent viroid (DLVd) for real-time RT-PCR, can be added to the homogenization buffer. Tubes are shaken by hand to obtain homogenous solutions. Each pair of *C. annuum* homogenates (out of six) are combined and mixed to make three subsamples for further processing.	C	*Category : TECHNICAL* **(235) United States of America (1 Apr 2024 7:44 PM)** Do you know why the "tubes upside down"?
197	After homogenization, one mL seed homogenate is transferred into a 1.5 mL tube and 30 µL 5 M dithiothreitol added, followed by incubation with shaking at 850 rpm and 65 °C for 15 min and centrifugation at 16 000 *g* for 10 min. For RNA extraction using the RNeasy Plant Mini Kit (QIAGEN),[1] 750 µL supernatant is transferred onto the QIAshredder (QIAGEN)[1] spin column. The manufacturer's instructions are followed. For high-throughput RNA extractions, a KingFisher KF96 system (Thermo Scientific)[1] can be used. In this system, 250 µL supernatant is transferred to a binding plate containing 600 µL binding buffer (kit) and 50 µL sbeadex Maxi Plant Kit (LGC Biosearch Technologies),[1] and RNA is extracted following the manufacturer's instructions.	C	*Category : TECHNICAL* **(350) EPPO (30 Apr 2024 7:53 PM)** DTT is optional

197	After homogenization, one mL seed homogenate is transferred into a 1.5 mL tube and 30 µL 5 M dithiothreitol added, followed by incubation with shaking at 850 rpm and 65 °C for 15 min and centrifugation at 16 000 *g* for 10 min. For RNA extraction using the RNeasy Plant Mini Kit (QIAGEN),[1] 750 µL supernatant is transferred onto the QIAshredder (QIAGEN)[1] spin column. The manufacturer's instructions are followed. For high-throughput RNA extractions, a KingFisher KF96 system (Thermo Scientific)[1] can be used. In this system, 250 µL supernatant is transferred to a binding plate containing 600 µL binding buffer (kit) and 50 µL sbeadex Maxi Plant Kit (LGC Biosearch Technologies),[1] and RNA is extracted following the manufacturer's instructions.	C	*Category : TECHNICAL* **(349) EPPO (30 Apr 2024 7:53 PM)** They have never use DTT at GEVES and their method was validated but they have no validation data to compare without or with DTT.
197	After homogenization, one mL seed homogenate is transferred into a 1.5 mL tube and 30 µL 5 M dithiothreitol added, followed by incubation with shaking at 850 rpm and 65 °C for 15 min and centrifugation at 16 000 *g* for 10 min. For RNA extraction using the RNeasy Plant Mini Kit (QIAGEN),[1] 750 µL supernatant is transferred onto the QIAshredder (QIAGEN)[1] spin column. The manufacturer's instructions are followed. For high-throughput RNA extractions, a KingFisher KF96 system (Thermo Scientific)[1] can be used. In this system, 250 µL supernatant is transferred to a binding plate containing 600 µL binding buffer (kit) and 50 µL sbeadex Maxi Plant Kit (LGC Biosearch Technologies),[1] and RNA is extracted following the manufacturer's instructions.	C	*Category : SUBSTANTIVE* **(348) EPPO (30 Apr 2024 7:53 PM)** Please provide this very useful deviation from the manufacturers protocol also for leaves (as mentioned before).
197	After homogenization, one mL seed homogenate is transferred into a 1.5 mL tube and 30 µL 5 M dithiothreitol added, followed by incubation with shaking at 850 rpm and 65 °C for 15 min and centrifugation at 16 000 *g* for 10 min. For RNA extraction using the RNeasy Plant Mini Kit (QIAGEN),[1] 750 µL supernatant is transferred onto the QIAshredder (QIAGEN)[1] spin column. The manufacturer's instructions are followed. For high-throughput RNA extractions, a KingFisher KF96 system (Thermo Scientific)[1] can be used. In this system, 250 µL supernatant is transferred to a binding plate containing 600 µL binding buffer (kit) and 50 µL sbeadex Maxi Plant Kit (LGC Biosearch Technologies),[1] and RNA is extracted following the manufacturer's instructions.	C	*Category : EDITORIAL* **(347) EPPO (30 Apr 2024 7:53 PM)** The first steps are not the same as in the manual. So only from the QIAshredder onwards you shoulf follow the manufacturers protocol
197	After homogenization, one mL seed homogenate is transferred into a 1.5 mL tube and 30 µL 5 M dithiothreitol added, followed by incubation with shaking at	P	*Category : EDITORIAL* **(346) EPPO (30 Apr 2024 7:53 PM)**

	850 rpm and 65 °C for 15 min and centrifugation at 16 000 *g* for 10 min. For RNA extraction using the RNeasy Plant Mini Kit (QIAGEN),[1] 750 µL supernatant is transferred onto the QIAshredder (QIAGEN)[1] spin column. ~~The~~ Thereafter, the manufacturer's instructions are followed. For high-throughput RNA extractions, a KingFisher KF96 system (Thermo Scientific)[1] can be used. In this system, 250 µL supernatant is transferred to a binding plate containing 600 µL binding buffer (kit) and 50 µL sbeadex Maxi Plant Kit (LGC Biosearch Technologies),[1] and RNA is extracted following the manufacturer's instructions.		
198	**Homogenization in phosphate buffer.** For both *C. annuum* and *S. lycopersicum*, 12 subsamples of 250 seeds are each immersed in 10 mL 0.1 M phosphate buffer (Na_2HPO_4 and KH_2PO_4, pH 7.2), incubated at 4 °C overnight, and then ground (e.g. with a FastPrep homogenizer (MP Biomedicals)[1] at speed 5 for 40 s).[1] After centrifugation at 10 000 *g* at 4 °C for 10 min, RNA is extracted using the RNeasy Plant Mini Kit (QIAGEN),[1] following the manufacturer's instructions with some minor modifications. In brief, 600 µL supernatant is added to 600 µL RLT Buffer (QIAGEN)[1] without β-mercaptoethanol. Two 600 µL aliquots of this mixture are successively loaded onto the same RNeasy Mini Spin Column (QIAGEN)[1] and centrifuged. RNA is eluted from the RNeasy Mini Spin Columns (QIAGEN)[1] by applying 50 µL of ribonuclease-free warm water (65 °C) followed by centrifugation. To maximize RNA recovery, an additional elution step is performed using the same conditions (i.e. adding another 50 µL of ribonuclease-free warm water or applying the eluate on the same RNeasy Mini Spin Column (QIAGEN)).[1] RNA extracts may be processed separately or may be combined.	C	*Category : EDITORIAL* **(354) EPPO (30 Apr 2024 7:53 PM)** consider deleting or rephrasing the sentence 'RNA extracts may be processed separately or may be combined'
198	**Homogenization in phosphate buffer.** For both *C. annuum* and *S. lycopersicum*, 12 subsamples of 250 seeds are each immersed in 10 mL 0.1 M phosphate buffer (Na_2HPO_4 and KH_2PO_4, pH 7.2), incubated at 4 °C overnight, and then ground (e.g. with a FastPrep homogenizer (MP Biomedicals)[1] at speed 5 for 40 s).[1] After centrifugation at 10 000 *g* at 4 °C for 10 min, RNA is extracted using the RNeasy Plant Mini Kit (QIAGEN),[1] following the manufacturer's instructions with some minor modifications. In brief, 600 µL supernatant is added to 600 µL RLT Buffer (QIAGEN)[1] without β-mercaptoethanol. Two 600 µL aliquots of this mixture are successively loaded onto the same RNeasy Mini Spin Column (QIAGEN)[1] and centrifuged. RNA is eluted from the RNeasy Mini Spin Columns (QIAGEN)[1] by	C	*Category : TECHNICAL* **(353) EPPO (30 Apr 2024 7:53 PM)** Is it an optional step? For the ToBRFV detection (ISF protocol) there is no incubation overnight.

	applying 50 µL of ribonuclease-free warm water (65 °C) followed by centrifugation. To maximize RNA recovery, an additional elution step is performed using the same conditions (i.e. adding another 50 µL of ribonuclease-free warm water or applying the eluate on the same RNeasy Mini Spin Column (QIAGEN)).[1] RNA extracts may be processed separately or may be combined.		
198	**Homogenization in phosphate buffer.** For both *C. annuum* and *S. lycopersicum*, 12 subsamples of 250 seeds are each immersed in 10 mL 0.1 M phosphate buffer (Na_2HPO_4 and KH_2PO_4, pH 7.2), incubated at 4 °C overnight, and then ground (e.g. with a FastPrep homogenizer (MP Biomedicals)[1] at speed 5 for 40 s).[1] After centrifugation at 10 000 *g* at 4 °C for 10 min, RNA is extracted using the RNeasy Plant Mini Kit (QIAGEN),[1] following the manufacturer's instructions with some minor modifications. In brief, 600 µL supernatant is added to 600 µL RLT Buffer (QIAGEN)[1] without β-mercaptoethanol. Two 600 µL aliquots of this mixture are successively loaded onto the same RNeasy Mini Spin Column (QIAGEN)[1] and centrifuged. RNA is eluted from the RNeasy Mini Spin Columns (QIAGEN)[1] by applying 50 µL of ribonuclease-free warm water (65 °C) followed by centrifugation. To maximize RNA recovery, an additional elution step is performed using the same conditions (i.e. adding another 50 µL of ribonuclease-free warm water or applying the eluate on the same RNeasy Mini Spin Column (QIAGEN)).[1] RNA extracts may be processed separately or may be combined.	C	*Category : TECHNICAL* **(352) EPPO (30 Apr 2024 7:53 PM)** Why 250 seeds ? Subsample size for seeds was recommended at 1000 above.
198	**Homogenization in phosphate buffer.** For both *C. annuum* and *S. lycopersicum*, 12 subsamples of 250 seeds are each immersed in 10 mL 0.1 M phosphate buffer (Na_2HPO_4 and KH_2PO_4, pH 7.2), incubated at 4 °C overnight, and then ground (e.g. with a FastPrep homogenizer (MP Biomedicals)[1] at speed 5 for 40 s).[1] After centrifugation at 10 000 *g* at 4 °C for 10 min, RNA is extracted using the RNeasy Plant Mini Kit (QIAGEN),[1] following the manufacturer's instructions with some minor modifications. In brief, 600 µL supernatant is added to 600 µL RLT Buffer (QIAGEN)[1] without β-mercaptoethanol. Two 600 µL aliquots of this mixture are successively loaded onto the same RNeasy Mini Spin Column (QIAGEN)[1] and centrifuged. RNA is eluted from the RNeasy Mini Spin Columns (QIAGEN)[1] by applying 50 µL of ribonuclease-free warm water (65 °C) followed by centrifugation. To maximize RNA recovery, an additional elution step is performed	C	*Category : EDITORIAL* **(351) EPPO (30 Apr 2024 7:53 PM)** Not clear. Why combining? How many can be combined? Is it related to the test to be used? Etc.. Condider to delete this phrase

	using the same conditions (i.e. adding another 50 µL of ribonuclease-free warm water or applying the eluate on the same RNeasy Mini Spin Column (QIAGEN)).[1] RNA extracts may be processed separately or may be combined.		
198	**Homogenization in phosphate buffer.** For both *C. annuum* and *S. lycopersicum*, 12 subsamples of 250 seeds are each immersed in 10 mL 0.1 M phosphate buffer (Na_2HPO_4 and KH_2PO_4, pH 7.2), incubated at 4 °C overnight, and then ground (e.g. with a FastPrep homogenizer (MP Biomedicals)[1] at speed 5 for 40 s).[1] After centrifugation at 10 000 *g* at 4 °C for 10 min, RNA is extracted using the RNeasy Plant Mini Kit (QIAGEN),[1] following the manufacturer's instructions with some minor modifications. In brief, 600 µL supernatant is added to 600 µL RLT Buffer (QIAGEN)[1] without β-mercaptoethanol. Two 600 µL aliquots of this mixture are successively loaded onto the same RNeasy Mini Spin Column (QIAGEN)[1] and centrifuged. RNA is eluted from the RNeasy Mini Spin Columns (QIAGEN)[1] by applying 50 µL of ribonuclease-free warm water (65 °C) followed by centrifugation. To maximize RNA recovery, an additional elution step is performed using the same conditions (i.e. adding another 50 µL of ribonuclease-free warm water or applying the eluate on the same RNeasy Mini Spin Column (QIAGEN)).[1] RNA extracts may be processed separately or may be combined.	C	*Category : TECHNICAL* **(236) United States of America (1 Apr 2024 7:47 PM)** Include seed grinding media for FastPrep homogenizer
199	In critical cases where the viroid concentration is expected to be low, increasing the pospiviroid RNA concentration may be desirable (Mehle *et al*., 2017). This can be achieved by transferring 4.5 mL supernatant to a 5 mL tube containing 0.5 g Amberlite IRA-900 anion-exchange resin (Polysciences).[1] The RNA is then bound to the resin by continuous shaking (at approximately 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 Mini Kit, QIAGEN)[1] to the pelleted Amberlite beads, followed by incubation and occasional agitation at room temperature for 10 min.[1] 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[1] column, ~~which is~~ washed and processed according to the manufacturer's instructions. Finally, the RNA is eluted from the QIAamp[1] column in 45 µL ribonuclease-free water prewarmed to 65 °C. The QIAamp Viral RNA Mini Kit (QIAGEN)[1] can be also used for RNA extraction from seed homogenate.	P	*Category : EDITORIAL* **(355) EPPO (30 Apr 2024 7:53 PM)**
200	For samples consisting of <100 seeds, a TissueLyser (e.g. QIAGEN or Retsch)[1] can be used. For larger numbers of seeds, a paddle blender (e.g. MiniMix,	C	*Category : EDITORIAL* **(357) EPPO (30 Apr 2024 7:53 PM)**

	Interscience)[1] or homogenizer (e.g. HOMEX 6)[1] 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*., 2015) 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.		Delete or rephrase the last sentence to indicate that one should be careful to avoid crosscontamination
200	For samples consisting of <100 seeds, a TissueLyser (e.g. QIAGEN or Retsch)[1] can be used. For larger numbers of seeds, a paddle blender (e.g. MiniMix, Interscience)[1] or homogenizer (e.g. HOMEX 6)[1] 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*., 2015) 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.	C	*Category : EDITORIAL* **(356) EPPO (30 Apr 2024 7:53 PM)** Delete? Depending on your lab/situation it might or might not be practical.
202	The RNeasy Plant Mini Kit (QIAGEN),[1] CTAB method (Boonham *et al*., 2004) or sbeadex Maxi Plant Kit (LGC Biosearch Technologies)[1] can be used for RNA extraction from tubers. Extraction of RNA using the KingFisher Total RNA Kit (Thermo Scientific)[1] has ~~also~~ been validated in combination ~~with real-time RT-PCR (Roenhorst~~ with and facilitates~~*et al*.,~~ testing large numbers of samples (Roenhorst ~~2005). The KingFisher[1] method throughput makes it a suitable method for testing large number of samples (Roenhorst~~ *et al.*, 2005).[1]	P	*Category : EDITORIAL* **(358) EPPO (30 Apr 2024 7:53 PM)**
203	**Magnetic bead (KingFisher)[1] method.** The following automated procedure is based on the use of the KingFisher mL Magnetic Particle Processor (Thermo Scientific).[1] With appropriate adjustment of volumes, other KingFisher[1] models may be used. For each sample, at least 200 mg tuber tissue is macerated and then add 200 µL extraction buffer (~~200~~ tetrasodium pyrophosphate solution pH 10 (8% (w/v)), 100 µ~~L tetrasodium pyrophosphate solution pH 10 (8% (w/v)) and 100 µ~~L Antifoam B Emulsion (Sigma-Aldrich)[1] ~~added to~~ and 9.8 mL guanidine lysis buffer (GLB: 8 M guanidine hydrochloride, 20 mM Na_2EDTA, 3% (w/v) PVP-10, 25 mM citric acid monohydrate, 1 mM tri-sodium citrate, 0.5% Triton X-100, 25% ethanol)) is ~~added immediately at a ratio of 1 g tuber tissue to 10 mL buffer~~added. Maceration is continued until a 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 supernatant is	P	*Category : EDITORIAL* **(361) EPPO (30 Apr 2024 7:53 PM)**

	removed and placed in the first tube (A) of the KingFisher mL[1] rack, into which 50 µL vortexed MAP Solution A magnetic beads (Invitek)[1] is added. Tube B has 1 mL GLB added to it; tubes C and D, 1 mL 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[1] and nucleic acid extraction is performed following the Thermo Scientific KingFisher Total RNA kit[1] instruction manual.		
203	**Magnetic bead (KingFisher)[1] method.** The following automated procedure is based on the use of the KingFisher mL Magnetic Particle Processor (Thermo Scientific).[1] With appropriate adjustment of volumes, other KingFisher[1] models may be used. For each sample, at least 200 mg tuber tissue is macerated and then extraction buffer (200 µL tetrasodium pyrophosphate solution pH 10 (8% (w/v)) and 100 µL Antifoam B Emulsion (Sigma-Aldrich)[1] added to 9.8 mL guanidine lysis buffer (GLB: 8 M guanidine hydrochloride, 20 mM Na_2EDTA, 3% (w/v) PVP-10, 25 mM citric acid monohydrate, 1 mM tri-sodium citrate, 0.5% Triton X-100, 25% ethanol)) is added immediately at a ratio of 1 g tuber tissue to 10 mL buffer. Maceration is continued until a 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 supernatant is removed and placed in the first tube (A) of the KingFisher mL[1] rack, into which 50 µL vortexed MAP Solution A magnetic beads (Invitek)[1] is added. Tube B has 1 mL GLB added to it; tubes C and D, 1 mL 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[1] and nucleic acid extraction is performed following the Thermo Scientific KingFisher Total RNA kit[1] instruction manual.	C	*Category : EDITORIAL* **(360) EPPO (30 Apr 2024 7:53 PM)** This step is unclear. You add 200 ul extraction buffer, 100 ul antifoam B and 9.8 ml GLB? This is a ratio of 0.2g in 10.1 ml. If that is correct, what does the last part of the sentence mean ("… immediately at a ratio of 1 g tuber tissue to 10 mL buffer")? Is there another buffer to add?
203	**Magnetic bead (KingFisher)[1] method.** The following automated procedure is based on the use of the KingFisher mL Magnetic Particle Processor (Thermo Scientific).[1] With appropriate adjustment of volumes, other KingFisher[1] models may be used. For each sample, at least 200 mg tuber tissue is macerated and then extraction buffer (200 µL tetrasodium pyrophosphate solution pH 10 (8% (w/v)) and 100 µL Antifoam B Emulsion (Sigma-Aldrich)[1] added to 9.8 mL guanidine lysis buffer (GLB: 8 M guanidine hydrochloride, 20 mM Na_2EDTA, 3% (w/v) PVP-10, 25 mM citric acid monohydrate, 1 mM tri-sodium citrate, 0.5% Triton X-100, 25% ethanol)) is added immediately at a ratio of 1 g tuber tissue to 10 mL buffer. Maceration is continued until a cell lysate with minimal intact tissue debris is obtained. Approximately 2 mL lysate is decanted into a fresh microcentrifuge	C	*Category : EDITORIAL* **(359) EPPO (30 Apr 2024 7:53 PM)** This refers to sample preparation

	tube, which is centrifuged at approximately 5 000 *g* for 1 min. One mL supernatant is removed and placed in the first tube (A) of the KingFisher mL[1] rack, into which 50 µL vortexed MAP Solution A magnetic beads (Invitek)[1] is added. Tube B has 1 mL GLB added to it; tubes C and D, 1 mL 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[1] and nucleic acid extraction is performed following the Thermo Scientific KingFisher Total RNA kit[1] instruction manual.		
205	There are several RT-PCR methods for the generic detection (a subset) of pospiviroids using combinations of different primer sets (Table 2). Some of these tests have been evaluated in an ~~Reverse transcription PCR using generic pospiviroid primers is an efficient and sensitive method to detect pospiviroids. There are several RT-PCR methods for the generic detection of pospiviroids using combinations of different primer sets (Table 2). Several real-time RT-PCR methods have been developed to detect a subset of pospiviroids. Some of these tests have been evaluated in an~~ interlaboratory comparison (Olivier *et al*., 2016). Validation data for the recommended methods are given in EPPO (2021a). A list of recommended methods for pospiviroid detection are presented in this section (3.4.3) and summarized in Table 2. Additional tests for pospiviroid detection are summarized in Table 3.	P	*Category : EDITORIAL* **(362) EPPO (30 Apr 2024 7:53 PM)**
205	Reverse transcription PCR using generic pospiviroid primers is an efficient and sensitive method to detect pospiviroids. There are several RT-PCR methods for the generic detection of pospiviroids using combinations of different primer sets (Table 2). Several real-time RT-PCR methods have been developed to detect a subset of pospiviroids. Some of these tests have been evaluated in an interlaboratory comparison (Olivier *et al*., 2016). Validation data for the recommended methods are given in EPPO (2021a). A list of recommended methods for pospiviroid detection are presented in this section (3.4.3) and summarized in Table 2. Additional tests for pospiviroid detection are summarized in Table 3.	C	*Category : TECHNICAL* **(237) United States of America (1 Apr 2024 7:49 PM)** To be specific, change 'real-time' to TaqMan real-time
207	**Table 2.** Recommended methods for the detection or identification of listed viroids in the genus *Pospiviroid*	C	*Category : SUBSTANTIVE* **(363) EPPO (30 Apr 2024 7:53 PM)** PLVd is missing. Verhoeven et al., 2015 suggests that PLVd is also detected by pospi1 en genpospi

207	**Table 2.** Recommended methods for the detection or identification of listed viroids in the genus *Pospiviroid*	C	*Category : TECHNICAL* **(258) Canada (22 Apr 2024 4:41 PM)** This table is really good to summarize the information about the selected methods here.
223	**3.4.3.1**	C	*Category : SUBSTANTIVE* **(274) China (24 Apr 2024 9:08 AM)** The suggested RT-PCR methods is the best primer sets for screening pospiviroids and detecting CLVd is our lab. pCLV would not detect PSTVd, PCFVd, TCDVd, and TPMVd, according to our results.
264	n/a	C	*Category : EDITORIAL* **(364) EPPO (30 Apr 2024 7:53 PM)** Suggestion to add this abbreviation in the legend of table 2 and 3
269	CLVd	C	*Category : SUBSTANTIVE* **(275) China (24 Apr 2024 9:08 AM)** This CLVd RT-qPCR method is valid by our test, it is the daily used ER-qPCR assay for us. Note the CLVd primer/probe set is the same one of 3.4.3.3 and 3.4.3.4
314	Mix A	C	*Category : SUBSTANTIVE* **(276) China (24 Apr 2024 9:09 AM)** According to our test results from 2021 to 2024, and many voice from local customs and companies, this PSTVd-231F/296R/251T was very solid on the detection of potatoes. However, in many tomatoes and peppers, there were always an no expected curve on healthy plants, especially seed samples. The healthy samples were confirmed by high-throughput sequencing and other molecular test assays. PCFVd, TPMVd, TASVd, and CLVd primer/probe are appliable for their sensitivities and specificities.
323	+[e]	C	*Category : EDITORIAL* **(365) EPPO (30 Apr 2024 7:53 PM)** * PLease list footnote e and f in the legend. * Furthermore footnote c and d seem to be lacking. * I guess that e) indicates that not all isolates of TPMVd are detected.
374	ected; -, not detected; bp, base pairs; CEVd, citrus exocortis viroid; CLVd, Columnea latent viroid; CSVd, rysanthemum stunt viroid; EPPO, European and Mediterranean Plant Protection Organization; IrVd-1, iresin oid 1; nt, not tested; PCFVd, pepper chat fruit viroid; PCR, polymerase chain reaction; PSTVd, potato spindl	C	*Category : SUBSTANTIVE* **(277) China (24 Apr 2024 9:09 AM)** Please add the definition of nt

	ber viroid; RT-PCR, reverse transcription PCR; TASVd, tomato apical stunt viroid; TCDVd, tomato chlorotic oid; TPMVd, tomato planta macho viroid.		
376	**Table 3.** Overview of additional PCR-based methods suitable for detection or identification of listed viroids in the genus *Pospiviroid*	C	*Category : TECHNICAL* **(270) Japan (23 Apr 2024 7:50 AM)** Japan proposes adding a method using RT-qPCR to identify individual viroid species. Reference: Yanagisawa et al. (2017, EJPP, 149;11–23)
376	**Table 3.** Overview of additional PCR-based methods suitable for detection or identification of listed viroids in the genus *Pospiviroid*	C	*Category : TECHNICAL* **(259) Canada (22 Apr 2024 4:42 PM)** There are more methods available in other publications, Can the authors specify the reasons for selecting only these methods and the recommended ones? and what are their criteria for selection?
379	**CSVd**	C	*Category : SUBSTANTIVE* **(366) EPPO (30 Apr 2024 7:53 PM)** The CSVd real-time RT-PCR described in the PM7-138 should be added to this table (Appendix 7).
625	If reagents other than those recommended are used (as, for some methods, the original reagents are no longer available), the reverse-transcription or cycling steps may perform differently and should be adapted accordingly and validated. For all reaction mixes and primer and probe dilutions, molecular grade nuclease-free water should be used. The storage temperature at the end of the PCR cycles should be between 4 °C and 20 °C.	C	*Category : EDITORIAL* **(369) EPPO (30 Apr 2024 7:53 PM)** An amplicon can typically be kept at 4-10 °C for short time. In cases where the gel is not done shortly after the PCR, usually amplicons are kept frozen at -20°C. We suggest to delete this sentence since this is routine procedure.
625	If reagents other than those recommended are used (as, for some methods, the original reagents are no longer available), the reverse-transcription or cycling steps may perform differently and should be adapted accordingly and validated. For all reaction mixes and primer and probe dilutions, molecular grade nuclease-free water should be used. The storage temperature at the end of the PCR cycles should be between 4 °C and 20 °C.	C	*Category : EDITORIAL* **(368) EPPO (30 Apr 2024 7:53 PM)** Delete, it is mentioned at with the specific tests/PCR programs
625	If reagents other than those recommended are used (as, for some methods, the original reagents are no longer available), the reverse-transcription or cycling steps may perform differently and should be adapted accordingly and validated. For all reaction mixes and primer and probe dilutions, molecular grade nuclease-free water should be used. The storage temperature at the end of the PCR cycles should be	C	*Category : EDITORIAL* **(367) EPPO (30 Apr 2024 7:53 PM)** Suggestion todelete this, as many reasons can be thought of to use other reagents, e.g.: - not available in your country - you prefer to use the same reagent as you already use for other tests….

	between 4 °C and 20 °C.		
626	If appropriate, an independent test should be conducted to confirm detection (i.e~~.~~. a ~~test using a~~ different test or method or ~~conducted~~ confirmation by a different ~~laboratory) should be conducted to confirm detection~~laboratory). The ~~methods~~ tests recommended or available for ~~such confirmatory tests~~ confirmation are the same as for the initial ~~test~~ testing (as described in the following subsections of 3.4.3, Table 2 and Table 3).	P	*Category : EDITORIAL* **(371) EPPO (30 Apr 2024 7:53 PM)**
626	If appropriate, an independent test (i.e. a test using a different method or conducted by a different laboratory) should be conducted to confirm detection. The methods recommended or available for such confirmatory tests are the same as for the initial test (as described in the following subsections of 3.4.3, Table 2 and Table 3).	C	*Category : EDITORIAL* **(370) EPPO (30 Apr 2024 7:53 PM)** Consider "in critical cases " or "applicable", also considering the wrding in the last box of fig. 1
628	The primer set Pospi1 allows the detection of all known pospiviroids except ~~for~~ CLVd (Verhoeven *et al*., 2004). The Pospi1 primer set is therefore combined with the pCLV4 primer set described by Spieker (1996), which specifically detects CLVd (Olivier *et al*., 2014).	P	*Category : EDITORIAL* **(372) EPPO (30 Apr 2024 7:53 PM)**
629	For the master mix, the OneStep RT-PCR Kit (QIAGEN)[1] has been shown to be reliable when used for the detection of PSTVd, PCFVd, TPMVd, CEVd, CLVd, CSVd, TASVd and TCDVd (Euphresco, 2010).	C	*Category : TECHNICAL* **(452) New Zealand (3 May 2024 5:26 AM)** We would like to know whether Invitrogen RT-PCR master mix is tested?
629	For the master mix, the OneStep RT-PCR Kit (QIAGEN)[1] has been shown to be reliable when used for the detection of PSTVd, PCFVd, TPMVd, CEVd, CLVd, CSVd, TASVd and TCDVd (Euphresco, 2010).	C	*Category : SUBSTANTIVE* **(374) EPPO (30 Apr 2024 7:53 PM)** What is meant here?
629	T~~For the master mix,~~he ~~the~~ OneStep RT-PCR Kit (QIAGEN)[1] has been shown to be reliable when used for the detection of PSTVd, PCFVd, TPMVd, CEVd, CLVd, CSVd, TASVd and TCDVd (Euphresco, 2010).	P	*Category : EDITORIAL* **(373) EPPO (30 Apr 2024 7:53 PM)**
635	**Viroids detected**	C	*Category : SUBSTANTIVE* **(375) EPPO (30 Apr 2024 7:53 PM)** Suggestion to add PLVd (Vased on Verhoeven et al 2015)
660	pCLVR4 (reverse)	C	*Category : EDITORIAL* **(376) EPPO (30 Apr 2024 7:53 PM)** suggestion to list the forward primer first
672	bp, base pair; CEVd, citrus exocortis viroid; CLVd, Columnea latent viroid; CSVd, chrysanthemum stunt viroid; IrVd-1, iresine viroid 1; PCFVd, pepper chat fruit viroid; PLVd, pospiviroid plvd; PSTVd, potato spindle tuber viroid; RT-PCR, reverse transcription–polymerase chain reaction; TASVd, tomato apical stunt viroid; TCDVd, tomato chlorotic dwarf viroid; TPMVd, tomato planta macho viroid.	C	*Category : EDITORIAL* **(378) EPPO (30 Apr 2024 7:53 PM)** PLVd is not listed in the table above

672	bp, base pair; CEVd, citrus exocortis viroid; CLVd, Columnea latent viroid; CSVd, chrysanthemum stunt viroid; IrVd-1, iresine viroid 1; PCFVd, pepper chat fruit viroid; PLVd, pospiviroid plvd; PSTVd, potato spindle tuber viroid; RT-PCR, reverse transcription–polymerase chain reaction; TASVd, tomato apical stunt viroid; TCDVd, tomato chlorotic dwarf viroid; TPMVd, tomato planta macho viroid.	C	*Category : EDITORIAL* **(377) EPPO (30 Apr 2024 7:53 PM)** Is a explanatory list needed for every table in this document (especially in the appendices)? One may assume that most abbreviations are well known to the readers and repeating many of these in bevery table oscures to whole document. Pls check throughout
681	-†	C	*Category : EDITORIAL* **(379) EPPO (30 Apr 2024 7:53 PM)** As the total volume of the reaction mixture is indicated, it is unneccesary to blur the amount of PCR-grade water. To make these tables (check throughout the whole document) more clear, it is strongly advised to write down the amount of water needed for every table in this document, so individual readers do not have to calculate ths themselves.
703	RNA Total	P	*Category : EDITORIAL* **(380) EPPO (30 Apr 2024 7:53 PM)**
705	1.0 25.0	P	*Category : EDITORIAL* **(381) EPPO (30 Apr 2024 7:53 PM)**
707	*Notes:* † For a final reaction volume of 25 µL.	C	*Category : EDITORIAL* **(382) EPPO (30 Apr 2024 7:53 PM)** Delete: see remark above. Check throughout the remainder of the document.
758	**Information on validation.** ~~Validation data (NVIP~~The pospi1 test was validated using the SuperScript One-Step RT-PCR kit (QIAGEN)1 (NIVIP, 2014) ~~were generated~~ according to EPPO (2021a) at ~~the Netherlands Institute for Vectors, Invasive Plants and Plant health (NIVIP)~~NIVIP, ~~Kingdom of~~ the Netherlands (Botermans *et al.*, 2013; EPPO, 2021a).	P	*Category : EDITORIAL* **(383) EPPO (30 Apr 2024 7:53 PM)**
759	Pospi1 Method The Pospi1 method was validated with the SuperScript One-Step RT-PCR (QIAGEN)[1] kit at NIVIP (NVIP, 2014; Botermans *et al.*, 2013; EPPO, 2021a).	P	*Category : EDITORIAL* **(441) Brazil (2 May 2024 4:44 PM)** For better understanding, assuming this sub section is related to this method (analytical sensitivity, analytical specificity, selectivity, repeatability and reproductibility), separate from pCLV4 method.
759	~~The Pospi1 method was validated with the SuperScript One-Step RT-PCR~~	P	*Category : SUBSTANTIVE*

	~~(QIAGEN)[1] kit at NIVIP (NVIP, 2014; Botermans *et al.*, 2013; EPPO, 2021a).~~The pCLV4 test was validated with the SuperScript One-Step RT-PCR kit with Platinum Taq DNA polymerase (Invitrogen)1 according to EPPO (2021a) at the Plant Health Laboratory, French Agency for Food, Environmental and Occupational Health & Safety (LSV ANSES), France (EPPO, 2021a). Both tests, pospi1 and pCLV4, have been compared for detection of pospiviroids in S. lycopersicum leaves and seeds by interlaboratory comparison (Olivier et al., 2016).		**(384) EPPO (30 Apr 2024 7:53 PM)**
760	**Analytical sensitivity.** Pospi1 primers detected all pospiviroids (except CLVd) up to a dilution in the range 102–105, depending on the viroid species and concentration in the original plant material. (Note that this performance criterion is expressed as a relative infection rate in EPPO (2021a), but both values are based on the same data.) Amplicons could be successfully sequenced up to a dilution of 102.	C	*Category : TECHNICAL* **(387) EPPO (30 Apr 2024 7:53 PM)** This is unclear. What is meant with dilution range 102-105
760	**Analytical sensitivity.** Pospi1 primers detected all pospiviroids (except CLVd) up to a dilution in the range 102–105, depending on the viroid species and concentration in the original plant material. (Note that this performance criterion is expressed as a relative infection rate in EPPO (2021a), but both values are based on the same data.) Amplicons could be successfully sequenced up to a dilution of 10^{2}.	C	*Category : TECHNICAL* **(386) EPPO (30 Apr 2024 7:53 PM)** In this paragraph superscript can somehow not be used, thus the powers of 10 are not written correctly. (3x times in this paragraph alone. Check throughout the whole document)
760	**Analytical sensitivity.** ~~Pospi1~~ The pospi1 primers detected all pospiviroids (except CLVd) up to a dilution in the range ~~102–105~~of 102–105 dilution, depending on the viroid species and concentration in the original plant material. (Note that this performance criterion is expressed as a relative infection rate in EPPO (2021a), but both values are based on the same data.) Amplicons could be successfully sequenced up to a dilution of 102.	P	*Category : EDITORIAL* **(385) EPPO (30 Apr 2024 7:53 PM)**
760	**Analytical sensitivity.** Pospi1 primers detected all pospiviroids (except CLVd) up to a dilution in the range 102–105, depending on the viroid species and concentration in the original plant material. (Note that this performance criterion is expressed as a relative infection rate in EPPO (2021a), but both values are based on the same data.) Amplicons could be successfully sequenced up to a dilution of 102.	C	*Category : EDITORIAL* **(248) Canada (19 Apr 2024 2:59 PM)** 102
760	**Analytical sensitivity.** Pospi1 primers detected all pospiviroids (except CLVd) up to a dilution in the range 102–105, depending on the viroid species and concentration in the original plant material. (Note that this performance criterion is expressed as a relative infection rate in EPPO (2021a), but both values are based on	C	*Category : TECHNICAL* **(247) Canada (19 Apr 2024 2:57 PM)** I do not see any value in giving this range as it is sample dependent i.e. will change based on titre of viroid in a sample. Rather absolute sensitivity (number of minimum number of viroid particle

	the same data.) Amplicons could be successfully sequenced up to a dilution of 102.		detectable in a matrix) is a better metrics
760	**Analytical sensitivity.** Pospi1 primers detected all pospiviroids (except CLVd) up to a dilution in the range 102–105, depending on the viroid species and concentration in the original plant material. (Note that this performance criterion is expressed as a relative infection rate in EPPO (2021a), but both values are based on the same data.) Amplicons could be successfully sequenced up to a dilution of 102.	C	*Category : EDITORIAL* **(246) Canada (19 Apr 2024 2:55 PM)** Correct to 102 - 105
760	**Analytical sensitivity.** Pospi1 primers detected all pospiviroids (except CLVd) up to a dilution in the range 102–105, depending on the viroid species and concentration in the original plant material. (Note that this performance criterion is expressed as a relative infection rate in EPPO (2021a), but both values are based on the same data.) Amplicons could be successfully sequenced up to a dilution of 102.	C	*Category : TECHNICAL* **(238) United States of America (1 Apr 2024 7:55 PM)** Change '102-105' to 10 degree 2-10 degree 5. Also, were you referring to in-vitro transcript copy number or dilutions of plant tissue extract? The last sentence in this para: the same change to 10 degree 2, and the same question applies there.
764	pCLV4 Method The pCLV4 method was validated with the SuperScript One-Step RT-PCR kit with Platinum Taq DNA polymerase (Invitrogen)[1] at the Plant Health Laboratory, French Agency for Food, Environmental and Occupational Health & Safety (LSV ANSES), France (EPPO, 2021a).	P	*Category : EDITORIAL* **(442) Brazil (2 May 2024 4:45 PM)** For better understanding, assuming this sub section is related to this method (analytical sensitivity, analytical specificity, selectivity, repeatability and reproductibility), separate from Pospi1 method.
764	~~The pCLV4 method was validated with the SuperScript One-Step RT-PCR kit with Platinum Taq DNA polymerase (Invitrogen)[1] at the Plant Health Laboratory, French Agency for Food, Environmental and Occupational Health & Safety (LSV ANSES), France (EPPO, 2021a).~~	P	*Category : EDITORIAL* **(388) EPPO (30 Apr 2024 7:53 PM)**
765	**Analytical sensitivity.** The pCLV4 primers detected all tested CLVd isolates up to at least a relative infection rate of 1% (i.e. 10^{-2}) for dilution of infected *S. lycopersicum* leaves in healthy *S. lycopersicum* leaves (six replicates for each sample).	P	*Category : EDITORIAL* **(389) EPPO (30 Apr 2024 7:53 PM)**
766	**Analytical specificity.** ~~As at~~ At the validation date, pCLV4 primers had been found to detect all CLVd isolates encountered at LSV ANSES. No cross-reactions were obtained for isolates of other viroids in the genus *Pospiviroid*. *In silico* analysis did not reveal cross-reactions with other *S. lycopersicum*-infecting viruses and host plant sequences (six replicates for each sample).	P	*Category : EDITORIAL* **(390) EPPO (30 Apr 2024 7:53 PM)**
769	~~Pospi1 and pCLV4 methods have been compared for detection of pospiviroids in *S. lycopersicum* leaves and seeds by interlaboratory comparison (Olivier *et al.*,~~	P	*Category : EDITORIAL* **(391) EPPO (30 Apr 2024 7:53 PM)**

	~~2016).~~		
770	***3.4.3.2 Real-time RT-PCR for the detection of pospiviroids on all tissues except seed: the GenPospi ~~method~~ test (Botermans* et al.*, 2013)***	P	*Category : EDITORIAL* **(392) EPPO (30 Apr 2024 7:53 PM)** again, check the use of the term "method" vs "test" throughout the whole document
771	The GenPospi ~~method~~ test (Botermans *et al*., 2013) detects all known pospiviroids in leaves, tubers and fruits. However, the method is not recommended for testing seeds because of its lack of sensitivity in this matrix. The ~~GenPospi method~~ test consists of two reactions running ~~concurrently~~ in ~~separate tubes~~parallel : the first targets all known pospiviroids except CLVd; the second specifically targets CLVd. In both reactions, the mitochondrial *NADH dehydrogenase subunit 5* (*nad5*) gene is included as an internal (extraction) control.	P	*Category : EDITORIAL* **(393) EPPO (30 Apr 2024 7:53 PM)**
920	*Notes:* * The use of reagents from the TaqMan RNA-to-C_T *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).	C	*Category : TECHNICAL* **(453) New Zealand (3 May 2024 6:05 AM)** It says that TaqMan RNA-to-Cr-1-step Kit (applied Biosystem) is critical for pospiviroid qPCR for all type except seeds. Ct values found to increase by 8-10 when using other kits. Ultraplex 1-step ToughMix (Quanta) is only used for samples from seeds. Were the authors tested these samples using ToughMix (Quanta)?
974	*Notes:* *TaqMan RNA-to-C_T *1-Step* Kit (Applied Biosystems).‡ Note that the use of this reagent can be critical, as Ct values have been found to increase by 8–10 when using other kits (Botermans *et al.*, 2013).	C	*Category : EDITORIAL* **(394) EPPO (30 Apr 2024 7:53 PM)** Above is written "is critical". Maybe change above also to can be? Maybe other tested kits, where the Ct value increases were observed, should be named here? There maybe other kits where no increase in Ct value occurs?
980	**Information on validation.** Validation data were generated according to EPPO (2021b) at NIVIP (Botermans *et al*., 2013; EPPO, 2021a). ~~Validation data are provided in Botermans *et al*. (2013) and NIVIP (2014).~~ Nucleic acid extraction was performed using the RNeasy Plant Mini Kit (QIAGEN).[1]	P	*Category : EDITORIAL* **(395) EPPO (30 Apr 2024 7:53 PM)**
982	**Analytical specificity.** The GenPospi method 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), PLVd (1), PSTVd (10), TASVd (3), TCDVd (5) and TPMVd (2)). No reactions were obtained for isolates of the following viroids: avocado sunblotch viroid (genus *Avsunviroid*), chrysanthemum chlorotic mottle viroid (genus	P	*Category : EDITORIAL* **(396) EPPO (30 Apr 2024 7:53 PM)** Based on Verhoeven et al (2015)

	Pelamoviroid) and eggplant latent viroid (genus *Elaviroid*) in the family *Avsunviridae*; apple scar skin viroid (genus *Apscaviroid*), coleus blumei viroid 1 (genus *Coleviroid*), hop latent viroid (genus *Cocadviroid*) and hop stunt viroid (genus *Hostuviroid*) in the family *Pospiviroidae*; and the tomato (*S. lycopersicum*) viruses alfalfa mosaic virus, cucumber mosaic virus, pepino mosaic virus, potato virus Y, tomato mosaic virus, tobacco mosaic virus, tomato chlorosis virus and tomato yellow leaf curl virus.		
986	The PospiSense method (Botermans *et al*., 2020) allows sensitive detection in seeds of all pospiviroids known to naturally infect *C. annuum* and *S. lycopersicum*. It makes use of a single fluorophore and does not discriminate between different pospiviroids. The method is described for samples of approximately ~~3~~ 3,000 seeds, tested in three subsamples of ~~1~~ 1,000 seeds. The method 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 can be used as an internal (extraction or spike) control. When present in high concentrations, individual pospiviroids may produce a signal in both reactions.	P	*Category : EDITORIAL* **(249) Canada (19 Apr 2024 3:01 PM)**
987	The primers and probes ~~for the PospiSense method~~ are listed in Table 11 and the mixes are described in Table 12 to Table 17.	P	*Category : EDITORIAL* **(397) EPPO (30 Apr 2024 7:53 PM)**
1041	Probe ~~PopspiP5~~PospiP5	P	*Category : EDITORIAL* **(398) EPPO (30 Apr 2024 7:53 PM)**
1185	**Table 16.** Composition of PospiSense reaction mix 1 for detection of CLVd, PCFVd, PSTVd, TCDVd, TPMVd and DLVd	C	*Category : EDITORIAL* **(399) EPPO (30 Apr 2024 7:53 PM)** Maybe a note can be made in the legend that when DLVd is not included, volumes can be adapted accordingly.
1214	Subtotal	C	*Category : EDITORIAL* **(400) EPPO (30 Apr 2024 7:53 PM)** Delete subtotal from all tables - or for consitency, include this in tables where it was omitted. Check throughout
1224	CLVd, Columnea latent viroid; DLVd, ~~dahlia~~ Dahlia latent viroid; PCFVd, pepper chat fruit viroid; PCR, polymerase chain reaction; PSTVd, potato spindle tuber viroid; TCDVd, tomato chlorotic dwarf viroid; TPMVd, tomato planta macho viroid.	P	*Category : EDITORIAL* **(260) Canada (22 Apr 2024 4:43 PM)**
1265	CEVd, citrus exocortis viroid; DLVd, ~~dahlia~~ Dhlia latent viroid; PCR, polymerase chain reaction; TASVd, tomato apical stunt viroid; TCDVd.	P	*Category : EDITORIAL* **(261) Canada (22 Apr 2024 4:44 PM)**

1269	**Analytical sensitivity.** For both *S. lycopersicum* and *C. annuum* seeds, one infected seed in a sample of ~~1~~ 1,000 seeds could be detected for CEVd, CLVd, PCFVd, PSTVd, TASVd, TCDVd and TPMVd.	P	*Category : EDITORIAL* **(250) Canada (19 Apr 2024 3:03 PM)**
1272	No cross-reactions were observed for ~~the~~ hop stunt viroid (genus *Hostuviroid*) and the following viruses of *C. annuum* and *S. lycopersicum*: alfalfa mosaic virus, cucumber mosaic virus, pepino mosaic virus, pepper mild mottle 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*.	P	*Category : EDITORIAL* **(401) EPPO (30 Apr 2024 7:53 PM)**
1275	**Diagnostic sensitivity and diagnostic specificity.** Comparison of the PospiSense method with the real-time RT-PCR method for seed testing of Naktuinbouw (section 3.4.3.4), by testing 40 pospiviroid-infected samples and four healthy samples, showed 100% concordance. It should be noted, however, that the PospiSense (Botermans *et al*., 2020) method appeared less sensitive for CEVd and TASVd than the ~~Naktuibouw method~~Naktuinbouw method (see paragraph 3.4.3.4).	P	*Category : EDITORIAL* **(402) EPPO (30 Apr 2024 7:53 PM)**
1276	***3.4.3.4 Real-time RT-PCR for the detection of pospiviroids in ~~seeds: the method of Naktuinbouw (2021~~seeds (Naktuinbouw 2021, 2022)***	P	*Category : EDITORIAL* **(403) EPPO (30 Apr 2024 7:53 PM)**
1277	The method developed by Naktuinbouw (2021, 2022) allows sensitive detection in seeds of all pospiviroids known to naturally infect *C. annuum* and *S. lycopersicum*. The method is described for samples of approximately 3 000 seeds, tested in three subsamples of 1 000 seeds. The method consists of four reactions running in parallel: A (to detect PCFVd, PSTVd, TCDVd and TPMVd (not all isolates)), B (to detect CEVd and CLVd), C (to detect TPMVd genotypes not detected by reaction A; GenBank accession number NC_001558) and D (to detect TASVd). In reactions A and B, DLVd can be used as an internal (extraction or spike) control. In reaction C, the *nad5* gene is used as an internal control. In reaction D, no internal control is used.	C	*Category : EDITORIAL* **(405) EPPO (30 Apr 2024 7:53 PM)** Rephrase, because the use and choice of internal controls depends on the lab.
1277	The method developed by Naktuinbouw (2021, 2022) allows sensitive detection in seeds of all pospiviroids known to naturally infect *C. annuum* and *S. lycopersicum*. The method is described for samples of approximately 3 000 seeds, tested in three subsamples of 1 000 seeds. The method consists of four reactions running in parallel: A (to detect PCFVd, PSTVd, TCDVd and TPMVd (not all isolates)), B (to	P	*Category : EDITORIAL* **(404) EPPO (30 Apr 2024 7:53 PM)**

	detect CEVd and CLVd), C (to detect TPMVd ~~genotypes~~ isolates not detected by reaction A; GenBank accession number NC_001558) and D (to detect TASVd). In reactions A and B, DLVd can be used as an internal (extraction or spike) control. In reaction C, the *nad5* gene is used as an internal control. In reaction D, no internal control is used.		
1277	The method developed by Naktuinbouw (2021, 2022) allows sensitive detection in seeds of all pospiviroids known to naturally infect *C. annuum* and *S. lycopersicum*. The method is described for samples of approximately ~~3~~ 3,000 seeds, tested in three subsamples of 1 000 seeds. The method consists of four reactions running in parallel: A (to detect PCFVd, PSTVd, TCDVd and TPMVd (not all isolates)), B (to detect CEVd and CLVd), C (to detect TPMVd genotypes not detected by reaction A; GenBank accession number NC_001558) and D (to detect TASVd). In reactions A and B, DLVd can be used as an internal (extraction or spike) control. In reaction C, the *nad5* gene is used as an internal control. In reaction D, no internal control is used.	P	*Category : EDITORIAL* **(251) Canada (19 Apr 2024 3:04 PM)**
1278	The primers and probes ~~for the method of Naktuinbouw (2021, 2022)~~ are listed in Table 18 and the mixes are described in Table 19 to Table 28.	P	*Category : EDITORIAL* **(406) EPPO (30 Apr 2024 7:53 PM)**
1282	**Table 18.** Real-time RT-PCR primers and probes for the method of Naktuinbouw (2021, 2022)	C	*Category : EDITORIAL* **(407) EPPO (30 Apr 2024 7:53 PM)** Check the font of all tables thoughout the document. These are not uniform.
1285	~~Ref~~**Reference**	P	*Category : EDITORIAL* **(408) EPPO (30 Apr 2024 7:53 PM)** Please use the full word. It saves a footnote.
1600	DLVd, ~~dahlia~~ Dahlia latent viroid; PCFVd, pepper chat fruit viroid; PCR, polymerase chain reaction; PSTVd, potato spindle tuber viroid; TCDVd, tomato chlorotic dwarf viroid; TPMVd, tomato planta macho viroid.	P	*Category : EDITORIAL* **(262) Canada (22 Apr 2024 4:45 PM)**
1633	CEVd, citrus exocortis viroid; CLVd, Columnea latent viroid; DLVd, ~~dahlia~~ Dahlia latent viroid; PCR, polymerase chain reaction.	P	*Category : EDITORIAL* **(263) Canada (22 Apr 2024 4:45 PM)**
1706	**Information on validation.** Validation data were generated according to EPPO (2021b) by Naktuinbouw~~, the Kingdom of ,~~ The Netherlands (Naktuinbouw, 2016). Nucleic acid was extracted ~~was~~ using the sbeadex Maxi Plant Kit (LGC Biosearch Technologies).[1]	P	*Category : EDITORIAL* **(409) EPPO (30 Apr 2024 7:53 PM)**

1707	**Analytical sensitivity.** For CEVd, CLVd, PCFVd, PSTVd, TASVd and TCDVd, one infected seed could be detected in a sample of 1 000 seeds. Detection of TPMVd was shown to be 10× less sensitive (e.g. one infected seed could be detected in a sample of 100 seeds).	C	*Category : TECHNICAL* **(454) New Zealand (3 May 2024 6:10 AM)** It has been shown that one infected seed in 1000 seeds could be detected. Do we know what is the limited detection in copy number and/or corresponding Ct value?
1707	**Analytical sensitivity.** For CEVd, CLVd, PCFVd, PSTVd, TASVd and TCDVd, one infected seed could be detected in a sample of 1 000 seeds. Detection of TPMVd was shown to be 10× less sensitive (e.g. one infected seed could be detected in a sample of 100 seeds).	C	*Category : EDITORIAL* **(410) EPPO (30 Apr 2024 7:53 PM)** Mix A or Mix C isolates?
1708	**Analytical specificity.** No cross-reactions were observed with 29 isolates of other ~~viruses and~~ viroids and virusses 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 and CLVd primer mix B was observed.	P	*Category : EDITORIAL* **(412) EPPO (30 Apr 2024 7:53 PM)**
1708	**Analytical specificity.** No cross-reactions were observed 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 and CLVd primer mix B was observed.	C	*Category : SUBSTANTIVE* **(411) EPPO (30 Apr 2024 7:53 PM)** Please be clearer: what level of cross-reactivity should be accepted?
1712	**Hybridization with a digoxigenin (DIG)-labelled RNA probe.** Nucleic acid hybridization using probes that cross-hybridize with other pospiviroids under low-stringency conditions has been used for viroid detection (Owens and Diener, 1981; Singh, Nie and Singh, 1999). Nucleic acid hybridization using a DIG-labelled PSTVd complementary (c)RNA probe (Agdia, Inc.) is a sensitive detection method but less amenable to high-throughput sequencing (HTS) in comparison with 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. laxum*, *S. lycopersicum* and *S. tuberosum* (Torchetti, Navarro and Di Serio, 2012; Monger and Jeffries, 2015). The sensitivity of detection has been found to be at least 17 pg PSTVd (Jeffries and James, 2005). Probe preparation, sample preparation, test-membrane preparation and hybridization conditions are as described in EPPO (2021a) and DP 7.	C	*Category : TECHNICAL* **(415) EPPO (30 Apr 2024 7:53 PM)** (note that Agdia, Inc. no longer provide the PSTVd DIG-labelled probe
1712	**Hybridization with a digoxigenin (DIG)-labelled RNA probe.** Nucleic acid	P	*Category : TECHNICAL* **(414) EPPO (30 Apr 2024 7:53 PM)**

	hybridization using probes that cross-hybridize with other pospiviroids under low-stringency conditions has been used for viroid detection (Owens and Diener, 1981; Singh, Nie and Singh, 1999). Nucleic acid hybridization using a DIG-labelled PSTVd complementary (c)RNA probe ~~(Agdia, Inc.) (~~ is a sensitive detection method but less amenable to high-throughput sequencing (HTS) in comparison with 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. laxum*, *S. lycopersicum* and *S. tuberosum* (Torchetti, Navarro and Di Serio, 2012; Monger and Jeffries, 2015). The sensitivity of detection has been found to be at least 17 pg PSTVd (Jeffries and James, 2005). Probe preparation, sample preparation, test-membrane preparation and hybridization conditions are as described in EPPO (2021a) and DP 7.		DIG probe by Agdia has been discontinued. Suggestion for modification in the text.
1712	**Hybridization with a digoxigenin (DIG)-labelled RNA probe.** Nucleic acid hybridization using probes that cross-hybridize with other pospiviroids under low-stringency conditions has been used for viroid detection (Owens and Diener, 1981; Singh, Nie and Singh, 1999). Nucleic acid hybridization using a DIG-labelled PSTVd complementary (c)RNA probe (Agdia, Inc.) is a sensitive detection method but less amenable to high-throughput sequencing (HTS) in comparison with 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. laxum*, *S. lycopersicum* and *S. tuberosum* (Torchetti, Navarro and Di Serio, 2012; Monger and Jeffries, 2015). The sensitivity of detection has been found to be at least 17 pg PSTVd (Jeffries and James, 2005). Probe preparation, sample preparation, test-membrane preparation and hybridization conditions are as described in EPPO (2021a) and DP 7.	C	*Category : TECHNICAL* **(413) EPPO (30 Apr 2024 7:53 PM)** Suggested reference for inclusion. Zhang, Y.H., Li Z.X., Du, Y.J., Li, S.F., Zhang, Z.X. 2023. A universal probe for simultaneous detection of six pospiviroids and natural infection of potato spindle tuber viroid (PSTVd) in tomato in China. Journal of Integrative Agriculture, 22: 790–798. https://doi.org/10.1016/j.jia.2022.08.119
1713	A list of additional tests for detection of several or individual members of the genus ~~**Other methods and kits.** A list of additional methods for detection of several or individual members of the genus~~ *Pospiviroid* are listed in Table 3 (EPPO, 2021a) and peer-reviewed journals (Hammond and Zhang, 2016, Kovalskaya and Hammond, 2022). ~~A~~ In addition, a range of commercial kits ~~are~~ is available for the detection of specific pospiviroids using either real-time PCR, isothermal amplification or hybridization ~~methods~~tests. Testing laboratories should follow	P	*Category : EDITORIAL* **(417) EPPO (30 Apr 2024 7:53 PM)**

	recommendations for users and validate these tests for the specific use intended.		
1713	**Other methods and kits.** A list of additional methods for detection of several or individual members of the genus *Pospiviroid* are listed in Table 3 (EPPO, 2021a) and peer-reviewed journals (Hammond and Zhang, 2016, Kovalskaya and Hammond, 2022). A range of commercial kits are available for the detection of specific pospiviroids using either real-time PCR, isothermal amplification or hybridization methods. Testing laboratories should follow recommendations for users and validate these tests for the specific use intended.	C	*Category : EDITORIAL* **(416) EPPO (30 Apr 2024 7:53 PM)** Consider to move this section above the previous one.
1715	For the test result to be considered reliable, appropriate controls – which will depend on the type of method used for the test and the level of certainty required – should be considered for each series of nucleic acid ~~isolations~~ isolation and amplification of the target pest or target nucleic acid. For RT-PCR, a positive nucleic acid control and a negative amplification control (no template control) are the minimum controls that should be used.	P	*Category : EDITORIAL* **(418) EPPO (30 Apr 2024 7:53 PM)**
1715	For the test result to be considered reliable, appropriate controls – which will depend on the type of method used for the test and the level of certainty required – should be considered for each series of nucleic acid isolations and amplification of the target pest or target nucleic acid. For RT-PCR, a positive nucleic acid control and a negative amplification control (no template control) are the minimum controls that should be used.	C	*Category : TECHNICAL* **(264) Canada (22 Apr 2024 4:46 PM)** Why are these two controls are the minimum? Shouldn't positive extraction control be used too?
1715	For the test result to be considered reliable, appropriate controls – which will depend on the type of method used for the test and the level of certainty required – should be considered for each series of nucleic acid isolations and amplification of the target pest or target nucleic acid. For RT-PCR, a positive nucleic acid control and a negative amplification control (no template control) are the minimum controls that should be used.	C	*Category : TECHNICAL* **(253) Canada (19 Apr 2024 3:06 PM)** Minimum controls should also include "Negative Extraction Control".
1715	For the test result to be considered reliable, appropriate controls – which will depend on the type of method used for the test and the level of certainty required – should be considered for each series of nucleic acid isolations and amplification of the target pest or target nucleic acid. For RT-PCR, a positive nucleic acid control and a negative amplification control (no template control) are the minimum controls that should be used.	C	*Category : TECHNICAL* **(252) Canada (19 Apr 2024 3:05 PM)** Why not for both RT-PCR and Real-time RT-PCR?
1716	**Positive nucleic acid control.** This control is used to ensure that amplification of a	C	*Category : EDITORIAL*

	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[1] gene fragments for specific pospiviroids such as PSTVd (Adkar-Purushothama, Pierrick and Perreault, 2017; EPPO, 2021a) can be used as a positive nucleic acid control. An additional control at the limit of detection may also be used.		**(420) EPPO (30 Apr 2024 7:53 PM)** Should it be noted that such a DNA control does not control the RT step?
1716	**Positive nucleic acid control.** 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[1] gene fragments for specific pospiviroids such as PSTVd (Adkar-Purushothama, Pierrick and Perreault, 2017; EPPO, 2021a) can be used as a positive nucleic acid control. An additional control at the limit of detection may also be used.	C	*Category : EDITORIAL* **(419) EPPO (30 Apr 2024 7:53 PM)** It should be indicated that preferably an RNA from a deviating isolate is used, i.e. to enable to recognise cross contamination. This information is mentioned for PIC but is relevant here as well.
1716	**Positive nucleic acid control.** This control is used to ensure that amplification of a known target happens as expected (apart from the extraction). ~~Pospiviroid-infected~~ Target pospiviroid-infected RNA extract, target viroid RNA or a synthetic control (e.g. cloned PCR product) can be used. Furthermore, gBlocks[1] gene fragments for specific pospiviroids such as PSTVd (Adkar-Purushothama, Pierrick and Perreault, 2017; EPPO, 2021a) can be used as a positive nucleic acid control. An additional control at the limit of detection may also be used.	P	*Category : EDITORIAL* **(254) Canada (19 Apr 2024 3:08 PM)**
1717	**Internal control.** An internal control is recommended for the RT-PCR tests to reduce the possibility of false negative results occurring because of nucleic acid extraction failure or target degradation, or the presence of PCR inhibitors. For conventional and real-time RT-PCR, a plant housekeeping gene such as the *cytochrome oxidase* (*COX*) gene or *nad5* can be used. Although the *COX* target has been used as an internal control in this protocol, *COX* primers will amplify RNA and DNA and, therefore, the *COX* target is not a control for the RT step. The mitochondrial *nad5* target has been shown to be a reliable indicator of the performance of the RNA extraction and RT step for both conventional RT-PCR (Menzel, Jelkmann and Maiss, 2002) and real-time RT-PCR (Botermans *et al.*, 2013) as the nad5 primers span an exon–intron junction and will therefore not amplify DNA. It has been tested against many plant species, including	C	*Category : EDITORIAL* **(422) EPPO (30 Apr 2024 7:53 PM)** Is this true for all the plant species? It is surprising if the same exon intron junction would be conserved in all plant species. In case the exon-intron junction in the primer is not present in all species, maybe rephrase sentence?

	several *Solanum* species (*S. bonariense*, *S. dulcamara*, *S. laxum*, *S. nigrum*, *S. pseudocapsicum*, *S. rantonnetii*, *S. sisymbrifolium*), *Acnistus arborescens*, *Atropa belladonna*, *Brugmansia* spp., *Capsicum* spp., *Cestrum* spp., *Iochroma cyaneum*, *Nicotiana* spp. and *Physalis* spp. (Seigner *et al*., 2008). As an alternative, an external (unrelated) spiked target such as DLVd can be used to replace the internal control. The internal control primers can be used in a duplex reaction with the pospiviroid primers or as two separate (simplex) reactions, should the analytical sensitivity of the test be reduced in a duplex reaction.		
1717	**Internal control.** An internal control is recommended for the RT-PCR tests to reduce the possibility of false negative results occurring because of nucleic acid extraction failure or target degradation, or the presence of PCR inhibitors. For conventional and real-time RT-PCR, a plant housekeeping gene such as the *cytochrome oxidase* (*COX*) gene or *nad5* can be used. ~~Although the~~ *COX* ~~target has been used as an internal control in this protocol, *COX*~~ primers will amplify RNA and DNA and, therefore, the *COX* target is not a control for the RT step. The mitochondrial *nad5* target has been shown to be a reliable indicator of the performance of the RNA extraction and RT step for both conventional RT-PCR (Menzel, Jelkmann and Maiss, 2002) and real-time RT-PCR (Botermans *et al*., 2013) as the nad5 primers span an exon–intron junction and will therefore not amplify DNA. It has been tested against many plant species, including several *Solanum* species (*S. bonariense*, *S. dulcamara*, *S. laxum*, *S. nigrum*, *S. pseudocapsicum*, *S. rantonnetii*, *S. sisymbrifolium*), *Acnistus arborescens*, *Atropa belladonna*, *Brugmansia* spp., *Capsicum* spp., *Cestrum* spp., *Iochroma cyaneum*, *Nicotiana* spp. and *Physalis* spp. (Seigner *et al*., 2008). However, for seeds nad5 is not recommended because it shows variable results for this matrix. As an alternative, an external (unrelated) spiked target such as DLVd can be used to replace the internal control. The internal control primers can be used in a duplex reaction with the pospiviroid primers or as two separate (simplex) reactions, should the analytical sensitivity of the test be reduced in a duplex reaction.	P	*Category : EDITORIAL* **(421) EPPO (30 Apr 2024 7:53 PM)**
1718	**Negative amplification control (or no template control).** This control is necessary for conventional and real-time ~~PCR~~ RT-PCR to rule out false positives resulting from contamination with target RNA during preparation of the reaction	P	*Category : EDITORIAL* **(423) EPPO (30 Apr 2024 7:53 PM)**

	mixture. PCR-grade water that was used to prepare the reaction mixture, or sterile phosphate-buffered saline, is added instead of the target at the amplification stage.		
1736	A Ct cut-off value may be applied according to laboratory validation data.	C	*Category : TECHNICAL* **(424) EPPO (30 Apr 2024 7:53 PM)** Is this not rather verification, which is needed here?
1736	A Ct cut-off value may be applied according to laboratory validation data.	C	*Category : TECHNICAL* **(239) United States of America (1 Apr 2024 8:00 PM)** Ct cut-off value is a must for routine diagnostics
1737	**4. Identification**	C	*Category : TECHNICAL* **(241) United States of America (1 Apr 2024 8:03 PM)** There is the need to include a sentence or two in this section on the need to clone conventional RT-PCR products before sequencing if the initial sequence without cloning is noisy or sequencing failed due to mixed infection of pospiviroids.
1738	Members of the genus *Pospiviroid* (with the exception of CLVd: see Table 2 and Spieker (1996)) can ~~only~~ be identified by sequence analysis of the ~~amplicon~~ amplicon(s) obtained by the conventional RT-PCR ~~method~~ tests (section 3.4.3.~~1) or HTS~~1), followed by comparison of the sequence with sequences in public databases. Table 2 and Table 3 give an overview of primer sets that can be used for amplification and sequencing for the identification of the different pospiviroids. 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, such as the first finding in a country or in a new host, the laboratory may confirm the result by conducting another test or asking another laboratory to conduct a test.~~	P	*Category : EDITORIAL* **(425) EPPO (30 Apr 2024 7:53 PM)**
1739	Further information and recommendations on the use of HTS as a diagnostic tool for phytosanitary purposes have been published (CPM R-08, 2019; Lebas *et al*., 2022).	C	*Category : EDITORIAL* **(427) EPPO (30 Apr 2024 7:53 PM)** Also refer to EPPO Guidelines PM7/151 (1)
1739	Alternatively, high throughput sequencing (HTS) can be used for the identification of pospiviroids. Further information and recommendations on the use of HTS as a diagnostic tool for phytosanitary purposes have been published (CPM R-08, 2019; Lebas *et al*., 2022).	P	*Category : EDITORIAL* **(426) EPPO (30 Apr 2024 7:53 PM)**
1740	EPPO (2021c) provides general guidance on sequencing and sequence analysis. For the identification of pospiviroids, it is preferable that the sequence of the	C	*Category : TECHNICAL* **(443) Brazil (2 May 2024 4:48 PM)**

	complete genome is analysed. According to ICTV, the main criterion for viroid identification is a sequence identity of more than 90% (Owens *et al*., 2012). However, if the sequence identity obtained is close to 90%, additional parameters should be included, such as biological properties. The ICTV Viroid Study Group is currently discussing viroid classification and the criteria for species demarcation.		Which ones? Should be interesting to have some examples in this section)
1740	EPPO (2021c) provides general guidance on sequencing and sequence analysis. For the identification of pospiviroids, it is preferable that the sequence of the complete genome is analysed. According to ICTV, the main criterion for viroid identification is a sequence identity of more than 90% (Owens *et al*., 2012). However, if the sequence identity obtained is close to 90%, additional parameters should be included, such as biological properties. The ICTV Viroid Study Group is currently discussing viroid classification and the criteria for species demarcation.	C	*Category : TECHNICAL* **(240) United States of America (1 Apr 2024 8:01 PM)** What will the additional parameters be when testing seeds?
1741	The method using the Pospi1 primers (Verhoeven *et al*., 2004) has been found to be the most sensitive conventional RT-PCR test, in some cases being comparable to real-time RT-PCR. ~~Although~~ The Pospi2 primers (Verhoeven *et al*., 2017), which have the ~~amplicon only covers about~~ opposite orientation, can be used to obtain the sequence of the other half of the ~~pospiviroid genome~~genome for completion. However, ~~this~~ the pospi2 test is less sensitive than the Pospi1 test. Therefore, in some cases it is not feasible to obtain the complete genome sequence. In some cases, the partial sequence ~~appears to~~ obtained by the pospi1 primers, which covers about half of the pospiviroid genome might be suitable for the correct identification of isolates.	P	*Category : EDITORIAL* **(428) EPPO (30 Apr 2024 7:53 PM)**
1742	~~The Pospi2 primers (Verhoeven *et al*., 2017), which have the opposite orientation, can be used to obtain the sequence of the other half of the genome for completion. However, this method is less sensitive than the Pospi1 method. Therefore, in some cases it is not feasible to obtain the complete genome sequence. Table 2 and Table 3 give an overview of primer sets that can be used for amplification and sequencing for the identification of the different pospiviroids.~~	P	*Category : EDITORIAL* **(429) EPPO (30 Apr 2024 7:53 PM)**
1743	A positive sample detected by real-time RT-PCR should, if required, be ~~retested~~ confirmed using either a different real-time ~~PCR~~ RT-PCR method ~~for confirmation~~ or a conventional RT-PCR to enable the amplicon to be sequenced for viroid identification. ~~However, because~~ Choices for further testing will depend on the initial method used; examples of methods suitable for substantiating results are	P	*Category : SUBSTANTIVE* **(430) EPPO (30 Apr 2024 7:53 PM)** Revised change by Netherlands on 6 Mar 2024 10:06

	provided in Table 2 and Table 3. For example, due to the higher analytical sensitivity of the real-time RT-PCR, an amplicon may not be obtained with conventional RT-PCR. This is especially so in the case of seed testing, where viroid concentrations may be ~~low and conventional RT-PCR methods may lack the analytical sensitivity to produce an amplicon~~low. ~~Choices for further testing will depend on the initial method used; examples of methods suitable for substantiating results are provided in Table 2 and Table 3.~~		
1744	To obtain the complete genome sequence or sequences, forward and reverse RT-PCR primers are used for bi-directional 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 the GenBank non-redundant nucleotide database) using a local alignment tool (such as the Basic Local Alignment Search Tool for nucleotides (BLASTN), available at the National Center for Biotechnology Information ~~((~~(NCBI, https://www.ncbi.nlm.nih.gov)). ~~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~~ position 1 of the viroid genome for comparison with public nucleotide databases. Further sequence analysis can be performed by multiple sequence alignment and phylogenetic analysis using appropriate software (such as MEGA or CLUSTALW). ~~nucleotide databases.~~	P	*Category : EDITORIAL* **(431) EPPO (30 Apr 2024 7:53 PM)**
1745	In critical cases, for example when a sequence is to be submitted to a public database or when a new viroid in the genus ~~When 100% sequence accuracy is required, for example when a sequence is to be submitted to a public database or when a new viroid in the genus~~ *Pospiviroid* is suspected, it is recommended that an RT-PCR amplicon covering the region of the primers used for the first RT-PCR be sequenced to clear any potential sequencing ambiguity. Design of a new set of primers from the initial sequence may be required for this purpose.	P	*Category : TECHNICAL* **(432) EPPO (30 Apr 2024 7:53 PM)**
1749	if relevant and ~~still~~ available, material of the original sample, stored at −80 °C or freeze-dried;	P	*Category : EDITORIAL* **(433) EPPO (30 Apr 2024 7:53 PM)**
1756	Netherlands Institute for Vectors, Invasive Plants and Plant health (NIVIP), ~~National Plant Protection Organization (NPPO) of the Netherlands~~Netherlands	P	*Category : EDITORIAL* **(434) EPPO (30 Apr 2024 7:53 PM)**

	Food and Consumer Product Safety Authority (NVWA), PO Box 9102, 6700 HC Wageningen, ~~Kingdom of the~~ The Netherlands (Johanna W. Roenhorst; email: j.w.roenhorst@nvwa.nl; Carla Oplaat; email: a.g.oplaat@nvwa.nl; and Marleen Botermans; email: m.botermans@nvwa.nl).		
1760	The first draft of this protocol was written by Christophe Lacomme (SASA, The Scottish Government (see preceding section)), Johanna W. Roenhorst and Carla Oplaat (NIVIP, ~~NPPO~~NVWA, ~~Kingdom of the~~ The Netherlands (see preceding section)), and Rose Hammond (Agricultural Research Service, United States Department of Agriculture, United States of America).	P	*Category : EDITORIAL* **(435) EPPO (30 Apr 2024 7:53 PM)**
1761	In addition, Ellis ~~Meeks~~ Meekes and Harrie Koenraadt (Naktuinbouw, ~~Kingdom of the~~ The Netherlands) provided comments during the expert consultation.	P	*Category : EDITORIAL* **(436) EPPO (30 Apr 2024 7:53 PM)**
1763	**8. References**	C	*Category : EDITORIAL* **(226) CA (19 Mar 2024 6:09 PM)** Complete this references: Referencias Bibliográficas: Fernow KH, Peterson LC & Plaisted RL (1970) Spindle tuber virus in seeds and pollen of infected plants. American Potato Journal 47, 75–80. Singh RP (1970) Seed transmission of potato spindle tuber virus in tomato and potato. American Potato Journal 47, 225–227. Merriam D & Bonde R (1954) Dissemination of spindle tuber by contaminated tractor wheels and by foliage contact with diseased plants. Phytopathology 44, 111. Manzer FE & Merriam D (1961) Field transmission of potato spindle tuber virus and virus X by cultivating and hilling equipment. American Potato Journal 38, 346–352. Morris TJ & Smith EM (1977) Potato spindle tuber disease: procedures for the detection of viroid RNA and certification of disease-free potato tubers. Phytopathology 67, 145–150 Gos RW (1926) Transmission of potato spindle tuber disease by cutting knives and seed piece contact. Phytopathology 16, 299–304.
1768	**Bernad, L. & Duran-Vila, N.** 2006. A novel RT-PCR approach for detection and characterization of citrus viroids. *Molecular and Cellular Probes*, 20: 105–113. https://doi.org/10.1016/j.mcp.2005.11.001	C	*Category : EDITORIAL* **(242) United States of America (1 Apr 2024 8:04 PM)** This reference is not cited in the main text; suggest deleting.

1779	Eiras, M., Targon, M. L. P. N., Fajardo, T. V. M., Flores, R., & Kitajima, E. W.. 2006. Citrus exocortis viroid and Hop Stunt viroid Doubly infecting grapevines in Brazil. Fitopatologia Brasileira, 31(5), 440–446. https://doi.org/10.1590/S0100-41582006000500002 **EFSA (European Food Safety Authority)**. 2008. Pest risk assessment made by France on citrus exocortis viroid (CEVd) considered by France as harmful in French overseas department of Réunion – Scientific opinion of the Panel on Plant Health. *EFSA Journal*, 685: 1–17. https://doi.org/10.2903/j.efsa.2008.685	P	*Category : SUBSTANTIVE* **(444) Brazil (2 May 2024 6:10 PM)** Due to the suggestion on Table 1 section 3.1