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ISPM 27
Diagnostic protocols for regulated pests

DP 36: Genus *Pospiviroid*

Adopted 2025; published 2025

CONTENTS

1. Pest information

Viroids are subviral agents that infect plants. A viroid consists of a closed, circular, un-encapsidated, single-stranded RNA molecule that does not code for any protein. The genome size for individual species varies within a range of about 250–430 nucleotides (ICTV, n.d.); however, some isolates have been reported to have genomes outside this range. A viroid replication mechanism uses RNA polymerase, a host-cell enzyme associated with synthesis of 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.*, 2021).

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 *Pospiviroid impedichrysanthemi*), citrus exocortis viroid (CEVd; species *Pospiviroid exocortiscitri*), Columnea latent viroid (CLVd; species *Pospiviroid latenscolumneae*), iresine viroid 1 (IrVd-1; species *Pospiviroid alphairesinis*), pepper chat fruit viroid (PCFVd; species *Pospiviroid parvicapsici*), portulaca latent viroid (PLVd; species *Pospiviroid latensportulacae*; Verhoeven *et al*., 2015; Di Serio *et al*., 2021), potato spindle tuber viroid (PSTVd; species *Pospiviroid fusituberis*; type species), tomato apical stunt viroid (TASVd; species *Pospiviroid apicimpeditum*), tomato chlorotic dwarf viroid (TCDVd; species *Pospiviroid chloronani*) and tomato planta macho viroid (TPMVd; species *Pospiviroid machoplantae*, 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 (Martínez-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 latest information on classification of the genus *Pospiviroid* may be obtained from the ICTV (n.d.).

Pospiviroids have been reported worldwide (Faggioli *et al*., 2017). They can cause severe diseases in their hosts, particularly PSTVd in *Solanum tuberosum* (potato) (Pfannenstiel and Slack, 1980) and CEVd, CLVd, PSTVd, TCDVd in *Solanum lycopersicum* (tomato) crops (Verhoeven *et al*., 2004). Therefore, pospiviroids are regulated in many countries (EPPO, 2025a). Although pospiviroids can be experimentally transmitted to many plant species, 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, including grafting (Roistacher, 1991), 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 (Semancik, 1980; Roistacher, 2004; 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) or *Bombus terrestris* (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; Van Bogaert *et al*., 2016). However, mechanical transmission by wounding of the flowers has also been suggested.

2. Taxonomic information

Viroid name: chrysanthemum stunt viroid (acronym CSVd)
Species name: *Pospiviroid impedichrysanthemi*
Other scientific names: *Chrysanthemum stunt mottle virus*, *Chrysanthemum stunt pospiviroid*, *Chrysanthemum stunt viroid*
Taxonomic position: *Pospiviroidae*, *Pospiviroid*
Common name: measles of chrysanthemum

Viroid name: citrus exocortis viroid (acronym CEVd)
Species name: *Pospiviroid exocortiscitri*
Other scientific names: *Citrus exocortis pospiviroid*, *Citrus exocortis viroid*, *Indian tomato bunchy top viroid*
Taxonomic position: *Pospiviroidae*, *Pospiviroid*
Common name: citrus exocortis

Viroid name: Columnea latent viroid (acronym CLVd)
Species name: *Pospiviroid latenscolumneae*
Other scientific names: *Columnea latent pospiviroid*, *Columnea latent viroid*
Taxonomic position: *Pospiviroidae*, *Pospiviroid*
Common name: none

Viroid name: iresine viroid 1 (acronym IrVd-1)
Species name: *Pospiviroid alphairesinis*
Other scientific names: *Iresine pospiviroid*, *Iresine viroid*, *Iresine viroid 1*
Taxonomic position: *Pospiviroidae*, *Pospiviroid*
Common name: none

Viroid name: pepper chat fruit viroid (acronym PCFVd)
Species name: *Pospiviroid parvicapsici*
Other scientific names: *Pepper chat fruit pospiviroid*, *Pepper chat fruit viroid*
Taxonomic position: *Pospiviroidae*, *Pospiviroid*
Common name: none

Viroid name: portulaca latent viroid (acronym PLVd)
Species name: *Pospiviroid latensportulacae*
Other scientific names: *Portulaca latent pospiviroid*, *Portulaca latent viroid*, *Pospiviroid plvd*
Taxonomic position: *Pospiviroidae*, *Pospiviroid*
Common name: none

Viroid name: potato spindle tuber viroid (acronym PSTVd)
Species name: *Pospiviroid fusituberis*
Other scientific names: *Potato gothic virus*, *Potato spindle tuber pospiviroid*, *Potato spindle tuber viroid*, *Potato spindle tuber virus*, *Tomato bunchy top virus*
Taxonomic position: *Pospiviroidae*, *Pospiviroid*
Common name: spindle tuber of potato

Viroid name: tomato apical stunt viroid (acronym TASVd)
Species name: *Pospiviroid apicimpeditum*
Other scientific names: *Tomato apical stunt pospiviroid*, *Tomato apical stunt viroid*
Taxonomic position: *Pospiviroidae*, *Pospiviroid*
Common name: none

Viroid name: tomato chlorotic dwarf viroid (acronym TCDVd)
Species name: *Pospiviroid chloronani*
Other scientific names: *Tomato chlorotic dwarf pospiviroid*, *Tomato chlorotic dwarf viroid*
Taxonomic position: *Pospiviroidae*, *Pospiviroid*
Common name: none

Viroid name: tomato planta macho viroid (acronym TPMVd)
Species name: *Pospiviroid machoplantae*
Other scientific names: *Tomato planta macho pospiviroid*, *Tomato planta macho viroid*
Taxonomic position: *Pospiviroidae*, *Pospiviroid*
Common name: tomato planta macho

3. Detection

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 can also detect other pospiviroids 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) No. 7 (*Potato spindle tuber viroid* (Annex 7 to ISPM 27 (*Diagnostic protocols for regulated pests*)).

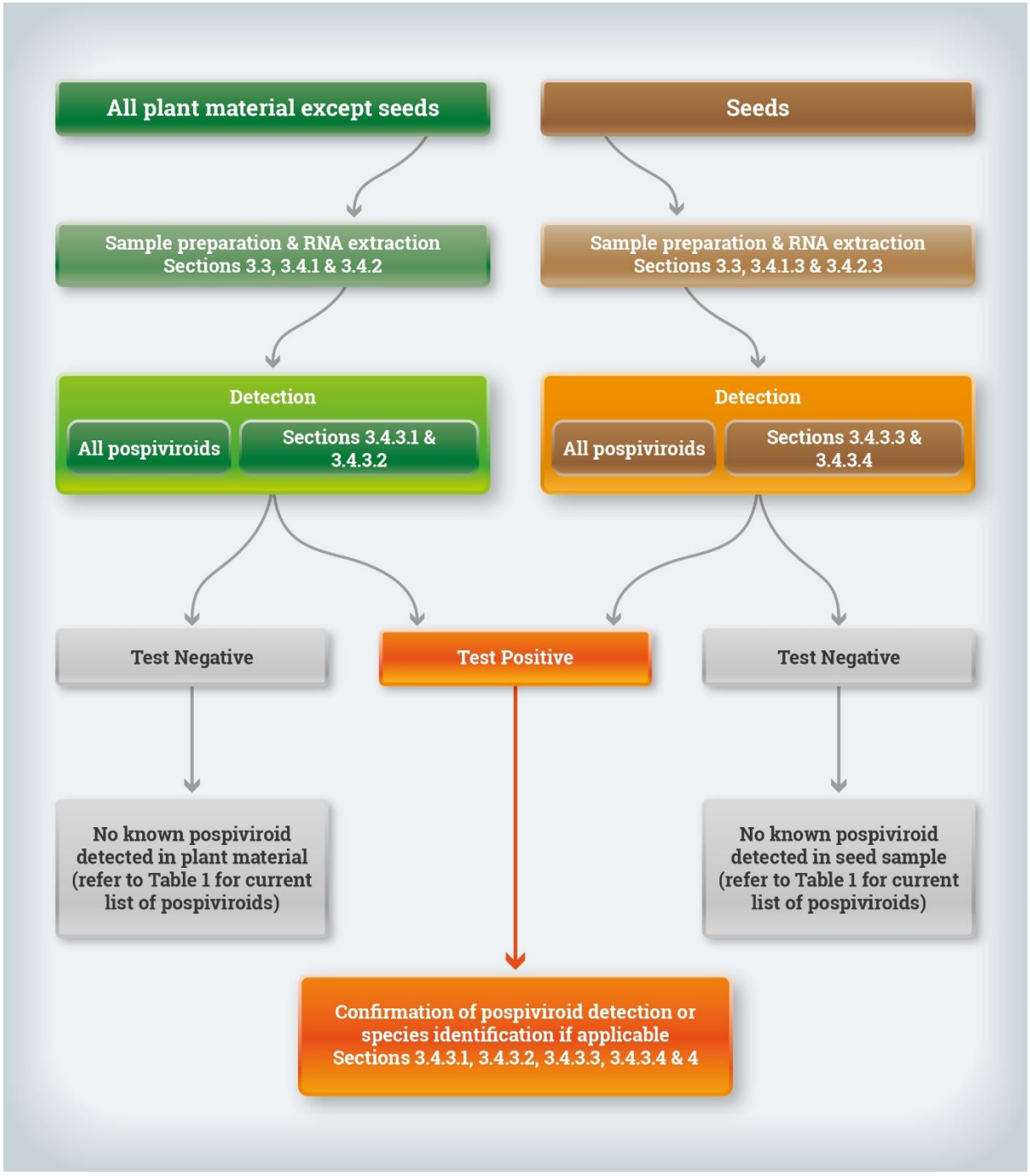

Figure 1. Decision scheme for testing plant samples for pospiviroids.

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

Source (see section 8.2): Adapted from EPPO, 2021a.

3.1 Host range and symptoms

Pospiviroids are generally distributed within most tissues of the plant (i.e. leaves, flowers, pollen, fruits including seed). Meristem tissues have not been reported to be infected by PSTVd; and viroid-free plants can be generated by meristem tip culture (Lizárraga *et al*., 1980; Zhu *et al.*, 2001).

The propensity of pospiviroids 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).

Table 1. Natural host range of members of the *Pospiviroid* genus

Name and acronym	Host range
Chrysanthemum stunt viroid (CSVd)	*Ageratum* spp., *Alkekengi officinarum* (*Physalis alkekengi*), *Argyranthemum frutescens*, *Chrysanthemum ×morifolium*, *Dahlia* spp., *Gerbera* spp., *Pericallis* spp., *Petunia* spp., *Solanum* spp., *Verbena* spp., *Vinca* spp.
Citrus exocortis viroid (CEVd)	*Cestrum* spp., *Citrus* spp., *Impatiens* spp., *Lycianthes rantonnetii, Petunia* spp., *Petunia* × *Calibrachoa*, *Solanum* spp., *Verbena* spp., *Vitis* spp.
Columnea latent viroid (CLVd)	*Brunfelsia* spp., *Columnea* spp., *Gloxinia* spp., *Nematanthus wettsteinii*, *Solanum* spp.
Iresine viroid 1 (IrVd-1)	*Alternanthera* spp., *Celosia* spp., *Iresine* spp., *Portulaca* spp., *Verbena* spp., *Vinca major*
Pepper chat fruit viroid (PCFVd)	*Capsicum* spp., *Solanum* spp.
Portulaca latent viroid (PLVd)	*Portulaca* spp.
Potato spindle tuber viroid (PSTVd)	*Brugmansia* spp., *Calibrachoa* spp., *Capsicum* spp., *Cestrum* spp., *Chrysanthemum* spp., *Dahlia* spp., *Datura* spp., *Ipomoea* spp., *Lycianthes rantonnetii*, *Nicandra* spp., *Nicotiana* spp., *Persea* spp., *Petunia* spp., *Physalis* spp., *Solanum* spp., *Streptosolen jamesonii*
Tomato apical stunt viroid (TASVd)	*Brugmansia* spp., *Capsicum annuum* (seed), *Cestrum* spp., *Lycianthes rantonnetii*, *Solanum* spp., *Streptosolen jamesonii Verbena* spp.
Tomato chlorotic dwarf viroid (TCDVd)	*Brugmansia* spp., *Calibrachoa* spp., *Dahlia pinnata*, *Petunia* spp., *Pittosporum* spp., *Solanum* spp., *Verbena* spp., *Vinca* spp.
Tomato planta macho viroid (TPMVd)	*Solanum lycopersicum*

Source (see section 8.2): Based on EPPO, 2021a, 2025a, with additional information from Eiras *et al.*, 2006, Verhoeven *et al.,* 2015, Wang *et al.*, 2024; POWO (2025).

On their main hosts, the following symptoms have been observed (see also EPPO (2021a) for additional information, and photos of symptoms in the European and Mediterranean Plant Protection Organization (EPPO) Global Database (EPPO, 2025b)).

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

Chrysanthemum ×morifolium (chrysanthemum). The main symptom of CSVd in *C. ×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 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. Symptoms are often variable and dependent on environmental conditions, especially temperature and light.

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).

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

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

3.2 Biological detection

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) can 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). 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.

Mechanical inoculation (EPPO, 2022a) is usually performed using 200–500 mg infected plant material ground in 0.1 M phosphate buffer pH 7.4 (1:1 w/v) containing carborundum powder (400 mesh particle size) or Celite 545 (Thermo Scientific).[1] For inoculation of young *S. lycopersicum* plants, one or two fully expanded leaves are gently rubbed with the inoculum. Since the viroid concentration in plants is affected by temperature and light intensity, indicator plants should be grown under controlled

[1] The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable.

conditions, with a temperature of at least 24 °C and a photoperiod of 14 h (Grasmick and Slack, 1985). Lower temperatures and less light may reduce the transmission and multiplication of the viroid, thereby reducing the reliability of the method (Verhoeven *et al*, 2010). The inoculated plants are regularly inspected for symptoms for up to six weeks after inoculation.

Inoculation of *S. lycopersicum* plants (such as cultivars 'Rutgers', 'Moneymaker' or 'Sheyenne') will provide visual evidence of pathogenicity of many (but not all) pospiviroids. 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 viroids or viruses. Indeed, CLVd mild and severe strains have been described on solanaceous plants including *S. lycopersicum* (cultivar 'Rutgers'), *Solanum melongena* (aubergine) and *C. annuum* (Tangkanchanapas *et al.*, 2021).

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 ×taitensis* (*Citrus jambhiri*, rough lemon) rootstock 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).

3.3 Sampling for molecular-detection methods

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 bulk sample (the bulking rate) depends on the detection method, the tissue being tested and the purpose of testing. The bulking rate should also be adapted to 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. If present, symptomatic plants or plant parts should be sampled. In the case of asymptomatic plants, sample young tissue.

3.3.1 Bark and woody tissue

Samples of *Citrus* species should be collected from multiple locations around the tree canopy to account for any unequal distribution of the viroid in the plant.

Bark or woody tissue from *Citrus* species should be sampled from the young shoots of symptomatic or asymptomatic plants (when the plant is approximately ten months old) (Rizza *et al*., 2009). In the case of infected 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).

3.3.2 Leaves

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*, bulking 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*, bulking rates of up to 25 have been found to be adequate (Verhoeven *et al*., 2008, 2016; van Brunschot *et al*., 2014). Some plant species (e.g. *Calibrachoa* spp., *Solanum* spp. (Singh *et al*., 2002)) contain biochemicals that may inhibit amplification in RT-PCR tests. Dilution of RNA extract has been shown to alleviate inhibition, although this may have an impact on the analytical sensitivity.

3.3.3 Microplants

Microplants of solanaceous hosts, such as *S. tuberosum* and *Petunia ×atkinsiana* (petunia), should be at least 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.

3.3.4 Seeds

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 rate (Euphresco, 2010).

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 or six subsamples of 1 000 or 500 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, 2023). However, both subsample and sample size may be adapted to address technical restrictions or to meet specific phytosanitary import requirements.

3.3.5 *Solanum tuberosum* tubers

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 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 (Roenhorst *et al.*, 2006).

3.4 Molecular detection

Various molecular methods are available for the detection of pospiviroids. The subsections below describe sample preparation and RNA extraction methods for different host plants and tissue types. The molecular methods that are currently the most widely used for testing all tissue types, including seeds – conventional (end-point) RT-PCR and real-time RT-PCR – are described.

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these define the original level of analytical sensitivity, analytical specificity (inclusivity as well as exclusivity), selectivity, repeatability and reproducibility achieved. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories (e.g. using other critical reagents or instruments), provided they are adequately validated for the specific use intended. Guidelines on validation of methods for plant pest diagnostics are provided by EPPO (2021b).

3.4.1 Sample preparation

This section describes sample preparation for RNA extraction for different hosts and tissue types. These initial steps, combined with the RNA extraction, are critical for the outcome of a test and may differ between matrices. Therefore, sample preparation methods should be validated in combination with RNA-extraction and PCR methods (EPPO, 2021b).

To homogenize plant material, a variety of tools can be used, such as a mortar and pestle, a hammer, a homogenizer (e.g. HOMEX 6 with extraction bags (BIOREBA)) or a bead-beater instrument (e.g. FastPrep homogenizer (MP Biomedicals), Mixer Mill (e.g. Retsch), TissueLyser (e.g. QIAGEN or Retsch), Geno/Grinder (SPEX SamplePrep)).[1] For all tissues, freezing the sample (e.g. by using liquid nitrogen) may facilitate grinding and homogenization.

3.4.1.1 Bark (woody tissue) and roots

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):

- The phloem-rich bark tissue is peeled and then chopped into small pieces (4–5 mm) 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, a single sterile stainless-steel bead is placed into each tube. After chilling tubes in liquid nitrogen, samples are pulverized using a Geno/Grinder 2010 (SPEX Sample Prep).[1] Section 3.4.2.1 describes the next steps of the extraction (Dang *et al*., 2022).
- Alternatively, 100 mg young bark (approximately ten months old) can be ground to a fine powder using a mortar and pestle with liquid nitrogen; however, care should be taken to avoid cross-contamination (Rizza *et al.*, 2009).

3.4.1.2 Leaves and microplants

Before grinding, plant material is chopped and transferred to an appropriate container (grinding bag, tube or mortar). Water or buffer is added to the plant material before homogenization; the volume and composition of the buffer depends on the method to be used for RNA extraction (see section 3.4.2). If freezing the sample in liquid nitrogen, water or lysis buffer should be added after grinding.

3.4.1.3 Seeds

For seeds, sample preparation and RNA extraction are highly interdependent and are described together in section 3.4.2.3. Seeds sample size varies but for *S. lycopersicum* and *C. annuum* seeds generally follows the International Seed Federation guidelines. Each sample consists of 3 000 seeds divided into three or six subsamples of 1 000 or 500 seeds each, respectively (ISF, 2023).

3.4.1.4 Tubers

Tuber cores can be ground and homogenized in water or lysis buffer (about 1 g/mL; composition of the buffer depending on the method used for RNA extraction) by using a homogenizer (such as HOMEX 6 with extraction bags (BIOREBA)[1]). Freezing the cores before adding the water or lysis buffer may facilitate grinding and homogenization.

3.4.2 RNA extraction

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) and the sbeadex Maxi Plant Kit (LGC Biosearch Technologies) can be used following the manufacturers' instructions or the instructions described in this diagnostic protocol.[1] For high-throughput RNA extraction, the sbeadex Maxi Plant Kit (or MagMAX (Applied Biosystems) or other) can be used in combination with a KingFisher KF96 system (Thermo Scientific).[1] The Maxwell RSC Plant RNA Kit (Promega) can be used in combination with a Maxwell RSC Instrument (Promega).[1] Other extraction methods, including the cetyltrimethylammonium bromide (CTAB) method (Gambino, Perrone and Gribaudo, 2008), can also be used once validated.

Extracted RNA should be stored at approximately 4 °C if storing for less than 8 h, at approximately −20 °C for less than one month, or at approximately −80 °C for longer periods.

3.4.2.1 Bark and woody tissue

Method 1. Extraction of RNA is accomplished by combining guanidine lysis buffer with the RNeasy Plant Mini Kit (QIAGEN)[1] as described by Bernard and Duran-Vila (2006). Approximately 100 mg 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.

Method 2. Approximately 100–500 mg 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).

Method 3. The pulverized bark or woody tissue from citrus trees (250 mg) is homogenized in 750 µL 4M extraction buffer and extracted with the MagMAX-96 Viral RNA Isolation Kit (Applied Biosystems), using the MagMAX Express-96 Deep Well Magnetic Particle Processor (Applied Biosystems), following the manufacturer's recommendations and as described in Dang *et al.* (2022).[1]

3.4.2.2 Leaves and microplants

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 bulked 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) or the sbeadex Maxi Plant Kit (LGC Biosearch Technologies) according to the manufacturer's instructions.[1] For the sbeadex Maxi Plant Kit (LGC Biosearch Technologies),[1] 250 µL lysate is transferred to a plate containing 450 µL binding buffer and 50 µL particle suspension and RNA is extracted according to the manufacturer's instructions.

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 for *S. lycopersicum*, *S. tuberosum* and a range of ornamental plant species; however, as a result of its lower sensitivity, this method is more suitable for surveys rather than for first detection or samples of critical importance.

3.4.2.3 Seeds

Homogenization in GH+ extraction buffer. For both *C. annuum* and *S. lycopersicum*, three subsamples of (approximately) 1 000 seeds are soaked in 40 mL (*C. annuum*) or 20 mL (*S. lycopersicum*) GH+ extraction buffer (see section 3.4.2.2 for buffer composition) 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 can be used, with the time of homogenization being adjusted accordingly (EPPO, 2021a).

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 1 700 rpm for 7 min for *C. annuum* and 4 min for *S. lycopersicum* seeds. After grinding, GH+ buffer is added: 10 mL for *C. annuum* and 20 mL for *S. lycopersicum* samples. 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. Two *C. annuum* homogenates (out of six) are combined and mixed to make three subsamples for further processing.

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), 750 µL supernatant is transferred onto the QIAshredder (QIAGEN) spin column.[1] 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 plate

containing 600 µL binding buffer and 50 µL particle suspension (sbeadex Maxi Plant Kit (LGC Biosearch Technologies)),[1] and RNA is extracted following the manufacturer's instructions.

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), in 15 mL Lysing Matrix A tubes (MP Biomedicals), incubated at 4 °C overnight, and then ground (e.g. with a FastPrep homogenizer (MP Biomedicals) 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 (to minimize number of samples tested) (EPPO, 2021a).

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 5 000 *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) to the pelleted Amberlite beads, followed by incubation and occasional agitation at room temperature for 10 min.[1] After centrifugation at 5 000 *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, 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.

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)) or homogenizer (e.g. HOMEX 6, BIOREBA) with a suitable quantity of lysis buffer (composition depending on the method used for nucleic acid extraction) can be used.[1] 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.

3.4.2.4 Tubers

The RNeasy Plant Mini Kit (QIAGEN), CTAB method (Boonham *et al*., 2004) or sbeadex Maxi Plant Kit (LGC Biosearch Technologies) can be used for RNA extraction from tubers.[1] Extraction of RNA using the KingFisher Total RNA Kit (Thermo Scientific) has been validated in combination with the KingFisher mL Magnetic Particle Processor (Thermo Scientific) for testing large numbers of samples (Roenhorst *et al.*, 2005).[1]

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 in extraction buffer (1 g tissue to 10 mL buffer). Extraction buffer consists of 200 µL 8.39% (w/v) tetrasodium pyrophosphate solution pH 10–10.9, 100 µL Antifoam B Emulsion (Sigma-Aldrich)[1] 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). 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 rack, into which 50 µL vortexed MAP Solution A magnetic beads (Invitek) is added.[1] 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 and nucleic acid extraction is performed following the Thermo Scientific KingFisher Total RNA Kit instruction manual.[1]

3.4.3 PCR-based detection

There are several RT-PCR methods for the general detection (i.e. detecting a subset) of pospiviroids using combinations of different primer sets (Table 2). Some of these methods 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 is presented in this section (3.4.3) and summarized in Table 2. Additional methods for pospiviroid detection are summarized in Table 3.

Table 2. Recommended methods for the detection or identification of listed viroids in the genus *Pospiviroid*

Section	Primers & probes	CSVd	CEVd	CLVd	IrVd-1	PCFVd	PSTVd	TASVd	TCDVd	TPMVd	Position of amplicon[a]	Size	References on validation	Source (see section 8.2)
3.4.3.1	Pospi1[b]	+	+	-	+	+	+	+	+	+	86–283 PSTVd NC_002030	197 bp	EPPO validation data (NPPO-NL, 2013a)	Verhoeven *et al.* (2004)
	pCLV	NT	-	+	-	NT	NT	-	NT	NT	102–101 CLVd NC_003538	370 bp	EPPO validation data (NPPO-NL, 2013a); Olivier *et al.* (2016)	Spieker (1996)
3.4.3.2	GenPospi	+	+	-	+	+	+	+	+	+	n/a	-	EPPO validation data (NPPO-NL, 2013c)	Botermans *et al.* (2013)
	CLVd	-	-	+	-	-	-	-	-	-	n/a	-	EPPO validation data (NPPO-NL, 2013c)	Monger *et al.* (2010)
3.4.3.3	PospiSense1	NT	-	+	NT	+	+	-	+	+	n/a	-	Botermans *et al.* (2020)	Botermans *et al.* (2020); Monger *et al.* (2010)
	PospiSense2	NT	+	-	NT	-	-	+	-	-	n/a	-	Botermans *et al.* (2020)	Botermans *et al.* (2020)
3.4.3.4	Mix A	NT	-	-	-	+	+	-	+	+[c]	n/a	-	Testa (2015); EPPO validation data (Naktuinbouw, 2021, 2022)	Boonham *et al.* (2004); Naktuinbouw, (2022, 2024)
	Mix B	-	+	+	NT	-	-	+[d]	-	-	n/a	-	Testa (2015); EPPO validation data (Naktuinbouw, 2021, 2022)	Monger *et al.* (2010); Naktuinbouw (2022, 2024)
	Mix C	NT	-	-	NT	-	-	-	-	+	n/a	-	Testa (2015); EPPO validation data (Naktuinbouw, 2021, 2022)	Botermans *et al.* (2013); Naktuinbouw, (2022, 2024)
	Mix D	NT	-	-	NT	-	-	+	-	-	n/a	-	Testa (2015); EPPO validation data (Naktuinbouw, 2021, 2022)	Monger *et al.* (2010)

Notes: Position and amplicon size are given for conventional RT-PCR methods only. [a] Position of amplicon in reference sequence of indicated species in GenBank (National Center for Biotechnology Information). [b] Sequence of PCR product can be used for identification. [c] At least one TPMVd isolate is not detected with this primer mix. [d] TASVd not the target, but primer mix may cross-react with TAVd isolates.

+, detected; -, not detected; bp, base pairs; CEVd, citrus exocortis viroid; CLVd, Columnea latent viroid; CSVd, chrysanthemum stunt viroid; EPPO, European and Mediterranean Plant Protection Organization; IrVd-1, iresine viroid 1; n/a, not applicable; NT, not tested; PCFVd, pepper chat fruit viroid; PCR, polymerase chain reaction; PSTVd, potato spindle tuber viroid; RT-PCR, reverse transcription PCR; TASVd, tomato apical stunt viroid; TCDVd, tomato chlorotic dwarf viroid; TPMVd, tomato planta macho viroid.

Table 3. Overview of additional PCR-based methods suitable for detection or identification of listed viroids in the genus *Pospiviroid*

Source (see section 8.2)	**Primers & probes**	**CSVd**	**CEVd**	**CLVd**	**IrVd-1**	**PCFVd**	**PSTVd**	**TASVd**	**TCDVd**	**TPMVd**	**Position of amplicon***	**Size**	**References on validation**
Boonham *et al.* (2004)	PSTV	-	-	-	-	-	+	-	+	+[a]	n/a	-	Naktuinbouw (2012a); Naktuinbouw (2015)
Hooftman *et al.* (1996)	CSVd h/c	+	NT	NT	NT	NT	NT	NT	NT	NT	62–112 CSVd NC_002015	complete genome[b]	Hooftman *et al.* (1996)
Mumford, Walsh and Boonham (2000)	Vir2/1	+	+	NT	NT	NT	+	NT	NT	NT	17–280 CSVd NC_002015	264 nucleotides	Fera (unp.)
Önelge (1997)	CEVd	NT	+	NT	NT	NT	NT	+[c]	NT	NT	80–117 CEVd NC_002015	complete genome[b]	Önelge (1997)
Shamloul *et al.* (1997)	3H1/2H1[d]	NT	NT	NT	NT	NT	+	NT	+	+[a]	69–113 PSTVd NC_002030	complete genome[b]	EPPO validation data (NPPO-NL, 2013d)
Spieker (1996)	pCLVR4/ pCLV4	NT	-	+	-	NT	NT	-	NT	NT	102–101 CLVd NC_003538	complete genome[b]	Spieker(1996); NPPO-NL (unp.)
Verhoeven *et al.* (2009)	AP-FW1/RE2	NT	NT	NT	NT	+	NT	NT	NT	NT	178–164 PCFVd NC_011590	*ca.* complete genome (−13 nucleotides)	Verhoeven *et al.* (2009)
Verhoeven *et al.* (2004)	Vid-FW/RW	-	-	+	-	-	+	-	+	-	355–354 PSTVd NC_002030	complete genome	EPPO validation data (NPPO-NL, 2013b)
Verhoeven *et al.* (2010)	IrVd-1	NT	NT	NT	+	NT	NT	NT	NT	NT	168–167 IrVd-1 NC_003613	complete genome[b]	Verhoeven *et al.* (2010)
Verhoeven *et al.* (2017)	Pospi2[e]	+	+	-	+	+	+	+	+	+	261–103 PSTVd NC_002030	*ca*. half genome	Verhoeven *et al.* (2017)
Monger *et al.* (2010); Naktuinbouw (unp.)	Generic	+	+	+	NT	NT	+	+	+	NT	n/a	-	Monger *et al.* (2010); Testa (2015)
	CEVd	-	+	-	NT	-	-	+[f]	-	-	n/a	-	
	CLVd	-	-	+	NT	-	-	-	-	-	n/a	-	
	TASVd	-	-	-	NT	-	-	+	-	-	n/a	-	
Naktuinbouw (unp.)	PCFVd	NT	-	-	NT	+	-	-	-	-	n/a	-	Testa (2015)
Mumford, Walsh and Boonham (2000)[g]	CSVd	+	-	NT	-	-	-	-	-	-	n/a	-	Fera (unp.); Naktuinbouw (unp.)

Notes: Position and size of amplicon are given for RT-PCR methods only; amplicon size is given where relevant for cloning and sequencing purposes. * Position of amplicon in reference sequence of indicated species in GenBank (National Center for Biotechnology Information). [a] It is known that at least one isolate of TPMVd (GenBank acc. no. K00817.1) will not be, or will only be poorly, detected (Testa, 2015; EPPO validation data; Naktuinbouw, 2022, 2024). [b] Complete sequence includes primer sequences (because of the circular genome, it might be advisable to include these sequences in BLAST searches). [c] All TASVd isolates tested at NPPO-NL were detected so far. [d] Primer names used in DP 7 (*Potato spindle tuber viroid*). [e] Primers complementary to Pospi1. [f] CEVd primers and probe cross-react with TASVd isolates. [g] Method described in EPPO (2002). +, detected; -, not detected; BLAST, Basic Local Alignment Search Tool; CEVd, citrus exocortis viroid; CLVd, Columnea latent viroid; CSVd, chrysanthemum stunt viroid; EPPO, European and Mediterranean Plant Protection Organization; IrVd-1, iresine viroid 1; n/a, not applicable; NT, not tested; PCFVd, pepper chat fruit viroid; PCR, polymerase chain reaction; PSTVd, potato spindle tuber viroid; RT-PCR, reverse transcription PCR; TASVd, tomato apical stunt viroid; TCDVd, tomato chlorotic dwarf viroid; TPMVd, tomato planta macho viroid; unp, unpublished.

If reagents other than those recommended are used, 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.

If applicable, an independent test (i.e. a test using a different method or performed by a different laboratory) should be conducted to confirm detection. The methods recommended or available for confirmation are the same as for the initial testing (as described in the following subsections of 3.4.3, Table 2 and Table 3).

3.4.3.1 Conventional RT-PCR

The primer set Pospi1 allows the detection of all known pospiviroids except CLVd (Verhoeven *et al*., 2004). The pCLV4 primer set described by Spieker (1996) is used to specifically detect CLVd (Olivier *et al.*, 2014).

The OneStep RT-PCR Kit (QIAGEN)[1] has been shown to be reliable when used for the detection of CEVd, CLVd, CSVd, PCFVd, PSTVd, TASVd, TCDVd and TPMVd (Euphresco, 2010).

The primers for conventional RT-PCR are listed in Table 4 and the master mixes are described in Table 5 and Table 6.

Table 4. Conventional RT-PCR primers and amplicons

Primer	Sequence (5′–3′)	Primer location	Viroids detected	Amplicon size (bp)
Pospi1-FW (forward)	GGG ATC CCC GGG GAA AC	86–102†	CEVd	197
Pospi1-RE (reverse)	AGC TTC AGT TGT WTC CAC CGG GT	283–261†	CSVd IrVd-1 PCFVd PSTVd TASVd TCDVd TPMVd	
pCLV4 (forward)	GGG GCT CCT GAG ACC GCT CTT G	101–80‡	CLVd	370
pCLVR4 (reverse)	GGG GCA ACT CAG ACC GAG C	102–120‡		

Notes: † Location in PSTVd NC_002030.

‡ Location in CLVd NC_003538.

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; 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.

Source (see section 8.2): Adapted from EPPO, 2021a.

Table 5. Composition of master mix for conventional RT-PCR for detection of viroids in the genus *Pospiviroid* (except CLVd) using Pospi1 primers

Reagents	Working concentration	Volume per reaction (µL)	Final concentration
PCR-grade water	-	-†	-
OneStep RT-PCR Buffer (QIAGEN)‡	5×	5.0	1×
dNTP mix (QIAGEN)‡	10 mM	1.0	0.4 mM
Primer Pospi1-FW (forward)	10 µM	1.0	0.4 µM
Primer Pospi1-RE (reverse)	10 µM	1.0	0.4 µM
OneStep RT-PCR Enzyme Mix (QIAGEN)‡	-	1.0	-
RNA	-	1.0	-

Notes: † For a final reaction volume of 25 µL.

‡ See page footnote 1.

CLVd, Columnea latent viroid; PCR, polymerase chain reaction; RT-PCR, reverse transcription–polymerase chain reaction.

Source (see section 8.2): Adapted from EPPO, 2021a.

Table 6. Composition of master mix for conventional RT-PCR for detection of CLVd using pCLV4 primers

Reagents	Working concentration	Volume per reaction (µL)	Final concentration
PCR-grade water	-	-†	-
OneStep RT-PCR buffer (QIAGEN)‡	5×	5.0	1×
dNTP mix (QIAGEN)‡	10 mM	1.0	0.4 mM
Primer pCLVR4 (reverse)	10 µM	0.5	0.2 µM
Primer pCLV4 (forward)	10 µM	0.5	0.2 µM
OneStep RT-PCR Enzyme Mix (QIAGEN)‡	-	1.0	-
RNA	-	2.0	-

Notes: † For a final reaction volume of 25 µL.

‡ See page footnote 1.

CLVd, Columnea latent viroid; PCR, polymerase chain reaction; RT-PCR, reverse transcription–polymerase chain reaction.

Source (see section 8.2): Adapted from EPPO, 2021a.

The cycling parameters are as follows:

- **Pospi1 primers.** Reverse transcription at 50 °C for 30 min; denaturation at 95 °C for 15 min; 14 cycles of denaturation at 94 °C for 30 s, annealing at 64 °C for 90 s and elongation at 72 °C for 45 s, followed by 29 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 90 s and elongation at 72 °C for 45 s; terminal elongation at 72 °C for 10 min; and stored at 20 °C.
- **pCLV4 primers.** Reverse transcription at 50 °C for 30 min; denaturation at 95 °C for 15 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 90 s and elongation at 72 °C for 45 s; terminal elongation at 72 °C for 10 min; and stored at 20 °C.

The PCR products (see Table 4 for amplicon size) should be analysed by gel electrophoresis (2% agarose gel).

Validation data

Note that the performance of a detection method may be different when using another nucleic acid- or RNA-extraction method or other PCR reagents, which implies that each laboratory needs to verify the performance of the method used. If not specified, PCR reagents were as specified in the respective protocols.

Information on validation. The Pospi1 method was validated using the OneStep RT-PCR Kit (QIAGEN)[1] at the Netherlands Institute for Vectors, Invasive plants and Plant health (NIVIP) NIVIP, 2014; EPPO, 2021a).

The pCLV4 method 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) (EPPO, 2021a).

Both methods, Pospi1 and pCLV4, have been compared for detection of pospiviroids in *S. lycopersicum* leaves and seeds by interlaboratory comparison (Olivier *et al.*, 2016).

Pospi1 method

Analytical sensitivity. The Pospi1 primers detected all pospiviroids (except CLVd) up to a dilution of 10^{-2}–10^{-5}, 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}.

Analytical specificity. Pospi1 primers had been found to detect all pospiviroid isolates (except CLVd) encountered at NIVIP as at the date of validation. No reactions were obtained for isolates of the following viroids: avocado sunblotch viroid (genus *Avsunviroid*), chrysanthemum chlorotic mottle viroid (genus *Pelamoviroid*) and eggplant latent viroid (genus *Elaviroid*) in the family *Avsunviroidae*; and apple scar skin viroid (genus *Apscaviroid*), coleus blumei viroid 1 (genus *Coleviroid*) and hop stunt viroid (genus *Hostuviroid*) in the family *Pospiviroidae*. *In silico* analysis did not reveal cross-reactions with other *S. lycopersicum*-infecting viruses and host-plant sequences. A cross-reaction was observed for an isolate of hop latent viroid (genus *Cocadviroid*). Portulaca latent viroid was detected using Pospi1 primers (Verhoeven *et al*., 2015); however, it was not included in the validation studies mentioned above.

Selectivity. No apparent matrix effects have been observed in a wide range of host plants, in particular in the families Apocynaceae, Gesneriaceae and Solanaceae.

Repeatability and reproducibility. The method was validated in both an intra- and interlaboratory comparison. Repeatability and reproducibility were shown to be 100% (six replicates for each sample).

pCLV4 method

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).

Analytical specificity. At the time of validation, 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).

Selectivity. No apparent matrix effects were observed in a wide range of host plants, in particular in the families Asteraceae, Chenopodiaceae and Solanaceae (six replicates for each sample).

Repeatability and reproducibility. The method was validated in both an intra- and interlaboratory comparison. Repeatability and reproducibility were shown to be 100% (six replicates for each sample).

3.4.3.2 Real-time RT-PCR for the detection of pospiviroids on all tissues except seed: the GenPospi method (Botermans et al.*, 2013)*

The GenPospi method (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 method consists of two reactions running in 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.

The primers for the GenPospi method are listed in Table 7 and the mixes are described in Table 8, Table 9 and Table 10.

The cycling parameters are 50 °C for 10 min, 95 °C for 2 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Table 7. Real-time RT-PCR primers and probes for the GenPospi method

Primers & probes	Sequence (5′–3′)	Reference
Reaction mix 1		
Primer TCR-F 1-1 (forward)	TTC CTG TGG TTC ACA CCT GAC C	1
Primer TCR-F 1-3 (forward)	CCT GTG GTG CTC ACC TGA CC	1
Primer TCR-F 1-4 (forward)	CCT GTG GTG CAC TCC TGA CC	1
Primer TCR-F IrVd (reverse)	AAT GGT TGC ACC CCT GAC C	1
Primer TCR-F PCFVd (forward)	TGG TGC CTC CCC CGA A	1
Primer TR-R1 (reverse)	GGA AGG GTG AAA ACC CTG TTT	1
Primer TR-R CEVd (reverse)	AGG AAG GAG ACG AGC TCC TGT T	1
Primer TR-R6 (reverse)	GAA AGG AAG GAT GAA AAT CCT GTT TC	1
Probe pUCCR	FAM-CCG GGG AAA CCT GGA-MGB	1
Reaction mix 2		
Primer CLVd-F (forward)	GGT TCA CAC CTG ACC CTG CAG	2
Primer CLVd-F2 (forward)	AAA CTC GTG GTT CCT GTG GTT	2
Primer CLVd-R (reverse)	CGC TCG GTC TGA GTT GCC	2
Probe CLVd-P	FAM-AGC GGT CTC AGG AGC CCC GG-BHQ1	2
Internal control		
Primer nad5-F (forward)	GAT GCT TCT TGG GGC TTC TTG TT	3
Primer nad5-R (reverse)	CTC CAG TCA CCA ACA TTG GCA TAA	3
Probe nad5-P	VIC-AGG ATC CGC ATA GCC CTC GAT TTA TGT G-BHQ1	1

Notes: RT-PCR, reverse transcription–polymerase chain reaction.

References: [1] Botermans *et al.*, 2013; [2] Monger *et al.*, 2010; [3] Menzel, Jelkmann and Maiss, 2002.

Source (see section 8.2): Adapted from EPPO, 2021a.

Table 8. Composition of GenPospi primer mix 1

Primers	Stock concentration (µM)	Volume (µL)	Final concentration (µM)
PCR-grade water	-	720	-
TCR-F 1-1 (forward)	100	10	1.25
TCR-F 1-3 (forward)	100	10	1.25
TCR-F 1-4 (forward)	100	10	1.25
TCR-F IrVd (forward)	100	10	1.25
TCR-F PCFVd (forward)	100	10	1.25
TR-R1 (reverse)	100	10	1.25
TR-R CEVd (reverse)	100	10	1.25
TR-R6 (reverse)	100	10	1.25
Total		800	

Note: PCR, polymerase chain reaction.

Source (see section 8.2): Adapted from EPPO, 2021a.

Table 9. Composition of GenPospi reaction mix for detection of viroids in the genus *Pospiviroid* (except CLVd) and *nad5*

Reagents	Working concentration	Volume per reaction (µL)	Final concentration
PCR-grade water	-	-†	-
TaqMan RNA-to-C_T *1-Step* Kit, 2X RT PCR mix* (Applied Biosystems)‡	2×	12.5	1×
TaqMan RT enzyme mix* (Applied Biosystems)‡	40×	0.6	approximately 1×
GenPospi primer mix (see Table 8)	1.25 µM	6.0	0.3 µM
Primer nad5-F (forward)	10 µM	0.75	0.3 µM
Primer nad5-R (reverse)	10 µM	0.75	0.3 µM
TaqMan probe pUCCR	10 µM	0.25	0.1 µM
TaqMan probe nad5-P	10 µM	0.5	0.2 µM
RNA		2.0	

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).

† For a final reaction volume of 25 µL.

‡ See page footnote 1.

CLVd, Columnea latent viroid; PCR, polymerase chain reaction.

Source (see section 8.2): Adapted from EPPO, 2021a.

Table 10. Composition of CLVd reaction mix for detection of CLVd and *nad5*

Reagents	Working concentration	Volume per reaction (µL)	Final concentration
PCR-grade water	-	-†	-
TaqMan RNA-to-C$_T$ *1-Step* Kit, 2X RT PCR mix* (Applied Biosystems)‡	2×	12.5	1×
TaqMan RT enzyme mix* (Applied Biosystems)‡	40×	0.6	approximately 1×
Primer CLVd-F (forward)	10 µM	0.75	0.3 µM
Primer CLVd-F2 (forward)	10 µM	0.75	0.3 µM
Primer nad5-F (forward)	10 µM	0.75	0.3 µM
Primer CLVd-R (reverse)	10 µM	0.75	0.3 µM
Primer nad5-R (reverse)	10 µM	0.75	0.3 µM
TaqMan probe CLVd-P	10 µM	0.25	0.1 µM
TaqMan probe nad5-P	10 µM	0.5	0.2 µM
RNA		2.0	

Notes: *TaqMan RNA-to-C$_T$ *1-Step* Kit (Applied Biosystems).‡ Note that the use of this reagent is critical, as Ct values have been found to increase by 8–10 when using other kits (Botermans *et al.*, 2013).

† For a final reaction volume of 25 µL.

‡ See page footnote 1.

CLVd, Columnea latent viroid; PCR, polymerase chain reaction.

Source (see section 8.2): Adapted from EPPO, 2021a.

Validation data

Information on validation. Validation data were generated according to EPPO (2021b) at NIVIP (Botermans *et al*., 2013; EPPO, 2021a). Nucleic acid extraction was performed using the RNeasy Plant Mini Kit (QIAGEN).[1]

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

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), 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 *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, tobacco mosaic virus, tomato chlorosis virus, tomato mosaic virus and tomato yellow leaf curl virus.

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

Repeatability and reproducibility. The method was validated in both an intra- and interlaboratory comparison for IrVd-1, PSTVd, TASVd and TCDVd, and repeatability and reproducibility were shown to be 100%.

3.4.3.3 Real-time RT-PCR for the detection of pospiviroids in seeds: the PospiSense method (Botermans et al.*, 2020)*

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 3 000 seeds, tested in three subsamples of 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.

The primers and probes are listed in Table 11 and the mixes are described in Table 12 to Table 17.

The method has been successfully performed on different real-time PCR systems, including the CFX96 (Bio-Rad Laboratories) and the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems).[1]

The cycling parameters are 50 °C for 10 min, 95 °C for 2 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Table 11. Real-time RT-PCR primers and probes for the PospiSense method

Primers & probes	Sequence (5′–3′)	Reference
PospiSense 1		
Primer PospiFW1 (forward)	TGC GCT GTC GCT TCG	1
Primer PospiFW5a (forward)	CCT TCC TTT CTT CGG GTT TC	1
Primer PospiRV1 (reverse)	AGA AAA AGC GGC GCT TG	1
Primer PospiRV2 (reverse)	TAG AGA AAA AGC GGT TCT CGG	1
Primer PospiRV5a (reverse)	GAA AAA GCA CCT CTG TCA GTT GTA	1
Primer CLVd-F (forward)	GGT TCA CAC CTG ACC CTG CAG	2
Primer CLVd-F2 (forward)	AAA CTC GTG GTT CCT GTG GTT	2
Primer CLVd-R (reverse)	CGC TCG GTC TGA GTT GCC	2
Probe PospiP1a	FAM-CGG TGG AAA CAA CTG-MGB	1
Probe PospiP3a	FAM-CGG CCT TCT CGC GCA-MGB	1
Probe CLVd-P	FAM-AGC GGT CTC AGG AGC CCC GG-BHQ1	2
PospiSense 2		
Primer PospiFW6a (forward)	GGA TCT TTC TTG AGG TTC CTG T	1
Primer PospiFW6b (forward)	GGA ACT TTC TTG AGG TTC CTG T	1
Primer PospiFW6c (forward)	TCT TTC CTT GTG GTT CCT GTG	1
Primer PospiRV6a (reverse)	CGA CTT CCT CCA GGT TTC C	1
Probe PospiP5	FAM-CTG CAG GGT CAG GTG-MGB	1
Internal control		
DaVd1-FT (forward)	GCT CCG CTC CTT GTA GCT TT	3
DaVd1-RT (reverse)	AGG AGG TGG AGA CCT CTT GG	3
Probe DaVd1-P	Texas Red-CTG ACT CGA GGA CGC GAC CG-BHQ2	3

Notes: RT-PCR, reverse transcription–polymerase chain reaction.

References: [1] Botermans *et al.*, 2020; [2] Monger *et al.*, 2010; [3] Naktuinbouw, 2022, 2024.

Source (see section 8.2): Adapted from EPPO, 2021a.

Table 12. Composition of PospiSense 1 primer mix

Primers	Stock concentration (µM)	Volume (µL)	Final concentration (µM)
PCR-grade water	-	20	-
PospiFW1 (forward)	100	10	10
PospiFW5a (forward)	100	10	10
PospiRV1 (reverse)	100	10	10
PospiRV2 (reverse)	100	10	10
PospiRV5a (reverse)	100	10	10
CLVd-F (forward)	100	10	10
CLVd-F2 (forward)	100	10	10
CLVd-R (reverse)	100	10	10
Total		100	

Note: PCR, polymerase chain reaction.
Source (see section 8.2): Adapted from EPPO, 2021a.

Table 13. Composition of PospiSense 1 probe mix

Probes	Stock concentration (µM)	Volume (µL)	Final concentration (µM)
PCR-grade water	-	70	-
PospiP1a	100	10	10
PospiP3a	100	10	10
CLVd-P	100	10	10
Total		100	

Note: PCR, polymerase chain reaction
Source (see section 8.2): Adapted from EPPO, 2021a.

Table 14. Composition of PospiSense 2 primer mix

Primers	Stock concentration (µM)	Volume (µL)	Final concentration (µM)
PCR-grade water	-	60	-
PospiFW6a (forward)	100	10	10
PospiFW6b (forward)	100	10	10
PospiFW6c (forward)	100	10	10
PospiRV6a (reverse)	100	10	10
Total		100	

Note: PCR, polymerase chain reaction.
Source (see section 8.2): Adapted EPPO, 2021a.

Table 15. Composition of DLVd primer mix (internal control) for the PospiSense method

Probes	Stock concentration (µM)	Volume (µL)	Final concentration (µM)
PCR-grade water	-	80	-
DaVd1-FT (forward)	100	10	10
DaVd1-RT (reverse)	100	10	10
Total		100	

Note: PCR, polymerase chain reaction.
Source (see section 8.2): Adapted from EPPO, 2021a.

Table 16. Composition of PospiSense reaction mix 1 for detection of CLVd, PCFVd, PSTVd, TCDVd, TPMVd and DLVd internal control

Reagents	Working concentration	Volume per reaction (µL)	Final concentration
PCR-grade water	-	-†	-
UltraPlex 1-Step ToughMix (Quanta Biosciences)‡	4×	5.0	1×
PospiSense 1 primer mix (see Table 12)	10 µM each	0.6	0.24 µM
PospiSense 1 probe mix (see Table 13)	10 µM each	0.2	0.08 µM
DLVd primer mix (see Table 15)	10 µM each	0.6	0.24 µM
Probe DaVd1-P	10 µM	0.4	0.16 µM
RNA		2.0	

Notes: † For a final reaction volume of 20 µL.

‡ See page footnote 1.

CLVd, Columnea latent viroid; DLVd, 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.

Source (see section 8.2): Adapted from EPPO, 2021a.

Table 17. Composition of PospiSense reaction mix 2 for detection of CEVd, TASVd and DLVd

Reagents	Working concentration	Volume per reaction (µL)	Final concentration
PCR-grade water	-	-†	-
UltraPlex 1-Step ToughMix (Quanta Biosciences)‡	4×	5.0	1×
PospiSense 2 primer mix (see Table 14)	10 µM each	0.6	0.24 µM
Probe PospiP5	10 µM	0.2	0.08 µM
DLVd primer mix (see Table 15)	10 µM each	0.6	0.24 µM
Probe DaVd1-P	10 µM	0.4	0.16 µM
RNA		2.0	

Notes: † For a final reaction volume of 20 µL.

‡ See page footnote 1.

CEVd, citrus exocortis viroid; DLVd, dahlia latent viroid; PCR, polymerase chain reaction; TASVd, tomato apical stunt viroid; TCDVd.

Source (see section 8.2): Adapted from EPPO, 2021a.

Validation data

Information on validation. Validation data were generated according to EPPO (2021b) at NIVIP (Botermans *et al*., 2020). Nucleic acid was extracted using the RNeasy Plant Mini Kit (QIAGEN) or using the Kingfisher KF96 system (Thermo Scientific) and the sbeadex Maxi Plant Kit (LGC Biosearch Technologies).[1]

Analytical sensitivity. For both *S. lycopersicum* and *C. annuum* seeds, one infected seed in a sample of 1 000 seeds could be detected for CEVd, CLVd, PCFVd, PSTVd, TASVd, TCDVd and TPMVd.

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

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

No cross-reactions were observed with 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*.

Selectivity. No apparent matrix effects were observed for *C. annuum* and *S. lycopersicum* seeds.

Repeatability and reproducibility. The method was validated in both an intra- and interlaboratory comparison. For *C. annuum* seeds infected with PSTVd, TASVd and both PCFVd and CLVd, as well as *S. lycopersicum* seeds infected with TASVd, TCDVd and TPMVd, both repeatability and reproducibility were 100%.

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 the detection of CEVd and TASVd than the Naktuinbouw method (see section 3.4.3.4).

3.4.3.4 Real-time RT-PCR for the detection of pospiviroids in seeds (Naktuinbouw 2022, 2024)

The method developed by Naktuinbouw (2022, 2024) 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 (*S. lycopersicum*) or in six subsamples of 500 seeds (*C. annuum)*. 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 isolates not detected by reaction A; GenBank accession number NC_001558) and D (to detect TASVd). In reactions A and B, DLVd is 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.

The primers and probes are listed in Table 18 and the mixes are described in Table 19 to Table 28.

All reactions can be performed with UltraPlex 1-Step ToughMix (Quanta Biosciences), a 4× concentrated master mix, and AgPath-ID One-step RT-PCR mix (Ambion, product no. 4387424).[1] These mixes have been shown to improve the reaction performance in comparison with qScript XLT Multiplex One-Step RT qPCR ToughMix (Quanta Biosciences),[1] a 2× concentrated master mix, which was used for validation of the original protocol (Testa, 2015). If using the AgPath-ID One-step RT-PCR mix (Ambion), replace the UltraPlex 1-Step ToughMix in Table 26, Table 27 and Table 28 with AgPath-ID One-step RT-PCR mix.[1]

The method has been successfully performed on different real-time PCR systems, including the CFX96 (Bio-Rad Laboratories) and the QuantStudio 5 and QuantStudio 7 Flex Real-Time PCR Systems (Applied Biosystems).[1]

The cycling parameters are 50 °C for 10 min, 95 °C for 2 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Table 18. Real-time RT-PCR primers and probes for the method of Naktuinbouw (2022, 2024)

Primers & probes	Sequence (5′–3′)	Reference
Primer mix A		
Primer PSTV-231F (forward)	GCC CCC TTT GCG CTG T	1
Primer PSTV-296R (reverse)	AAG CGG TTC TCG GGA GCT T	1
Probe PSTV-251T	FAM-CAG TTG TTT CCA CCG GGT AGT AGC CGA-BHQ1	1
Primer PCFVd-F (forward)	TCT TCT AAG GGT GCC TGT GG	2
Primer PCFVd-R (reverse)	GCT TGC TTC CCC TTT CTT TT	2
Probe PCFVd-P	VIC-CTC CCC CGA AGC CCG CTT AG-BHQ1	2
Primer mix B		
Primer CLVd-F (forward)	GGT TCA CAC CTG ACC CTG CAG	3
Primer CLVd-F2 (forward)	AAA CTC GTG GTT CCT GTG GTT	3
Primer CLVd-R (reverse)	CGC TCG GTC TGA GTT GCC	3
Probe CLVd-P	FAM-AGC GGT CTC AGG AGC CCC GG-BHQ1	3
Primer CEVd-F2-304 (forward)	CTC CAC ATC CGR TCG TCG CTG A	3
Primer CEVd-R2-399 (reverse)	TGG GGT TGA AGC TTC AGT TGT	3
Probe CEVd-P2-337	FAM-CCC TCG CCC GGA GCT TCT CTC TG-BHQ1	3
Primer mix C		
Primer TPMVd-F1 (forward)	AAA AAA GAA TTG CGG CCA AA	2
Primer TPMVd-R (reverse)	GCG ACT CCT TCG CCA GTT C	2
Probe pUCCR	FAM-CCG GGG AAA CCT GGA-MGB	4
Primer mix D		
Primer TASVd-F2-200 (forward)	CKG GTT TCC WTC CTC TCG C	3
Primer TASVd-R2-269 (reverse)	CGG GTA GTC TCC AGA GAG AAG	3
Probe TASVd-P2-228	FAM-TCT TCG GCC CTC GCC CGR-BHQ1	3
Internal controls		
Primer DaVd1-FT (forward)	GCT CCG CTC CTT GTA GCT TT	2
Primer DaVd1-RT (reverse)	AGG AGG TGG AGA CCT CTT GG	2
Probe DaVd1-P	Texas red-CTG ACT CGA GGA CGC GAC CG-BHQ2	2
Primer nad5-F (forward)	GAT GCT TCT TGG GGC TTC TTG TT	5
Primer nad5-R (reverse)	CTC CAG TCA CCA ACA TTG GCA TAA	5
Probe nad5-P	VIC-AGG ATC CGC ATA GCC CTC GAT TTA TGT G-BHQ1	4

Notes: RT-PCR, reverse transcription–polymerase chain reaction.

References: [1] Boonham *et al.*, 2004; [2] Naktuinbouw, 2022, 2024; [3] Monger *et al.*, 2010; [4] Botermans *et al.*, 2013; [5] Menzel, Jelkmann and Maiss, 2002.

Source (see section 8.2): Adapted from EPPO 2021a.

Table 19. Composition of primer mix A for the method of Naktuinbouw (2022, 2024)

Primers	Stock concentration (µM)	Volume (µL)	Final concentration (µM)
PCR-grade water	-	400	-
PSTV-231F (forward)	100	100	10
PSTV-296R (reverse)	100	100	10
PCFVd-F (forward)	100	100	10
PCFVd-R (reverse)	100	100	10
DaVd1-FT (forward)	100	100	10
DaVd1-RT (reverse)	100	100	10
Total		1 000	

Note: PCR, polymerase chain reaction.
Source (see section 8.2): Adapted from EPPO, 2021a.

Table 20. Composition of probe mix A for the method of Naktuinbouw (2022, 2024)

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

Note: PCR, polymerase chain reaction.
Source (see section 8.2): Adapted from EPPO, 2021a.

Table 21. Composition of primer mix B for the method of Naktuinbouw (2022, 2024)

Primers	Stock concentration (µM)	Volume (µL)	Final concentration (µM)
PCR-grade water	-	300	-
CLVd-F	100	100	10
CLVd-F2	100	100	10
CLVd-R	100	100	10
CEVd-F2-304	100	100	10
CEVd-R2-399	100	100	10
DaVd1-FT	100	100	10
DaVd1-RT	100	100	10
Total		1 000	

Note: PCR, polymerase chain reaction.
Source (see section 8.2): Adapted from EPPO, 2021a.

Table 22. Composition of probe mix B for the method of Naktuinbouw (2022, 2024)

Probes	Stock concentration (µM)	Volume (µL)	Final concentration (µM)
PCR-grade water	-	350	-
CLVd-P	100	50	10
CEVd-P2-337	100	50	10
DaVd1-P	100	50	10
Total		500	

Note: PCR, polymerase chain reaction.

Source (see section 8.2): Adapted from EPPO, 2021a.

Table 23. Composition of primer mix C for the method of Naktuinbouw (2022, 2024)

Primers	Stock concentration (µM)	Volume (µL)	Final concentration (µM)
PCR-grade water	-	600	-
TPMVd-F1	100	100	10
TPMVd-R	100	100	10
nad5-F	100	100	10
nad5-R	100	100	10
Total		1 000	

Note: PCR, polymerase chain reaction.

Source (see section 8.2): Adapted EPPO, 2021a.

Table 24. Composition of probe mix C for the method of Naktuinbouw (2022, 2024)

Probes	Stock concentration (µM)	Volume (µL)	Final concentration (µM)
PCR-grade water	-	400	-
pUCCR	100	50	10
nad5-P	100	50	10
Total		500	

Note: PCR, polymerase chain reaction.

Source (see section 8.2): Adapted from EPPO, 2021a.

Table 25. Composition of reaction mix A for the detection of PCFVd, PSTVd, TCDVd, TPMVd and DLVd using the method of Naktuinbouw (2022, 2024)

Reagents	Working concentration	Volume per reaction (µL)	Final concentration
PCR-grade water	-	-[†]	-
UltraPlex 1-Step ToughMix (Quanta Biosciences)[‡]	4×	6.25	1×
Primer mix A (see Table 19)	10 µM (each)	0.75	0.3 µM (each)
Probe mix A (see Table 20)	10 µM (each)	0.5	0.2 µM (each)
RNA		6.0	

Notes: [†] For a final reaction volume of 25 µL.

[‡] See page footnote 1.

DLVd, 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.

Source (see section 8.2): Adapted from EPPO, 2021a.

Table 26. Composition of reaction mix B for the detection of CEVd, CLVd and DLVd using the method of Naktuinbouw (2022, 2024)

Reagents	Working concentration	Volume per reaction (µL)	Final concentration
PCR-grade water	-	-†	-
UltraPlex 1-Step ToughMix (Quanta Biosciences)‡	4×	6.25	1×
Primer mix B (see Table 21)	10 µM each	0.75	0.3 µM each
Probe mix B (see Table 22)	10 µM each	0.5	0.2 µM each
RNA		6.0	

Notes: † For a final reaction volume of 25 µL.

‡ See page footnote 1.

CEVd, citrus exocortis viroid; CLVd, Columnea latent viroid; DLVd, dahlia latent viroid; PCR, polymerase chain reaction.

Source (see section 8.2): Adapted from EPPO, 2021a.

Table 27. Composition of reaction mix C for the detection of TPMVd and *nad5* using the method of Naktuinbouw (2022, 2024)

Reagents	Working concentration	Volume per reaction (µL)	Final concentration
PCR-grade water	-	-†	-
UltraPlex 1-Step ToughMix (Quanta Biosciences)‡	4×	6.25	1×
Primer mix C (see Table 23)	10 µM each	0.75	0.3 µM each
Probe mix C (see Table 24)	10 µM each	0.5	0.2 µM each
RNA		6.0	

Notes: † For a final reaction volume of 25 µL.

‡ See page footnote 1.

PCR, polymerase chain reaction; TPMVd, tomato planta macho viroid.

Source (see section 8.2): Adapted from EPPO, 2021a.

Table 28. Composition of reaction mix D for the detection of TASVd using the method of Naktuinbouw (2022, 2024)

Reagents	Working concentration	Volume per reaction (µL)	Final concentration
PCR-grade water	-	-†	-
UltraPlex 1-Step ToughMix (Quanta Biosciences)‡	4×	6.25	1×
Primer TASVd-F2-200 (forward)	10 µM	0.75	0.3 µM
Primer TASVd-R2-269 (reverse)	10 µM	0.75	0.3 µM
Probe TASVd-P2-228	10 µM	0.5	0.2 µM
RNA		6.0	

Notes: † For a final reaction volume of 25 µL.

‡ See page footnote 1.

PCR, polymerase chain reaction; TASVd, tomato apical stunt viroid.

Source (see section 8.2): Adapted from EPPO, 2021a.

Validation data

Information on validation. Validation data were generated according to EPPO (2021b) by Naktuinbouw (Naktuinbouw, 2021). Nucleic acid was extracted using the sbeadex Maxi Plant Kit (LGC Biosearch Technologies).[1]

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).

Analytical specificity. No cross-reactions were observed with 29 isolates of other viroids and viruses 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 cross-reactivity of TASVd isolates with the CEVd and CLVd primer mix B was observed.

Selectivity. No apparent matrix effects were observed for *C. annuum* and *S. lycopersicum* seeds.

Repeatability and reproducibility. Repeatability and reproducibility were 100% for all target species.

3.4.3.5 Other detection methods

A list of selected 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; Zhang *et al.*, 2023). Testing laboratories should follow recommendations for users and validate these methods for the specific use intended.

3.5 Controls for molecular tests

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 isolation and amplification of the target pest or target nucleic acid. For RT-PCR, a positive nucleic acid control and a negative amplification control (no template control) are the minimum controls that should be used.

Positive nucleic acid control. This control is used to ensure that amplification of a known target happens as expected (apart from the extraction). 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.

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. However, as *COX* primers will amplify RNA and DNA, 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*, *Lycianthes rantonnetii* (*S. rantonnetii*), *S. sisymbrifolium*), *Atropa belladonna*, *Brugmansia* spp., *Capsicum* spp., *Cestrum* spp., *Iochroma arborescens* (*Acnistus arborescens*), *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.

Negative amplification control (or no template control). This control is necessary for RT-PCR to rule out false positives resulting from contamination with target RNA during preparation of the reaction 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.

Positive extraction control. This control is used to ensure that the target viroid nucleic acid extracted is of sufficient quantity and quality for PCR-based detection. Viroid nucleic acids are extracted from infected host tissue or healthy plant tissue that has been spiked with the viroid. Care needs to be taken to avoid cross-contamination resulting from aerosols from the positive control. The sequence of the positive control used in the laboratory should be known so that this sequence can be readily compared

with the sequence obtained for the samples to trace potential cross-contamination by the positive control. Alternatively, synthetic positive controls can be used.

Negative extraction control. This control is used to monitor both contamination during nucleic acid extraction and cross-reaction with the host tissue. The control comprises nucleic acid that is extracted from uninfected host tissue and subsequently amplified. Alternatively, extraction blanks (e.g. sterile water, clean extraction buffer) can be processed with the samples to be tested if sufficient uninfected host tissue is not available. This will allow contamination of extraction reagents and cross-contamination between samples to be identified. It is recommended that multiple negative extraction controls be included when large numbers of positive samples are expected.

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

3.6.1 Conventional RT-PCR

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

- the negative extraction control and negative amplification control produce no band corresponding to the expected amplicon size; and
- the positive extraction control and the positive nucleic acid control, as well as the internal control if applicable, produce bands that correspond to the expected amplicon size (note that, in the case of a positive sample, the internal control may produce no band or only a faint band).

When these conditions are met:

- a sample will be considered negative if it produces no band or a band that corresponds to an amplicon size that is different than expected; and
- a sample will be considered positive if it produces a band corresponding to the expected amplicon size.

3.6.2 Real-time RT-PCR

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

- the negative extraction control and negative amplification control produce no target viroid amplification curve; and
- the positive extraction control and the positive nucleic acid control, as well as the internal control if applicable, produce exponential amplification curves (note that, in the case of a positive sample, the amplification curve of the internal control may not be produced or the curve may not be exponential).

When these conditions are met:

- a sample will be considered negative if it produces no amplification curve or produces a curve that is not exponential; and
- a sample will be considered positive if it produces an exponential amplification curve.

A Ct cutoff value may be applied according to laboratory validation data.

4. Identification

Members of the genus *Pospiviroid* can be identified by sequence analysis of the amplicon or amplicons obtained by the conventional RT-PCR method (section 3.4.3.1), 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. Columnea latent viroid can be identified without sequencing because of the specificity of the primer set (see Table 2).

Alternatively, high-throughput sequencing can be used for the identification of pospiviroids. Further information and recommendations on the use of high-throughput sequencing as a diagnostic tool for phytosanitary purposes have been published (CPM R-08, 2019; EPPO, 2022b; Lebas *et al*., 2022).

EPPO (2021c) provides general guidance on Sanger 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 (i.e. type of symptoms, host range, see section 3.1). The ICTV Viroid Study Group is currently discussing viroid classification and the criteria for species demarcation.

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. 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, the Pospi2 method is less sensitive than the Pospi1 method. Therefore, in some cases it is not feasible to obtain the complete genome sequence. In such cases, the partial sequence obtained by the Pospi1 primers, which covers about half of the pospiviroid genome, can be used in the sequence comparisons, as it may be sufficient to correctly identify some isolates.

A positive sample detected by real-time RT-PCR should, if required, be confirmed using either a different real-time RT-PCR method or a conventional RT-PCR to enable the amplicon to be sequenced for viroid identification. Examples of methods suitable for substantiating results are provided in Table 2 and Table 3; the choice of method will depend on the initial method used. For example, in the case of seed testing where viroid concentrations may be low, conventional RT-PCR may not be suitable to confirm a positive result from real-time RT-PCR, because the latter has a higher analytical sensitivity and therefore may produce an amplicon in cases where an amplicon may not be obtained with conventional RT-PCR.

To obtain the complete genome sequence or sequences, forward and reverse RT-PCR primers are used for bi-directional Sanger sequencing (EPPO, 2021c). The 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)). For identification, it is advisable to use the consensus sequence starting at 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).

In critical cases, 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.

5. Records

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

In instances where other contracting parties may be affected by the results of the test results, in particular in cases of non-compliance (ISPM 13 (*Guidelines for the notification of non-compliance and emergency action*)) or when a regulated pospiviroid is found in an area for the first time or in a new host, the following materials should be kept in a manner that ensures traceability:

- if relevant and available, material of the original sample, stored at −80 °C or freeze-dried;

- RNA extracts stored at −80 °C;
- RT-PCR amplicons stored at −20 °C to −80 °C; and
- DNA sequence trace files used to generate the consensus sequence.

6. Contact points for further information

Further information on this protocol can be obtained from:

SASA, The Scottish Government, Roddinglaw Road, Edinburgh EH12 9FJ, United Kingdom (Christophe Lacomme; email: christophe.lacomme@sasa.gov.scot).

Netherlands Institute for Vectors, Invasive plants and Plant health (NIVIP), Netherlands Food and Consumer Product Safety Authority (NVWA), PO Box 9102, 6700 HC Wageningen, 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).

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

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), who will forward it to the Technical Panel on Diagnostic Protocols (TPDP).

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This protocol is based, with permission, on EPPO (2021a) and includes information from DP 7. Potato spindle tuber viroid, which is the subject of DP 7, is included in this protocol because the available diagnostic methods do not allow discrimination between different members of the genus *Pospiviroid*.

8. References

The present annex may refer to ISPMs. ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/en/core-activities/standards-setting/ispms.

8.1 Main text

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8.2 Figures and tables

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Publication history

This is not an official part of the standard

2018-11 Standards Committee (SC) added *Pospiviroid* species (except *Potato spindle tuber viroid* (DP 7)) to work programme.

2022-10 Technical Panel on Diagnostic Protocols (TPDP) reviewed.

2023-01 TPDP approved for expert consultation (2023_eTPDP_Jan_01).

2023-03 Expert consultation.

2023-11 TPDP revised and recommended to SC for consultation.

2024-01 SC approved for consultation (2024_eSC_May_03).

2024-07 Consultation.

2024-10 TPDP revised.

2025-06 TPDP reviewed and recommended to SC for adoption (agreed by email).

2025-06 SC approved draft to be submitted to the 45-day DP notification period (2025_eSC_Nov_02).

2025-08 SC adopted DP on behalf of CPM (no objections received).

ISPM 27. **Annex 36**. Genus *Pospiviroid*. IPPC secretariat. FAO, Rome.

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