Euphresco Final Report

Detection and Epidemiology of Pospiviroids 2 (DEP2)

Introduction

Pospiviroids are plant pathogens, composed only of infectious circular RNA molecules, which are both latent on many ornamentals and harmful for cultivated plant species like tomato, potato, sweet pepper, citrus or chrysanthemum.

Throughout the world, these pathogens cause sporadic outbreaks on susceptible annual crops while they often remain undetected although widespread on ornamentals. To assess the risk posed by pospiviroids in the EU, the link between this potential ornamental reservoir and the outbreaks on solanaceous as well as the role of infected seeds in these contaminations have been recently reviewed by the EFSA panel on plant health (PLH) (EFSA, 2011). However, the continuing uncertainty over the role of insects and infected seeds in the spreading of pospiviroids complicated the likelihood assessment of these transmission routes.

On the other hand, the PLH concluded that disinfection measures and the accurate generic detection of pospiviroids are among the indispensable tools for the management of these diseases.

The project DEP2 (Detection and Epidemiology of Pospiviroids 2) thus has focused on these different elements to decrease the uncertainty about the routes of interspecific transmission (WP2) and seed transmission (WP3), compare available generic detection methods (WP4) and evaluate the efficacy of the only approved pospiviroid disinfectant in EU (WP5).

Overview of Work Packages

Work Packages (WP)	
No. of WP	**Title**
1	Project management and co-ordination
2	Assessment of the risk of transmission from ornamentals to tomato
3	Assessment of the transmission rate through tomato seeds
4	Ring-test for the detection of pospiviroids in tomato seeds
5	Evaluation of different physical and chemical pospiviroid inactivation procedures

Topic andResearch coordinator

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WP1 *Project Management and Co-ordination* (Lead: T. Olivier)

WP1.1 Research Consortium Coordination

- CRA-W served as contact for the EUPHRESCO Call Secretariat as well as for partners regarding project issues;
- CRA-W compiled the final project progress reports for EUPHRESCO Call Secretariat and organized the collaboration, meetings and steer activities.

WP1.2 Meetings and training courses

The project kick-off meeting was organized in Brussels the 3rd of May 2012.
A mid-term web meeting was held on June the 19th, 2013. The final web meeting was held on May the 3rd, 2014.

WP1.3 Technology Transfer and Dissemination

The dissemination of the results was carried out through scientific publications:

F. Faggioli, M. Luigi, V. Sveikauskas, T. Olivier, M. Virscek Marn, I. Mavric Plesko, K. De Jonghe, N. Van Bogaert, S. Grausgruber-Gröger (2015) An assessment of the transmission rate of four pospiviroid species through tomato seeds. *European Journal of Plant Pathology* DOI 10.1007/s10658-015-0707-7 Published online 11 July 2015.

T. Olivier, V. Sveikauskas, S. Grausgruber-Gröger, M. Virscek Marn, F. Faggioli, M. Luigi, E. Pitchugina, V. Planchon (2015) Efficacy of five disinfectants against Potato spindle tuber viroid. *Crop Protection* 67: 257-260.

WP2 Assessment of the risk of transmission from ornamentals to tomato (Lead: Kris De Jonghe)

Main Partners: N. Van Bogaert, K. De Jonghe, T.Olivier

In this study, insects from three different functional groups (i.e. pest species, pollinating insects and polyphagous biological control agents), all typically occurring in susceptible vegetable solanaceous species, were evaluated for their capacity to transmit the pospiviroids. To represent the pest insects, the green peach aphid *Myzus persicae* (Sulzer) was selected for its widespread and reccurent presence in susceptible crops and its well-established role of plant virus vector. Bradley and Harris (1972) showed that *M. persicae* and *Macrosiphum euphorbiae* (Thomas) can transmit the *Tobacco mosaic virus* (TMV) by simply piercing the leaf with contaminated "claws" at the end of their legs. This phenomenon would particularly occur when an aphid has difficulties in removing its stylet from inside the foliar cells during nutritional events, or when piercing is constrained by numerous hairs on the surface of the leaf (Bradley and Harris 1972). Even if the risk of mechanical transmission by insects could be considered as minor in comparison with other modes of dissemination in crops, this transmission mode should be taken into account when quarantine pathogens are concerned.

To represent the group of the pollinators, the buff-tailed bumblebee (*Bombus terrestris* L.) was chosen. These days, many European greenhouses and open-field crops rely on commercially reared bumblebees for their ability to provide a higher level of cross-pollination (Goulson 2010). Furthermore, foraging activity of pollinators has been implicated in plant virus transmission before (Shipp *et al.* 2008; Li *et al.* 2014). Additionally, bumblebees are increasingly being investigated in the context of entomovectoring, where pollinators are being used as carriers and disseminators of control agents against pests (Mommaerts et al., 2011). However, a converse scenario could be imagined where bumblebees are not functioning as "flying doctors", but as carriers of ominous cargo, i.e. pathogens.

The polyphagous bug species, *Macrolophus pygmaeus* (Rambur) , was selected to represent the biological control agents because of its increasing popularity as biological control agent in the commercial cultivation of many crops. Heteroptera, such as *M. pygmaeus*, are generally considered to be of minor importance as vectors, although they share similar feeding behaviors with Sternorrhyncha, like whiteflies and aphids (Mitchell 2004). Additionally, these bugs are potential candidates for pollen-mediated transmission, since they are able to complete their lifecycles by feeding onto this protein source (Vandekerkhove and De Clercq 2010).

To evaluate whether these three insect species could function as vectors for pospiviroids, four transmission experiments were organized for each insect. During these experiments, insects were placed in cages together with different species of pospiviroid-infected and healthy host plants.

Materials and methods

Experimental set-up

For each of the three insect species, i.e. *B. terrestris*, *M. persicae* and *M. pygmaeus*, four different transmission experiments were conducted (Table 1). The experiments vary from each other in terms of: number and species of host plants, inoculated pospiviroid, location of the experiment and ambient temperature. This variety in experimental setups was chosen to explore a diversity of different scenarios during which transmission could happen as well as to take some practical constraints into account (e.g. simultaneous flowering of the plants,). The pospiviroid isolates that were used in this study are: *Potato spindle tuber viroid* (PSTVd), *Tomato apical stunt viroid* (TASVd), *Tomato chlorotic dwarf viroid* (TCDVd) and *Pepper chat fruit viroid* (PCFVd) (Table 1). The different hosts plants used were tomato (*Solanum lycopersicum*, L.), chili pepper (*Capsicum chinense*, L.), Petunia (*Petunia x hybrida*) and tobacco (*N. benthamiana* L.) (Table 1). Two experiments (No. 1 & 2) were performed at the Walloon Agricultural Research Centre (CRA-W, Gembloux, Belgium), while all other experiments (No. 3-12) were conducted at the Institute for Agricultural and Fisheries Research (ILVO, Merelbeke, Belgium).

At the start of each experiment, adult insect individuals were placed onto, or in the case of bumblebee hives, in close proximity with pospiviroid infected plants. For *B. terrestris* (Exp. No 1-4, Table 1), a Mini-Hive (Biobest, Westerlo, Belgium) containing approximately 50 adult bumblebees was placed at one meter distance of the pospiviroid-infected host plants (e.g. Figure 1). Bumblebee experiments 1 and 2 (Table 1) were conducted in a greenhouse (2.65 x 2.3 m) of CRA-W. The other two bumblebee experiments (Exp. No. 3 & 4, Table 1) were carried out in a breeding cage (1.75 x 1.75 x 1.75 m, mesh size = 0.8 x 0.8 mm) that was placed inside a greenhouse compartment (4.7 x 4.7 m) located at ILVO.

In the experiments with *M. persicae* and *M. pygmaeus* (Exp. No. 5-12, Table 1), adult insects were placed onto leaves of pospiviroid-infected host plants in a medium-sized breeding cage (60 x 60 x 90 cm, mesh size = 0.8 x 0.8 mm) in a climate chamber (3 x 2 m). After an acquisition period of two days, healthy host plants were placed inside the cage, making sure that the distance between healthy and infected plants was large enough to avoid any contact. Insect movement from one plant to another was not influenced in any way.

To investigate the transmission of viroids by *M. pygmaeus*, 50 adults were fed with 1g of TASVd-infected pollen collected from *Petunia x hybrida*. The insects were allowed to feed on this pollen during two days in a small breeding cage (30 x 30 x 30 cm, mesh size = 0.8 x 0.8 mm). After this period, the individuals were placed onto healthy tomato plants.

During all experiments, insect activity (i.e. flying, foraging) was closely monitored throughout 6 weeks.

Plant sampling and testing

At the end of each transmission experiment, a random flower and leaf sample of each infected and (initially) healthy plant was taken. After crushing, total RNA was extracted from 100mg of plant material of each sample using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA). The pospi1-FW/RE primers (Verhoeven *et al.* 2004) and the pospi1deg-FW, pospi1s-RE and pCLV4s primers (Olivier *et al.* 2014) were used for the

generic detection of pospiviroids. After electrophoresis, amplicons were sent for sequencing (Macrogen Europe, Amsterdam, the Netherlands). When weak or ambiguous signals were detected during electrophoresis, viroid RNA was re-analyzed using an RT-qPCR with the Agpath-ID^{TM} one-step RT qPCR Kit (Applied Biosystems, Foster City, CA,USA) using the primers and probes of Botermans *et al.* (2013), Boonham *et al.* (2004) and Monger *et al.* (2010). All necessary diagnostic controls were taken into account during the analyses (i.e. a healthy tomato and a blank control). Based on validation data, Ct-values higher than 35 were considered as negative.

Insect sampling and testing

After an acquisition period of two days, five *M. persicae* and *M. pygmaeus* individuals that were seen probing onto the plants, were sampled from the cages (Experiment No. 5-12, Table 1). After putting the individual insects in 2ml microtubes and thorough crushing in liquid nitrogen, RNA was extracted using the RNeasy RNA extraction kit (Qiagen, Hilden, Germany). Additionally, *B. terrestris* individuals that were actively foraging on infected flowers were captured during experiment No. 3 and 4 (Table 1). In total 15 and 10 bumblebees were sampled from these experiments, respectively.

To avoid excess material for RNA-extraction, bumblebee body parts were first dissected using sterile micro-scissors (Vannas scissors No.14003, World Precision Instruments, Sarasota, FL, USA) and allocated to three groups: head, thorax plus abdomen and legs. The micro-scissors were decontaminated using 1% Virkon and rinsed three times with distilled water during the dissection of the body parts of an individual and between each individual. Legs of one bumblebee individual were pooled together in one reaction. Also thorax and abdomen of one individual were analyzed together in one reaction. Pospiviroid detection was done via RT-(q)PCR as described above.

Insect species	Exp. No.	Pospiviroid	No. of insects	AT	Infected plants (IP)	No. IP	Receiving plants (RP)	No. RP
B. terrestris	**1***	TCDVd (HG739070)	50	19°C	*Petunia x hybrida*	33	*S. lycopersicum* cv. Minibel	18
	2*	TCDVd (HG739070)	50	25°C	*Petunia x hybrida*	16	*S. lycopersicum* cv. Minibel	18
	3**	TASVd (KF484879)	50	20°C	*Solanum lycopersicum* cv. Marmande *Nicotiana benthamiana*	12 2	*S. lycopersicum* cv. Marmande	3
	4**	TASVd (KF484879)	50	23°C	*Solanum lycopersicum* cv. Marmande *Petunia x hybrida*	2 8	*Petunia x hybrida*	8
M. persicae	**5****	TASVd (KF484879)	100	23°C	*S. lycopersicum* cv. Marmande	1	*S. lycopersicum* cv. Marmande	3
	6**	TASVd (KF484879)	10	20°C	*Physalis* sp.	1	*Physalis* sp.	1
	7**	TASVd (KF484879)	10	20°C	*N. benthamiana*	1	*N. benthamiana*	1
	8**	TASVd (KF484879)	50	20°C	*Capsicum chinense* cv. Mme Jeanette	1	*C. chinense* cv. Mme Jeanette	3
M. pygmaeus	**9****	TASVd (KF484879)	100	23°C	*S. lycopersicum* cv. Marmande	1	*S. lycopersicum* cv. Marmande	3
	10**	PSTVd (FM998542)	100	23°C	*S. lycopersicum* cv. Marmande	1	*S. lycopersicum* cv. Marmande	1
	11**	TASVd (KF484879)	50	20°C	pollen of *Petunia x hybrida*	1g	*S. lycopersicum* cv. Marmande	1
	12**	PCFVd (FJ409044)	100	23°C	*C. chinense* cv. Mme Jeanette	1	*C. chinense* cv. Mme Jeanette	3

Table 1: Overview of the transmission experiments conducted for bumblebees (*Bombus terrestris*, L.), green peach aphids (*M. persicae*, Sulzer) and whitefly predatory bugs (*Macrolophus pygmaeus*, Rambur) in the period 2012-2015. Column headings (f.l.t.r.): Insect species = scientific species name, Exp. No = experiment number and location of the experiment: * = CRA-W, Gembloux, Belgium or ** = ILVO, Merelbeke, Belgium, Viroid isolate = inoculated viroid isolate with Genbank Accession number, No. of insects = number of insect individuals used in each experiment, AT = ambient temperature of each experiment in °C, Infected Plants (IP) = species name of infected host plants used in each experiment, No. IP = number of infected plants, Receiving Plants (RP) = species name of healthy plants presented after the acquisition period, No. RP= number of receiving plants .

Results

Bumblebees, aphids and whitefly predatory bugs were regularly observed feeding on infected and healthy plants during all transmission experiments. In the case of *M. persicae*, all 20 sampled individuals tested positive for pospiviroids throughout the experiment. For *B. terrestris*, one positive detection result was acquired in experiment No. 3 (Table 1) where the legs of one individual resulted in a Ct-value of 34,7 after conducting the one-step RT-qPCR assay designed by Botermans *et al.* (2013). The 24 other bumblebee samples taken throughout this experiment, however, were negative. All 20 samples of *M. pygmaeus* individuals tested negative.

In bumblebee experiment No. 3, two samples of (initially healthy) tomato flowers tested positive in a one-step RT-qPCR conducted after four weeks (Ct = 31,7 and Ct = 34,5; Botermans *et al.* 2013). However, when the same plants were resampled two weeks later, all samples were negative, indicating that systemic spread from the flowers to the rest of the plant did not occur. In bumblebee experiment No. 2 (Table 1; Figure 1) the flower sample of one of the 18 initially healthy tomato plants taken two months after the experiment had started, tested positive after an RT-PCR with primers of Olivier *et al.* (2014, Figure 2). After four months, both leaf and fruit samples of this plant tested positive using the same PCR-test as before (Lane No. 17, Figure 1A & B). For these samples, clear bands at the expected size ± 200nt were observed after gel-electrophoresis. The sequence of the amplicons obtained at two and four months showed a perfect similarity with the TCDVd isolate present in the petunias which served as experiment inoculum (Accession No. HG739070). None of the other tomato plants (Lane No. 1-16 and Lane 18) tested positive. In bumblebee experiment No. 1 (Table 1; Figure 1), the 18 tomato plants tested negative 70 days after the bumblebee release.

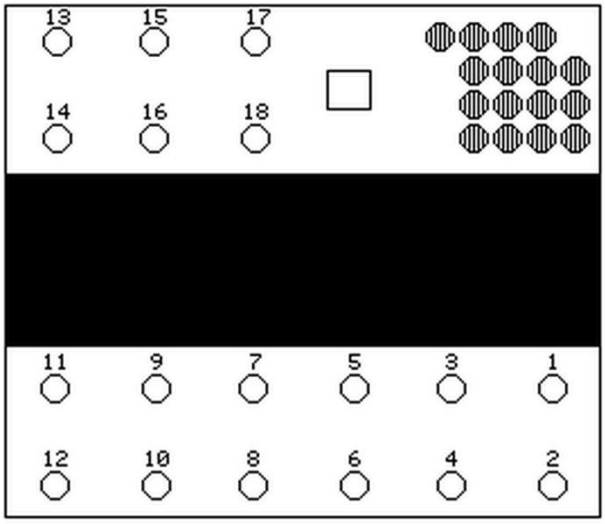

Figure 1: Experimental layout of Exp. No. 2 with *Bombus terrestris* conducted in a greenhouse at CRA-W. Empty circles = 18 initially healthy tomato plants in individual saucers placed onto two (white) benches separated by a (black) corridor, empty rectangle = bumblebee hive, lined circles = 16 TCDVd-infected Petunia plants.

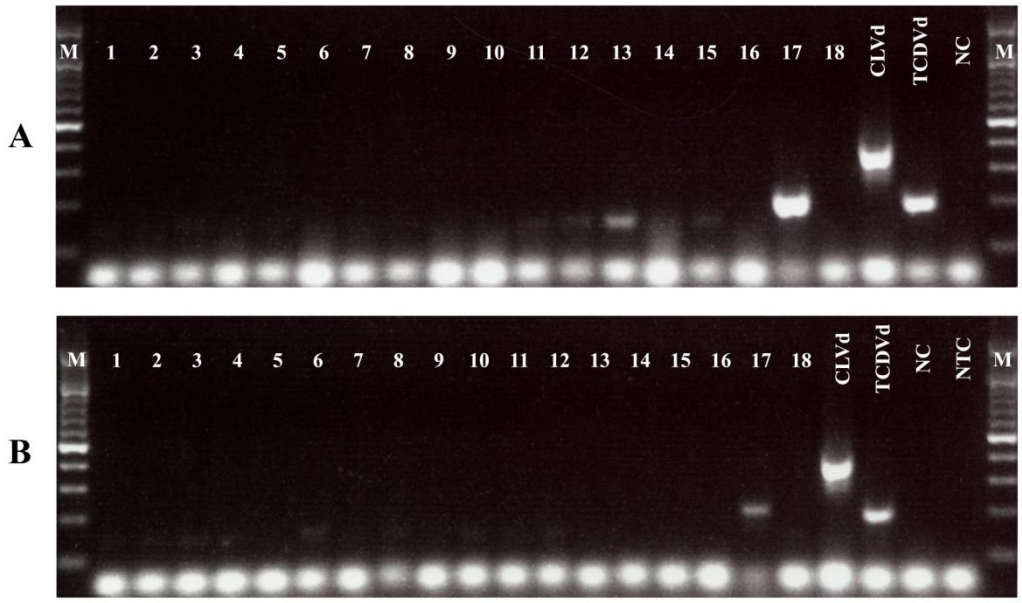

Figure 2: Gel pictures of A) leaf samples and B) fruit samples of 18 initially healthy tomato plants after four months of bumblebee transmission (amplicons obtained by RT-PCR, Olivier *et al.* 2014). "M" = Molecular weight markers (O' GeneRuler 100bp Plus DNA Ladder, Thermo Fisher Scientific, Waltham, MA, USA), Lane "1-18" = initially healthy tomato plants, "CLVd" = CLVd positive control (size 375nt), "TCDVd"= TCDVd positive control (size 195nt), NC = negative tomato control, NTC = blank control. Primer dimers (± 50nt) are observed in all lanes and slight nonspecific bands (± 165nt) in lanes 12, 13 and 15 of figure 1A.

Discussion

Little information is available on pospiviroid natural infections in susceptible crops. However, in several cases, the primary infections seem to appear either at a unique location or in patches throughout the crop. These primary infections then spread along rows of the crop as the growing season progresses (Verhoeven *et al.* 2004; Verhoeven *et al.* 2007; Mackie *et al.* 2002). While the latter spreading can be attributed to mechanical transmission during pruning or staking, the scattered pattern of primary infections may suggest a low transmission through seeds or insects.

Regarding insect transmission, the results of this study show that for *M. persicae*, although all of the sampled individuals tested positive, these contaminations did not lead to successful transmission of the viroids to healthy plants. The fact that aphid individuals themselves tested positive just after the acquisition period is in accordance with previous results (Van Bogaert *et al.* 2015). FISH and qPCR-experiments have indeed located viroids in aphid's stylets, legs and foreguts after feeding on viroid-infected plant material (Van Bogaert *et al.* 2015). These observations, along with those reported by De Bokx and Piron (1981), suggest that pospiviroids are degraded in the foregut of the green peach aphid preventing a circulative transmission. The reason why *M. persicae* seems unable to transmit pospiviroids, even in a non-persistent way, is unknown, but the fact that viroids could not be retained at the extreme tip of the stylet like non-persistent viruses (Uzest *et al.* 2007) and/or the double stranded RNA degradation activity observed in aphid saliva (Christiaens *et al.* 2014) could explain this phenomenon. In any case, the results of the present study are in line with those previously obtained by De Bokx and Piron (1981) for *M. persicae*. The risk posed by *M. persicae* regarding pospiviroid transmission is thus considered as negligible.

In the transmission experiments with *M. pygmaeus*, despite the fact that this bug partially shares the same feeding mode with aphids, the 20 sampled insects tested negative. Because it was observed that *M. pygmaeus* preferred *Capsicum* over tomatoes, the transmission experiment with PCFVd was performed on this plant. While it was hypothesized that the attractiveness of the food source could result in positive transmission results, this did not seem to be the case. Also the consumption of TASVd-infected pollen did not lead to transmission events. Although it cannot be completely excluded that insect extracts have some inhibitory effects on RT-PCR and/or that the pospiviroid concentrations were below the detection limit, an early degradation of viroid due to salivary enzymes could also be considered. This latter hypothesis is supported by the dsRNA degradation activity observed in the saliva of another member of the *Miridae* family : *Lygus lineolaris* (Palisot de Beauvois) (Allen and Walker 2012). It should be mentioned, however, that the tarnished plant bug was found to transmit PSTVd at a low rate on potato (Schumann *et al.* 1980). Considering the relatively high number of bugs used in the four experiments carried out in this study as well as the absence of positive plants and insects, we conclude that the importance of *M. pygmaeus* as a potential vector for pospiviroids is minimal.

Concerning the role of bumblebees, it can be assumed that one particular tomato plant (No.17 in experiment No. 2) got infected as a result of bumblebee activity. The infection could also be linked to the position of this particular plant and its proximity to the bumblebee hive and the infected petunia plants (Figure 1). Located closest to the hive, plant No. 17 may have had more bumblebee visits compared to any of the other receiving plants. Unfortunately this cannot be confirmed, since the number of bumblebee visits per plant was not assessed during

the experiment. In this experiment, the risk of mechanical contamination due to human handling of the plants or contact with infected plants was reduced to the minimum since all conceivable phytosanitary precautions were taken: i.e. infected and healthy plants were physically separated from each other (Figure 1), placed in separate saucers and attached to supporting sticks to prevent the plants from bending over. For each sampling, new sterile gloves were used. Additionally, diffuse spraying during watering of the plants was prevented.

While the inoculation through the germination of infected pollen on the receiving plant stigma could explain the observed intraspecific transmission of pospiviroids or viruses in tomato (Antignus *et al.* 2007; Shipp *et al.* 2008; Matsuura *et al.* 2010), another mechanism should be involved in interspecific transmission. The contamination of bumblebee mandibles with infected plant sap during nectar robbing activities or flower biting observed in the so-called 'buzz pollination' of tomato could, for instance, provide the necessary entry point for viroid inoculation.

It is worth noting that the only transmission event of this study was observed in the experiment performed at the highest temperature applied (25 °C). Because pospiviroid concentration and mechanical transmissibility seem to increase with temperature (Harris and Browning 1980, Schumann *et al.* 1980, Verhoeven *et al.* 2010), it could be reasonably postulated that the risk of transmission by insects would increase accordingly. Interestingly, successful pospiviroid insect transmission without heterologous encapsidation reported in the literature also happened at day temperature equals or greater than 25 °C (Schumann *et al.* 1980, De Bokx and Piron 1981, Antignus *et al.* 2007; Matsuura *et al.* 2010). In order to better understand the origin of pospiviroid natural contaminations in susceptible crops, one could speculate that an early expression of symptoms would indicate a seed-borne transmission while a late infection would rather suggest an insect transmission. However, because the temperature modulates the symptom expression, symptom appearance could be delayed making the distinction between both origins more difficult.

In conclusion, out of the twelve experiments performed in this study with three different commonly used or encountered insect species in the susceptible crops, only one TCDVd transmission event was recorded when bumblebees were used. Considering the high density of bumblebees used, the close proximity of infection source and the relatively low transmission efficiency for tomatoes observed (1/39 = 2.6 %), it is suggested that pospiviroid transmission by bumblebees can happen, but that the risk should be considered as low. This assumption is supported by the fact that pospiviroids are largely widespread in ornamental plants in Europe, but relatively few outbreaks were reported in susceptible crops, such as tomato and pepper, in the same area. Thus, our opinion is that the use of pollinating insects and biological control agents in these susceptible crops does not imply a major phytosanitary threat for viroid dispersal. Additionally, as previously stated by EFSA (*et al.*2011), not assisting pollination by bumblebees in glasshouse crops is not an option due to the consequential yield losses.
Yield losses due to pospiviroid infections in greenhouses can be prevented by a good observation of the crops, an early determination of the disease with reliable detection methods and the application of effective hygiene measures.

WP3 *Assessment of the transmission rate through tomato seeds (Lead: F. Faggioli)*

Main Partners: F. Faggioli, M. Luigi, V. Sveikauskas, T. Olivier, M. Virscek Marn, I. Mavric Plesko, K. De Jonghe, N. Van Bogaert, S. Grausgruber-Gröger

In recent years, different pospiviroid species, such as *Tomato apical stunt viroid* (TASVd; Antignus *et al.*, 2002), *Citrus exocortis viroid* (CEVd), *Columnea latent viroid* (CLVd) *Potato spindle tuber viroid* (PSTVd) and *Tomato chlorotic dwarf viroid* (TCDVd) (Verhoeven *et al.et al.*, 2004; Singh *et al.*, 1999) were found in tomato plants cultivated in greenhouses. In their Scientific Opinion on the assessment of the risk of solanaceous pospiviroids for the EU territory, the European Food Safety Authority (EFSA) identified the following three pathways related to plant propagation material for entry in the EU: plants for planting, potato tubers and seeds (EFSA, 2011).

Among these three pathways, transmission through seed is the most uncertain, although it has been observed in tomato for several pospiviroids e.g. PSTVd (EUPHRESCO, 2011), TASVd (Antignus *et al.*, 2007) and TCDVd (Singh and Dilworth, 2009). Seed transmission has also been associated with several outbreaks of CLVd (Sansford and Morris, 2010) but this link has not been proven. However some ambiguities remained when seed transmission was experimentally tested. Some unsuccessful experiments have been reported: in 1999 a total of 700 seeds and 400 seedlings, obtained from TCDVd-infected solanaceous plants, were analysed and no infection was detected in r-PAGE (Singh *et al.*, 1999); moreover, in 2009, 4.000 tomato seedlings obtained from TCDVd-infected seeds were analyzed in RT-PCR and all tested negative (Koenraadt *et al.*, 2009).

Moreover, many discrepancies were reported as regards to transmission rate of pospiviroids by seeds. For TASVd, 80% of seed transmission was reported using an unknown variety of tomato (Antignus *et al.*, 2007). PSTVd has showed a seed transmission rate on tomato ranging from 0.3% in naturally infected seeds (van Brunschot *et al.*, 2014) to 20% (Kryczyński *et al.*, 1988). Variable transmission rate for PSTVd was also connected to the differences in strain and/or variety (EUPHRESCO, 2011). The transmission rate of TCDVd ranged from 0.1% in naturally infected seeds (Candresse *et al.*, 2010) to 80% in tomato seedlings grown from seeds harvested from mechanically infected plants (Singh and Dilworth, 2009) whereas *Chrysanthemum stunt viroid* (CSVd) showed 100% transmission in tomato (Kryczyński *et al.*, 1988).

To assess the transmission rate through tomato seeds for TASVd, CEVd, PSTVd and CLVd, and to understand the true risk of pospiviroid spread through infected tomato seeds, a new EUPHRESCO Project : 'Detection and Epidemiology of Pospiviroids (DEP2)' was started and part of the activity and results are reported here.

Materials and methods

Plant material and viroid isolates

75 plants of tomato cv Roma and 90 of cv Minibel, obtained from healthy seeds, were infected by mechanical inoculation at flowering stage, using the same isolates of four

pospiviroids: CEVd (GenBank Accession No EU094208), TASVd (GenBank Accession No EF192395), CLVd (GenBank Accession No AY372392) and PSTVd (GenBank Accession No HQ452413;) in Italy (CRA-PAV laboratory) and Lithuania (State Plant Service laboratory). The CEVd, CLVd and TASVd isolates were kindly provided by Dr. J.Th.J. Verhoeven, the PSTVd isolate belongs to the CRA-PAV collection.

Plant inoculation, seed extraction and seedling growing

Sap for inoculum was prepared by grinding viroid-infected leaves in sterilised buffer (0.1M phosphate, pH 7.2) and celite. Two-three leaves, just below the flowers, have been rubbed with the preparation containing the viroidal RNA, the buffer and the celite; then the leaves were rinsed. From infected plants, fruits were collected and analysed. Seeds from infected fruits were fermented in their own flesh, then rinsed and dried. Most of the seeds has been treated with a disinfectant (solution at 2% bleach), while a smaller part was not treated, in order to verify if the eventual presence of pospiviroids was internal or external. The seeds were then either directly analysed or sown, in order to produce seedlings that were analysed from the second true leaf stage up to three months old, in different laboratories. All the experiments were carried out in glasshouses or growing chambers at 20-25°C and 12-14 hour photoperiods.

RNA extraction and PCR

For RNA extractions, leaves and fruits were grinded in liquid nitrogen using mortar and pestle; seeds were grinded using Bioreba (Bioreba, Switzerland) bags and 2 ml of sterilized water. RNA was extracted using Spectrum plant RNA extraction kit (Sigma-Aldrich, Saint Louis, MO, USA) according to manufacturer instructions. Plants, fruits, seeds and seedlings were analysed using different diagnostic protocols according to the pospiviroid species and the different laboratories involved. Specifically, for TASVd, protocols according to Botermans *et al.* (2013), Verhoeven *et al.*, (2004), Verhoeven *et al.*, (2008), Luigi *et al.*, (2014) or EPPO, (2002) were used; for CEVd, protocols according to Verhoeven *et al.*, (2004), Verhoeven *et al.*, (2008), Luigi *et al.*, (2014) or EPPO, (2002) were used; for PSTVd, protocol according to Di Serio, (2007) was used, and for CLVd protocols according to Spieker, (1996) or Luigi *et al.*, (2014) were used.

Results

After inoculation, all 75 'Roma' and 86 out of 90 'Minibel' tomato plants appeared infected with respective pospiviroid. TASVd was diagnosed in 60 plants, CLVd in 44, CEVd in 42 and PSTVd in 15 (Table 2). The plants of 'Minibel' have produced much more seeds than those of 'Roma' cultivar: about 7000 seeds versus 300 (Table 2). A part of seeds, about 1000, was analysed directly either singularly or pooled, with or without disinfection, and 100% of the samples have proved to be infected by the respective pospiviroid (Figure 3). About 1000 seeds were not used, whereas the remaining seeds were sown, and 4701 seedlings were obtained (242 from 'Roma' and 4459 from 'Minibel'). The seedlings were analysed, starting from the second true leaf stage up to three months of age, and all seedlings were always negative for pospiviroid infection (Table 2).

Table 2: Viroid species used for the inoculations, number of inoculated and infected plants, seeds produced from infected plants, analysed and infected seedlings. Summary of the plants/ seeds infected and analysed according to the different Pospiviroid species and tomato variety used.

	Viroid species	Infected plants/ inoculated plants	Seeds produced	Seedlings infected/ analysed
Roma	TASVd	30/30	160	0/114
	CEVd	15/15	68	0/54
	CLVd	15/15	60	0/52
	PSTVd	15/15	44	0/22
	Total	75/75	332	0/242
Minibel	TASVd	30/30	2000[a]	0/1118
	CEVd	27/30	2000[a]	0/1795
	CLVd	29/30	3000[a]	0/1546
	Total	86/90	7000[a]	0/4459

[a] Estimated value obtained according the total weight of the seeds collected

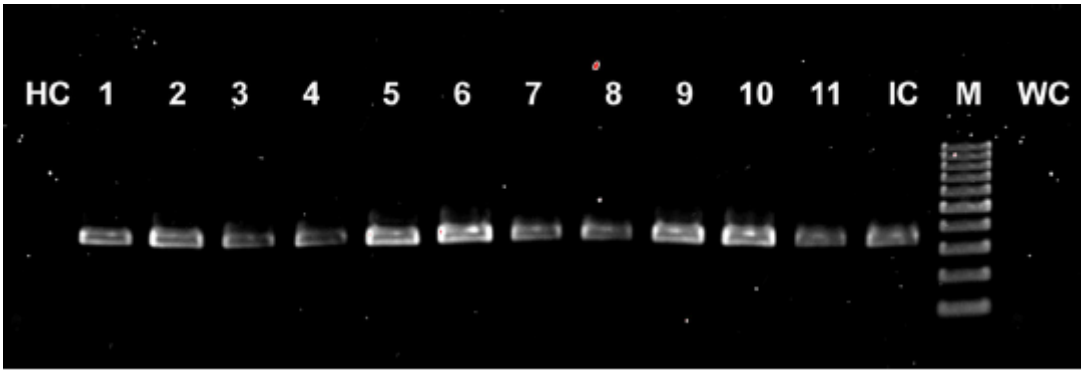

Figure 3: Results of singularly analyzed seeds, by RT-PCR (Luigi *et al.* 2014), infected by TCDVd. Lane HC=healthy control; lanes 1–11=seeds obtained from fruits harvested from TCDVd-infected tomato plants; lane IC=infected control; lane M=DNA size markers (Gene RulerTM100 bp DNA Ladder, Fermentas, Vilnius, Lithuania); lane WC=water control

Discussion

The strategy to inoculate plants at flowering stage has been very effective. It allowed for the successful infection of almost all plants (more than 97.5%), which, despite their infection, were able to produce fruits with high efficiency. In fact, all fruits tested positive for the relevant pospiviroid (data not shown).

Considering the high number of seedlings analysed, it seems noticeable that the tested strain of TASVd, CEVd, CLVd and PSTVd are not transmitted by seeds, at least in the tomato varieties 'Minibel' and 'Roma'. Moreover, the results recently obtained in the framework of the EU Project 'TESTA', confirmed our data; specifically, from 100% PSTVd and TCDVd infected seeds, none of, 1000 and 4000 of obtained seedlings, respectively, were found to be positive (Koenraadt, personal communication). Nonetheless these data do not fully clarify the actual role of seeds in the transmission of pospiviroids in tomato, in fact some uncertainty remains analysing the results of some studies which showed successful seeds transmission, as reported before.

Although there is no direct evidence for the strain/variety dependent viroid transmission through the seed, Singh and Dilworth (2009) recorded 26.7–80% seed transmission rate for a TCDVd strain (GenBank Accession No. EU62557) in tomato (cv. Sheyenne), but no seed transmission as well as infection of ovaries with another TCDVd strain (GenBank Accession No. AB329668) was revealed by Matsushita *et al.* (2011) in tomato (cv. Rutgers). Of course minor modifications in pospiviroid genome sequences or in plant varieties cannot solely explain all the variations of differences in seed transmission rates. Obviously, other factors also can affect the transmission of pospiviroids through the seed. There are some evidences that environmental conditions can be of importance as well. Recently the influence of crossing time on the rate of CSVd transmission through the seed in Chrysanthemum was demonstrated. Crosses made in December, showed a lower seed transmission rate (1.5%) compared to crosses performed in May (66.7%; Chung and Pak, 2008). It is interesting to note, that unusually high rate (80%) of TASVd transmission was obtained for the seeds collected from infected tomato, grown under high temperature conditions (22/35 ^{o}C night/day) (Antignus *et al.*, 2007). However, this is not an absolute rule, because another pospiviroid, *Pepper chat fruit viroid* (PCFVd) showed a relative high percentage (19%) of seed transmission in pepper plants, which were grown under quite moderate temperature conditions (20/25 ^{o}C) (Verhoeven *et al.*, 2009). The physiological stage of the plants at the time of infection could also play a role as suggested by the high transmission rate (80%) obtained by Singh and Dilworth (2009) who inoculated their plants at the seedling stage rather than in the flowering stage as in our experiments. Likewise, the time lapse between inoculation and seed harvesting could be of importance, as it was shown in the case of *Pepino mosaic virus* (PepMV) for which tomato seedling infection rate increased with post inoculation time (Hanssen *et al.*, 2010). Time could give the chance for pathogen particles to accumulate in threshold levels in right place and time for transmission, as shown with *Pea seed-borne mosaic virus* (PSbMV; Roberts *et al.*, 2003). Despite the high seed transmission rate sometimes reported in the literature, our results together with the results of the other authors show, that when the transmission by seeds is assessed using a very rigorous and controlled greenhouse experimental model, even if seeds themselves tested positive for pospiviroids, their transmission through tomato seeds is negligible. Nevertheless, significant differences in pospiviroid seed transmission rates sometimes observed most likely are determined by different genetic, physiological and environmental factors.

WP4 Ring-test for the detection of pospiviroids in tomato seeds (Lead: T. Olivier)

Main Partners: T. Olivier, K. De Jonghe, S. Grausgruber-Gröger, M. Virscek Marn, K. Petrutis and V. Gusina, F. Faggioli, V. Sveikauskas, P. Gentit.

Different methods are currently available for the generic detection of pospiviroids (Monger *et al.*, 2010 ; Torchetti *et al.*, 2012; Botermans *et al.*, 2013; Luigi *et al.*, 2014; Olivier *et al.*, 2014), but in Europe and at the EPPO level, still no official method has been designated. Therefore, the National Reference Laboratories have to carry out extensive performance comparisons of available methods in order to select the most efficient one on host plant leaves as well as on tomato seeds.

This work presents the results of an inter-laboratory comparison of four methods consisting of a pair of RT-PCR (ANSES 1-2; ANSES, 2013), a pair of RT-qPCR (Botermans 1-2; Botermans *et al.*, 2013) and two single RT-PCR methods: Luigi (Luigi *et al.*, 2014) and Olivier (Olivier *et al.*, 2014), which were tested on twenty-two tomato leaf and seed samples and their respective 100-fold water dilutions. The evaluation of these methods was performed using the performance criteria defined in the EPPO pest management standards PM 7/76 (2) and PM 7/98 (2) (EPPO, 2010; EPPO, 2014) and a statistical discrimination of methods using generalized linear models.

Materials and methods

Samples

Twenty-two samples prepared according to the same procedure and tested for their homogeneity were coded and randomly distributed to the eight laboratories to ensure a blind test. Thirteen samples consisted of finely chopped tomato leaves and nine samples consisted of crushed tomato seeds which were either healthy or harvested from mechanically inoculated plants. The samples were freeze dried, further reduced into powder using a mortar and pestle and liquid nitrogen when needed. In order to assess the analytical sensitivity of methods, three tomato leaf and four tomato seed samples at 25-fold dilutions in their respective matrix were prepared on a calibrated analytical balance and were included in the twenty-two sample set. Samples were conditioned in 2 mL microtubes and thoroughly mixed before sending. Moreover, labs were asked to dilute each of the twenty-two sample RNA extracts 100-fold in water (2 µL of RNA extract in 198 µL of RNase-free water). Four dilution levels: 1, 25, 100 and 2500-fold were therefore present in the comparison and in total.; Forty-four samples were tested in each laboratory for as much different protocols as possible (Table 3).

Because of the high number of protocols to be tested and because of the difficulty to produce infected seeds, only four out of the ten known pospiviroid species were included in the test: *Potato spindle tuber viroid* (PSTVd), *Tomato apical stunt viroid* (TASVd), *Citrus exocortis viroid* (CEVd) and *Columnea latent viroid* (CLVd). The choice of these species was guided by the regulatory status of the species, their prevalence in European ornamentals (EFSA, 2011), their aggressiveness towards tomatoes and the ability to test the analytical specificity of the selected methods. The selected pospiviroid isolates are presented in Table 3. Out of the nine seed samples, two were co-infected with CLVd and CEVd and two were co-

infected with CLVd and TASVd (Table 3). The homogeneity of samples was tested on five extra replicates prior to the sending using Olivier protocol (Olivier et al., 2014) and by placing the repetitions from one sample next to each other on agarose gels. To increase the stability of samples as much as possible, freeze dried material was used.

Table 3: Isolates and matrix/dilution combinations used in the inter-laboratory test

Status	Sequence id	Matrix	Dilution			
			Undiluted	1/25 [a]	1/100 [b]	1/2500 [a,b]
Negative control	-	Leaves	v		v	
Negative control	-	Seeds	v		v	
CEVd	HG739073	Leaves	v		v	
CEVd and CLVd	HG739076 and FM995507	Seeds	v	v	v	v
CLVd	AY372392	Leaves	v		v	
CLVd	FM995507	Leaves	v		v	
CLVd	FM995507	Seeds	v	v	v	v
PSTVd	FM998542	Leaves	v		v	
PSTVd	AY372400	Leaves	v	v	v	v
PSTVd	X17268	Leaves	v		v	
PSTVd	FM998542	Leaves	v	v	v	v
PSTVd	FM998542	Seeds	v	v	v	v
TASVd	HG739072	Leaves	v		v	
TASVd	HG739071	Leaves	v	v	v	v
TASVd and CLVd	HG739072 and FM995507	Seeds	v	v	v	v

[a] dilution in the corresponding matrix

[b] dilution in water

RT-PCR based methods

Each laboratory was invited to apply four different methods on the twenty-two RNA samples and their 100-fold dilutions. Two methods consisted of a pair of either end-point RT-PCR (ANSES 1-2) or real time RT-PCR (Botermans 1- 2) protocols (because a separate (real-time) RT-PCR for the detection of CLVd is required for these methods) and the two other methods consisted of two single end-point RT-PCR protocols (Luigi and Olivier).

To allow the use of extraction and PCR kits available in participating laboratories, this step was not considered as being part of the protocols. Therefore in this study protocols consisted of the following parameters specified in Table 4: primer and probe composition and concentration, PCR reaction volumes, quantity of RNA extract per reaction and (real time) RT-PCR cycling programs.

However, in order to be able to evaluate potential efficacy differences between extraction and RT-PCR kits, the kit choice was limited to ensure that at least two labs use the same combination of extraction and RT-PCR kits for the four end-point PCR protocols. The use of real time RT-PCR reagent kits was left to the appraisal of the laboratories. The kits used by each participating laboratory are presented in Table 5.

To minimize the variability between labs and to comply as much as possible with the original published procedures (ANSES, 2013; Botermans *et al.*, 2013; Luigi *et al.*, 2014; Olivier *et al.*, 2014), the instructions for each procedure were described in a common document.

Buffer and enzyme concentrations were used as recommended by the kit manufacturers, dNTPs were added at 200µM each and RNase inhibitor or DTT was not used.

Expected amplicon sizes were as follows: ANSES 1: around 200 bp for pospiviroids other than CLVd; ANSES 2: 359 bp for CLVd; Luigi: around 300 bp for all pospiviroids; Olivier: 359 bp for CLVd and around 200 bp for all other pospiviroids.

For real time RT-PCRs, the cut-off value was set at 32 Ct. Samples with Cts greater or equal to 32 and lower or equal to 37 were considered as undetermined and their final status (positive or negative) was left to the appraisal of the laboratories according to their available validation data. Samples with Ct above 37 were considered as negative following the recommendations of the corresponding publication (Botermans *et al.*, 2013).

Table 4: RT-PCR mix compositions and (real time) RT-PCR cycles of each protocol of the four tested methods

Primer/Probe names	Reference	Protocol/ Method code	Primer final concentration	Reaction Volume	RNA extract volume	Denaturation	RT step	PCR cycles	Final Elongation
TCR-F 1-1; TCR-F 1-3; TCR-F 1-4; TCR-F PCFVd; TCR-F IrVd; TR-R1; TR-R CEVd; TR-R6	Botermans et al., 2013	Botermans 1	0.3 µM	25 µL	2 µL		48°C for 30 min	40 cycles at 95°C for 15 s, 60°C for 1 min	
pUCCR			0.1 µM						
CLVd-F; CLVd-F2; CLVd-R		Botermans 2	0.3 µM						
CLVd-P			0.1 µM						
Pospi1deg-FW; Pospi1s-RE pCLV4s	Olivier et al., 2014	Olivier	0.24 µM		1 µL		50°C for 30 min	39 cycles at 94°C for 30 s, 60°C for 45 s and 72°C for 45 s	72°C for 2 min
POP1-FW	Luigi et al., 2014	Luigi	0.3 µM	50 µL	2 µL		50°C for 30 min	35 cycles at 95°C for 30 s, 62°C for 30 s and 72°C for 1 min	72°C for 7 min
POP3-FW			0.2 µM						
POP-REV			0.4 µM						
Pospi 1-FW	Verhoeven et al., 2004	ANSES 1	1 µM	25 µL	1 µL		50°C for 30 min	15 cycles at 95°C for 30 s, 62°C for 90 s and 72°C for 45 s and 30 cycles at 95°C for 30 s, 59°C for 90 s and 72°C for 45 s	72°C for 7 min
Pospi 1-RE									
pCLV4	Spieker, 1996	ANSES 2	1 µM	50 µL	1 µL	95 °C for 3 min, 4°C for 10 min	50°C for 30 min	30 cycles at 95°C for 1 min, 60°C for 1 min and 72°C for 1 min	72°C for 5 min
pCLV4R									

Table 5: Use of extraction and (real time) RT-PCR kits in the eight participating laboratories

Lab number	Extraction kit	RT-PCR kit	Real Time RT-PCR kit
1	Rneasy Plant Mini Kit (Qiagen)	SuperScript® One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Life technologies)	Master Mix (Applied Biosystem)
3			AgPath-ID™ One-Step RT-PCR (Life technologies)
7			SuperScript® III Platinum® One-Step Quantitative RT-PCR System (Invitrogen)
2		OneStep RT-PCR Kit (Qiagen)	AgPath-ID™ One-Step RT-PCR (Life technologies)
4			QuantiFast Probe RT-PCR Plus Kit (Qiagen)
6			M-MuLV Reverse Transcriptase (Thermo Scientific) and AmpliTaq Gold® Polymerase (Life Technologies).
5	Spectrum plant total RNA Kit (Sigma-Aldrich)	Titan One Tube RT-PCR System (Roche)	Precision OneStep qRT-PCR MasterMix (Primerdesign)
8			One step qRT-PCR MasterMix No ROX (Eurogentec)

Pospiviroid species identification

To identify the RT-PCR detected species, each laboratory was invited to sequence the amplicons of undiluted samples. *Pospiviroid* species were then identified using BLAST search against the NCBI viroid reference sequences. Alternatively, the amplicons obtained with Luigi protocol were digested with either Alu I or Sau 96I for 60 min (FastDigest enzymes) or for 12 hours (conventional restriction enzymes) at 37°C and separated on 5% polyacrylamide gels. Species identifications were performed by comparing the restriction band patterns with those available in the reference publication (Luigi *et al.*, 2014).

Evaluation of performance criteria and statistical analysis

The status of each sample (true positive or true negative) was determined according to the data available and the status of each of the 1980 results available (true/false positive or true/false negative) was assigned accordingly.

These latter statuses were used to determine diagnostic sensitivity, diagnostic specificity and relative accuracy of the individual protocols and the detection methods. The definitions of these performance criteria were taken from PM 7/76 (2) and PM 7/98 (2) standards (EPPO, 2010; EPPO, 2014) and are given in equations 1, 2 and 3 respectively:

Diagnostic sensitivity = nb of true positives / (nb of true positives + nb of false negatives) (1)

Diagnostic specificity = nb of true negatives / (nb of true negatives + nb of false positives) (2)

Relative accuracy = (nb of true positives + nb of true negatives) / (nb of true positives + nb of false positives + nb of true negatives + nb of false negatives) (3)

Because CLVd requires a specific (real-time) RT-PCR reaction in two methods and because Luigi's method is not able to distinguish this species without additional analysis, different results were taken into account to compare the three aforementioned performance criteria without bias.

For the comparison of the protocols dedicated to the detection of the same pospiviroid species, the results of singly infected samples were analyzed. ANSES 2, Botermans 2, Olivier and Luigi protocols were thus compared for CLVd singly infected and negative samples while ANSES 1, Botermans 1, Olivier and Luigi were compared for the samples singly infected with PSTVd, CEVd or TASVd and negative samples.

For the comparison of the four methods, the results of double (real time) RT-PCR methods (ANSES 1-2 and Botermans 1-2) and the results of the double band method (Olivier) were combined to allow comparison with the results of Luigi's method. For a given sample, the combination was carried out as follows: two correct results gave a correct combined result; one or two incorrect (false positive/negative) gave an incorrect combined result. However, for the eight co-infected samples, only the results for pospiviroids other than CLVd were taken into account to minimize the bias between Luigi's method and the three other detection methods.

Prior to statistical analyses, several laboratory/method combinations were withdrawn from the datasets. Along with not performed combinations (lab 5/ANSES 2; lab 6/Botermans 1; lab 6/Botermans 2), two experiments not complying with the common protocols of table 4 (lab 4/ Luigi's and Olivier's methods) because of higher primer concentrations were not taken into account.

To compare the three above-mentioned performance criteria between protocols and methods, mixed logit models or linear mixed models after an empirical logit transformation of the dependent variables were built on the corresponding dataset following the equation (4).

$PC_{i,j} = \beta_0 + \beta_1[\text{method/protocol}] + \gamma_j + \varepsilon_{i,j}$ (4)

where:

$PC_{i,j}$ is the performance criteria (diagnostic sensitivity, diagnostic specificity or relative accuracy) expressed in percent for the sample i and the laboratory j.

β_0 is the intercept

β_1 is the linear slope for the factor [method/protocol]

γ_j the random effect coefficient for laboratory j

$\varepsilon_{i,j}$ is the error for sample i for laboratory j

To assess the difference of accuracy between the two extraction kits and the three combinations of extraction and RT-PCR kits, these factors were alternatively added in the model following the equation 5. In this case, only conventional RT-PCR results were taken into account.

$$Accuracy_{i,j} = \beta_0 + \beta_1[method/protocol] + \beta_2[extraction\ (and\ PCR)\ kit(s)] + \gamma_j + \varepsilon_{i,j} \quad (5)$$

where:

$Accuracy_{i,j}$ is the relative accuracy expressed in percent for the sample i and the laboratory j.

β_2 is the linear slope for the factor [extraction (and PCR) kit(s)]

When the comparison of laboratories was needed, the laboratory factor was considered as fixed in the model according to equation 6.

$$Accuracy_{i,j} = \beta_0 + \beta_1[method/protocol] + \beta_2[laboratory] + \varepsilon_{i,j} \quad (6)$$

where:

β_2 is the linear slope for the factor [laboratory]

Along with the three above mentioned criteria, the analytical sensitivity and analytical specificity wereas also assessed. Analytical sensitivity was assessed by comparing the relative accuracy of each method at the four different dilutions (1, 25, 100 and 2500-fold). For each dilution, the model described in equation 4 was applied. Analytical specificity was evaluated on the basis of non-diluted samples for each strain/matrix combination.

The statistical significance of differences between factors' levels (i.e. protocols, methods, laboratory and extraction and/or RT-PCR kits) was computed by Tukey's multiple comparison test performed on the corresponding linear models.

The reproducibility was assessed by computing the Fleiss Kappa index (Fleiss *et al.*, 2003) for each detection method.

The repeatability was not assessed because no systematic repetition of a same sample/PCR method combination was performed by the participating laboratories, except for the homogeneity tests.

The statistical analyses were computed with R version 2.15.3 and the packages lme4, irr and multcomp (Hothorn *et al.*, 2008; Gamer *et al.*, 2012; Bates *et al.*, 2013; R Core Team 2013).

Results

Performance criteria

Table 6 shows the laboratory relative accuracy obtained for each individual protocol and method. As mentioned in the section 'Materials and methods', because two methods requires a specific (real-time) RT-PCR reaction for the detection of CLVd and because Luigi's method is not able to distinguish this species without additional analysis, different results were taken into account to compare the three aforementioned performance criteria without bias.

Table 6: Laboratory mean relative accuracy (expressed in percent) obtained for each individual protocol and method.

Laboratory	Pospiviroid species other than CLVd			CLVd			Pospiviroids			
	ANSES1	Botermans1	Olivier (other than CLVd band)	ANSES2	Botermans2	Olivier (CLVd band)	ANSES1-2	Botermans1-2	Luigi	Olivier
1	79.5	61.4	72.7	68.2	63.6	70.5	68.2	43.2	50	63.6
2	79.5	84.1	83.3	63.6	50	78.6	70.5	40.9	28.6	81
3	97.7	88.6	63.6	81.8	70.5	63.6	93.2	63.6	38.6	47.7
4	90.9	75	97.7 [a]	84.1	79.5	88.6 [a]	86.4	63.6	77.3 [a]	97.7 [a]
5	63.6	75	79.5	na	86.4	63.6	na	70.5	29.5	61.4
6	86.4	na	77.3	72.7	na	72.7	77.3	na	54.5	68.2
7	93.2	86.4	75	77.3	86.4	72.7	86.4	81.8	29.5	65.9
8	84.1	70.5	75	72.7	90.9	72.7	77.3	68.2	40.9	65.9
mean	84.4	77.3	78	74.3	75.3	72.9	79.9	61.7	43.6	68.9

na: data not available

[a] : value not taken into account in Tables 7 to 10

As shown in Table 7, no significant difference was observed between the accuracies of ANSES 2, Botermans 2, Luigi and Olivier protocol (CLVd band) dedicated to CLVd detection. However the four evaluated protocols differed in terms of diagnostic sensitivity and diagnostic specificity. ANSES 2 and Olivier protocols did not result in false positives (i.e. 100 % diagnostic specificity) while Botermans 2 method gave significantly less false negative (i.e. higher diagnostic sensitivity) than ANSES 2, Luigi and Olivier (CLVd band) protocols.

Table 7: Relative accuracy, diagnostic specificity and diagnostic sensitivity obtained for healthy samples or CLVd singly infected samples.

	Number of samples/Lab	ANSES 2			Botermans 2			Luigi			Olivier (CLVd band)		
		Mean	Sd	Group [a]	Mean	Sd	Group [a]	Mean	Sd	Group [a]	Mean	Sd	Group [a]
Accuracy	12	67.9	17	a	84.5	11.2	a	78.6	10.6	a	69	10.4	a
Specificity		100	0	a	67.9	34.5	b	89.3	19.7	b	100	0	a
Sensitivity		51.8	25.4	b	92.9	6.7	a	73.2	13.4	b	53.6	15.7	b

[a] Statistical group according to Tukey's test are mentioned for each performance criterion/protocol combination

The second analysis was performed on samples either healthy or singly infected by pospiviroid species other than CLVd to compare ANSES 1, Botermans 1, Luigi and Olivier (pospiviroids other than CLVd band) (real time) RT-PCR protocols.

Table 8 shows that there were significant differences between the relative accuracies of (real time) RT-PCR protocols dedicated to the detection of pospiviroid species other than CLVd. Olivier (pospiviroids other than CLVd band) method was significantly less accurate than ANSES 1 protocol and Luigi's method was significantly less accurate than the other three protocols. The analysis showed that the difference was due to diagnostic sensitivity. No significant differences in diagnostic specificity between tested protocols/methods were found.

The influence of matrix (seeds or leaves) was investigated by performing statistical analyses on three datasets: all samples, seed samples or leaf samples.

Table 8: Relative accuracy, diagnostic specificity and diagnostic sensitivity obtained for healthy samples or samples singly infected with a pospiviroid species other than CLVd.

	Number of samples/Lab	ANSES 1			Botermans 1			Luigi			Olivier (PSTVd group band)		
		Mean	Sd	Group [a]	Mean	Sd	Group [a]	Mean	Sd	Group [a]	Mean	Sd	Group [a]
Accuracy	28	88.4	10.6	a	79.6	10.9	ab	31.4	11.2	c	77.5	9.5	b
Specificity		87.5	26.7	a	78.6	22.5	a	89.3	19.7	a	89.3	19.7	a
Sensitivity		88.5	13.3	a	79.8	13.1	ab	21.5	15.8	c	75.5	10.8	b

[a] Statistical group according to Tukey's test are mentioned for each performance criterion/protocol combination

When combined results of the four methods were compared for their relative accuracy for the twenty-two RNA isolates and their 100-fold dilutions of the inter-laboratory comparison, three statistical groups appeared: ANSES 1-2 method gave significantly more accurate results than Botermans 1-2 and Olivier's methods, these two latter methods giving significantly more accurate results than Luigi's method (Table 9 top).

Results obtained for seed samples showed a similar pattern as for the whole set of samples. Botermans 1-2 method, however, statistically grouped with ANSES 1-2 and Olivier's method, the latter being not significantly different from Luigi's method (Table 9 middle).

For leaf samples, in terms of accuracy, ANSES 1-2 and Olivier's methods scored best, followed by Botermans 1-2 method (not significantly different from Olivier's method) which itself was significantly better than Luigi's method (Table 9 bottom).

For the three latter analyses on matrix effect, diagnostic sensitivity was, especially for Luigi's and Olivier's methods, the limiting performance criterion (Table 9).

Table 9: Relative accuracy, diagnostic specificity and diagnostic sensitivity obtained for either all samples, seed samples or leaf samples for the four tested methods.

		Number of samples/ Lab	ANSES 1-2			Botermans 1-2			Luigi			Olivier		
			Mean	Sd	Group [a]	Mean	Sd	Group [a]	Mean	Sd	Group [a]	Mean	Sd	Group [a]
All samples														
	Accuracy		79.9	9.1	a	61.7	14.8	b	38.8	10.4	c	64.8	9.8	b
	Specificity	44	85.7	28.3	ab	60.7	31.8	b	89.3	19.7	a	89.3	19.7	a
	Sensitivity		79.3	8.5	a	61.8	14.3	b	33.7	13.1	c	62.4	11.6	b
Seed samples														
			ANSES 1-2			Botermans 1-2			Luigi			Olivier		
			Mean	Sd	Group [a]	Mean	Sd	Group [a]	Mean	Sd	Group [a]	Mean	Sd	Group [a]
	Accuracy		68.3	10.5	a	54.8	14.1	ab	35.3	11.9	c	42.2	12.5	bc
	Specificity	18	92.9	18.9	a	57.1	34.5	b	100	0	a	85.7	24.4	a
	Sensitivity		65.2	11.3	a	54.5	16.8	a	27	13.5	b	36.7	16.5	b
Leaf samples														
			ANSES 1-2			Botermans 1-2			Luigi			Olivier		
			Mean	Sd	Group [a]	Mean	Sd	Group [a]	Mean	Sd	Group [a]	Mean	Sd	Group [a]
	Accuracy		89.7	9.9	a	66.5	22.2	b	41.2	12.3	c	80.2	13.8	ab
	Specificity	26	78.6	39.3	a	64.3	47.6	a	78.6	39.3	a	92.8	18.9	a
	Sensitivity		88.7	8.9	a	66.7	20.7	b	38.1	15.9	c	79.2	14.4	ab

[a] Statistical group according to Tukey's test are mentioned for each performance criterion/method combination

Analytical specificity showed that all pospiviroid isolates are detected by all protocols. However, limited amplification was obtained with Luigi's protocol for the three PSTVd isolates on leaf and seed samples and for one of the TASVd isolates provided only as leaf samples. A weak amplification was also observed for CLVd isolates on seeds both for ANSES 2 and Olivier's protocols (data not shown).

Analytical sensitivity was assessed using the relative accuracy of the four tested methods for each of the four dilutions: 1, 25, 100 and 2500-fold (Table 10). Overall, for leaf samples, ANSES 1-2 and Olivier's methods gave the best relative accuracy at each of the four levels of dilution. Botermans 1-2 gave a lower accuracy at 25-fold dilution whereas Luigi's method showed a significantly lower accuracy at all dilution levels. For seed samples, the accuracies

of all four detection methods were not significantly different for undiluted samples, Luigi's and Olivier's methods showed a significantly lower accuracy at 25 and 100-fold dilutions. The relative accuracy at 2500-fold was very low for each of the four methods.

Table 10: Analytical sensitivity investigated through relative accuracies obtained for the four tested methods at the four dilution levels.

	Number of samples/ Lab	ANSES 1-2			Botermans 1-2			Luigi			Olivier		
Seed samples		Mean	Sd	Group [c]	Mean	Sd	Group [c]	Mean	Sd	Group [c]	Mean	Sd	Group [c]
1	5	83	21	a	69	30	a	63	18	a	71	11	a
25 [a]	4	82	12	a	61	24	ab	33	28	b	38	34	b
100 [b]	5	77	8	a	63	31	ab	37	14	b	43	29	b
2500 [b]	4	25	32	a	21	37	a	0	0	a	8	14	a
Leaf samples													
1	10	87	17	a	70	32	ab	54	17	b	86	18	a
25 [a]	3	90	16	a	43	37	b	10	16	b	90	16	a
100 [b]	10	89	11	a	77	21	a	49	16	b	79	17	a
2500 [b]	3	86	18	a	43	32	ab	5	13	b	57	32	a

[a] dilution in the corresponding matrix

[b] dilution in water

[c] Statistical group according to Tukey's test are mentioned for each performance criterion/method combination

Extraction and (real time) RT-PCR kit comparison

No significant difference was observed between the two extraction kits ($P = 0.9$) or between the three extraction/RT-PCR kit combinations ($P \geq 0.39$) when the results of the four (real time) RT-PCR tested methods or of the three RT-PCR tested protocols were respectively considered (data not shown).

Pospiviroid species identification

A total of 169 amplicons were sequenced and allowed a correct identification of pospiviroid species in 91.1 % of cases, a wrong assignment in 8.3 % and an undetermined result in 0.6 %. Wrong assignments in sequencing were probably due to contamination with positive controls, considering the best hits in blast for these sequences and the fact that this errors came from only one laboratory.

Twelve amplicons from Luigi protocol were digested with endonuclease. The restriction of these amplicons allowed a correct assignment for 83.3 %, led to a wrong assignment in 8.3 % and did not allow any identification in 8.3 % of cases. Fragmentary data did not allow conducting a statistical analysis to compare both identification methods.

Variability between laboratories and reproducibility

Because the laboratory number four obtained the best relative accuracy for Luigi and Olivier methods using 1µM of each primer rather than the lower recommended concentrations (Table 4), reason why these lab/method combinations were initially withdrawn from the dataset, the significance of differences between this laboratory and the seven other laboratories was evaluated. Tukey's test showed that relative accuracy of the laboratory number 4 for Luigi

and Olivier methods was significantly higher compared to each of the seven other laboratories ($P \leq 0.002$) while these latter were not significantly different from each other ($P \geq 0.35$).

The calculation of Fleiss Kappa indexes for the relative accuracy allowed to compare the reproducibility of the four tested methods and to obtain the following ranking: Luigi, ANSES 1-2, Olivier and Botermans 1-2 with the indexes of 0.6 (moderate agreement), 0.368 and 0.303 (fair agreement) and 0.132 (slight agreement) respectively.

Discussion

Although participating laboratories did not use the same extraction and real time RT-PCR kits and despite the fact that two-thirds of samples were diluted at least 25-fold and more than a quarter of samples were close to the detection limit of the six tested protocols, the values of the performance criteria obtained were satisfactory and allowed a comparison of the different pospiviroid detection methods.

Method ANSES 1-2, derived from Verhoeven *et al.* (2004) and Spieker (1996) provides the best results for almost all performance criteria tested, except for reproducibility and diagnostic sensitivity of ANSES 2 for CLVd infected samples.

Olivier 1-2 method also derived from Verhoeven *et al.* (2004) and Spieker (1996) but consisting of a single RT-PCR, allows an accurate detection of pospiviroid in leaf samples. Nevertheless, a general lack of diagnostic sensitivity in tomato seed samples, especially for CLVd infected samples, decreased its accuracy. However, when the concentration of primers for this method was raised to 1 µM, the best combined relative accuracy of the inter-laboratory comparison (97.7 %) was obtained (Table 6).

Botermans 1-2 method showed relative accuracy not significantly different from ANSES 1-2 for seed samples, although 13.5 % lower (Table 9 middle), but significantly lower for leaf samples (Table 9 bottom). Even if Botermans 1 and 2 protocols grouped with the most sensitive protocols/methods individually (Table 7 and Table 8) the generally accepted advantage of real time RT-PCR over conventional RT-PCR regarding the sensitivity could not be demonstrated when the results of both protocols were combined (Table 9 top). The analysis of raw data showed that the substantial loss of relative accuracy between the two individual protocols and the combined Botermans 1-2 method (Table 6) was mainly due to the non-overlapping conjunction of false negatives of Botermans 1 protocol and false positives of Botermans 2 protocol.

Luigi method was significantly less accurate than other protocols or methods due to its lack of specificity against PSTVd isolates and one of the two TASVd isolates. This problem was probably linked, at least for PSTVd isolates, to mismatches between the last seven nucleotides of POP1-FW primer 5' end and the targeted region in the genome of the concerned isolates. It should be stressed, however, that Luigi method was the only one allowing the confirmation of CLVd in co-infected samples through the sequencing of the amplicons. Again, because Luigi protocol does not provide a distinct band for CLVd it was not possible to fully compare the different performance criteria for doubly infected samples. A slight bias is then possible when the protocols or methods were compared with one another for seed samples (Tables 9 and 10).

Although a trend of decreased accuracy for seed samples was observed, it was not possible to make conclusions about the matrix effect (leaves or seeds), since different pospiviroid isolates were used for the individual matrices and pospiviroid concentrations in the samples were unknown.

The reproducibility of methods was rather low ranging from slight agreement (Botermans 1-2) to fair agreement (ANSES 1-2 and Olivier) and moderate agreement (Luigi). The general

lack of reproducibility can be explained by the great proportion of samples which were close to the limit of detection of the different protocols/methods and which were consequently either positive or negative depending on the laboratory.

Considering that sequencing data were too fragmentary and RFLP data were only provided by one lab and only for Luigi method, no statistical analysis could be performed to compare both identification methods. However, because RFLP identification was linked to Luigi method, which proved to be less accurate than other tested methods and because sequencing proved to be effective and allowed detecting PCR contaminations, sequencing should be preferred. The capacity of sequencing to correctly assign species, and thus distinguish between regulated and non-regulated species, as well as genotyping the isolates involved in outbreaks should also be considered as an advantage of RT-PCR over real time RT-PCR.

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WP5 Evaluation of different physical and chemical pospiviroid inactivation procedures: (Lead: T.Olivier)

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As pospiviroid affected plants are often cultivated in greenhouses or are subjected to a lot of handling, effective disinfection measures to clean the cultivating tools, machineries and facilities have to be found and approved by the plant protection authorities (EFSA, 2011).
Although bleach proved to be an effective disinfectant against different pospiviroids (Garnsey and Jones, 1967; Garnsey and Whidden, 1971; Singh *et al.*, 1989; Matsuura *et al.*, 2010) only the commercial disinfectant, Menno Florades®, based on benzoic acid had been tested against viroids (Timmermann *et al.*, 2001). Many European authorities thus approved the Menno Florades® as well as MENNO® clean, containing the same active compound at the same concentration as Menno Florades®, for disinfection of viroid contaminated surfaces. In this study, *Potato spindle tuber viroid* (PSTVd), the type species of the genus *Pospiviroid* and probably the most studied viroid species, was chosen to assess five commercial virucids. Two different experimental setups were used to test and discriminate the disinfectants according to their effectiveness. Firstly, the products were tested on dried droplets of PSTVd infected tomato plant sap deposited on glass panes at the concentration and contact time recommended

by the manufacturer. Secondly, the products were evaluated on aqueous solutions of fresh saps at the manufacturer's recommended concentration. For this second experimental setup, products were assessed for their effectiveness at the manufacturer's recommended minimum contact time and for their relative effectiveness at a same contact time of 15 min. The aim of this study was to assess and compare the effectiveness of 5 commercial disinfectants: Virkon®, Hyprelva™ SL, Jet 5 ®, MENNO® clean and Virocid™ against PSTVd through an interlaboratory blind test.

Materials and methods

Healthy and PSTVd infected saps production

On the one hand, the infected saps which served to assess the effectiveness of the five disinfectants were produced from PSTVd positive tomato plants grown in favorable conditions for viroid multiplication. Each lab used a different strain of PSTVd characterized by either its Genbank accession number or its name: FM998542 (CRAW), HQ452399 (CRA-PAV), HQ454919 (AIS), EF192393 (AGES), Mumford (LT-MoA). The healthy saps which served as controls for detecting accidental contaminations and potential disinfectant phytotoxicity were produced from healthy tomato. Each individual plant was tested for PSTVd by RT-PCR prior to every experiment. For each experiment described hereafter the saps were produced by grinding 7.5 g of either PSTVd infected leaves or healthy leaves with a mortar and pestle in 30 mL of distilled water. In order to obtain a homogenous mixture, the saps were centrifuged for 4 min at 1000 g and the supernatants were stored at 4°C until used for the experiment.

Disinfectants

A random sample of 5 disinfectants: Virkon®, Hyprelva™ SL, Jet 5 ®, MENNO® clean and Virocid™ were either directly used in the laboratory or sent to four other laboratories in numbered and sealed Falcon tubes for a blind test. A bleach control and a water control were added for each experiment to check that the experiments were properly performed and that the PSTVd infected saps were infectious.

Assessment on dried droplets

In this series of two experiments which were performed in the same laboratory (CRA-W) and with the same PSTVd isolate (see Section Healthy and PSTVd infected saps production), 40 µL of either PSTVd infected or healthy tomato sap was deposited on glass panes in individual numbered cells following a completely randomized design. Sap droplets were allowed to dry overnight at around 25 °C in the dark. Forty µL of disinfectants were then deposited at the manufacturer's recommended concentration and for the recommended contact time (see Table 11) in the predetermined cell. The mix of sap and disinfectant from an individual cell was inoculated on a single tomato plantlet cv. Minibel at the first true-leaf stage. Six and three plantlets were used for each sap/disinfectant combination in the first and second experiment respectively. For the inoculation, swabs soaked in the mix were rubbed on plantlet leaves previously dusted with carborundum. Plantlets were then rinsed with water and grown at a photoperiod of 12 h and a temperature around 25 °C. Plants were checked for the presence of PSTVd about 6 weeks after inoculation using RT-PCR as described in Section RT-PCR.

Table 11 List of disinfectants

Disinfectant	Main active compounds	Incubation time			Applied final concentration	Approved targets at the applied concentration
		Dried droplets (min)	Aqueous solutions 1 (min) Contact time recommended by manufacturer	Aqueous solutions 2 (min) Contact time 15 min for each product		
Virkon®	Potassium monopersulphate Sulphamic and malic acids	30	30	15	1%[a]	Animal viruses, bacteria, pathogenic fungi, mycoplasm
Hyprelva™ SL	Alkyldimethylbenzylammonium chloride 82 g/L Dimethyldidecylammonium chloride 15 g/L Glutaraldehyde 133 g/L	30	30	15	0.75%[a]	Animal viruses and bacteria
Jet 5®	Peracetic acid 5% Hydrogen peroxide 20%	20	20	15	1%[a]	Animal viruses, bacteria, pathogenic fungi, yeast
MENNO® clean	Benzoic acid 90 g/L	15	3	15	1%[a]	*Potato spindle tuber viroid* (PSTVd)
Virocid™	Alkyldimethylbenzylammonium chloride 170 g/L Dimethyldidecylammonium chloride 78 g/L Glutaraldehyde 107 g/L	15	15	15	0.5%[a]	Animal viruses, bacteria, pathogenic fungi
Household bleach control	Sodium hypochlorite	10	10	15	0.8%[b]	
Water control	–	30	30	15	–	

[a] Dilution of the commercial product.
[b] Concentration of sodium hypochlorite expressed in percent active chlorine.

Assessment in aqueous solutions

For each sap/disinfectant combination, aqueous solutions of infected and healthy sap (3 ml) were mixed separately with the same volume (3 ml) of disinfectant in glass test tubes. The concentrations of disinfectants were doubled, so that the final concentration of disinfectant in the mixture was as recommended by the producer (Table 11). In a first series of experiments, the effectiveness of the disinfectants was assessed at the manufacturer's recommended concentration and for the minimum recommended contact time. In a second series, the effectiveness of products was compared at the manufacturer's recommended concentration and at the same contact time (15 min). The final concentrations and incubation times are presented in Table 11. During the incubation, the temperature was controlled to be within the range of 20 °C ± 3 °C. The mixes were vortexed at the beginning of the incubation period and every 5 min. The order of processing of the different disinfectants was chosen at random.

After the incubation period, the mixes were inoculated to Minibel tomato plantlets as previously described in Section Assessment on dried droplets. Inoculated plantlets were grown and checked according to the same procedure as in Sections Assessment on dried droplets and RT-PCR. Both series of experiments on aqueous solutions were performed in five laboratories and the experimental unit consisted of five inoculated tomato plantlets for PSTVd infected sap and two plantlets for healthy leaves sap.

RT-PCR

The laboratories used different detection protocols according to laboratory practices. RNA was extracted using the Spectrum Plant Total RNA kit (Sigmae-Aldrich) or the RNeasy kit (Qiagen). The RNA extracts were then tested according to one of the two following RT-PCR protocols: i) 1 µL of RNA extracts was tested by one-step RT-PCR using VIR 1/VIR 2 primer pair (EPPO, 2002) and the Titan One Tube RT-PCR kit (Roche) in a reaction volume of 25 µL or the OneStep RT-PCR Kit (Qiagen) in a 10 µL reaction volume. ii) 2 µL of RNA extracts was tested by one-step RT-PCR using PSTVd 32C/33H (Di Serio, 2007) and the amplification mixture previously described (Faggioli et al., 2013). All the RT-PCR products

obtained were separated by 1.5 % agarose gel electrophoresis and visualized using DNA staining on a transilluminator under ultraviolet light.

Statistical analysis

The statistical analysis was performed on arcsine square root transformed proportion of positive RT-PCR for each experimental unit in order to stabilize the variance and to normalize the data. A batch of plants treated with the same mix of sap/disinfectant, by the same laboratory and in the same experiment was considered as an experimental unit. For each of the three different series of experiments, transformed data were analyzed using two-way ANOVA's with disinfectant and experiment considered as fixed factors. Household bleach and water controls were alternatively used as baseline levels in order to check the significance of the difference between them and the tested products. For the assignment of statistical groups, all products were compared to both controls using Dunnett's test. The software R version 2.15.3 and the package multcomp (Hothorn *et al.*, 2008; R Core Team, 2013) were used to perform ANOVA's and Dunnett's tests.

Results

The results of two-way variance analysis for the three series of experiments are presented in Table 12. The results of the two experiments performed on glass panes showed that no significant difference could be observed between disinfectants (P = 0.143) and experiments (P = 0.065) at the 0.05 level. The results of the two series of experiments in aqueous solution indicate that the effect of disinfectants is significant: P = 1.41e-05 was for the first series of aqueous solution experiments and P = 6.26e-06 for the second series of aqueous solution experiments. The results of the first series of experiments in aqueous solution where disinfectants were used at their minimum recommended contact times and concentrations showed that Hyprelva™ SL, Virocid, Virkon®, and Jet 5® were not significantly different from the bleach control (Dunnett's test) (Table 13). MENNO® clean however was clearly less effective than bleach with a highly significant probability (P = 0.003) according to Dunnett's test. When compared with water control, only MENNO® clean was not significantly different (P = 0.31). For the second series of experiments in aqueous solution, where the effectiveness of the 5 disinfectants was compared at the same contact time (15 min), again two groups of significantly different product appeared. Virocid, Hyprelva™ SL, Virkon® and Jet 5® grouped with the bleach control, whereas MENNO® clean grouped with water control (P = 0.051) and was significantly different from bleach control (P = 0.007). For both series of experiments in aqueous solution a significant difference was found for the experiment factor. P = 0.028 was for the first series of experiments in aqueous solutions. However, the effect of experiments was only slightly significant (P = 0.045) for the second series of experiments.

Table 12: Two-way ANOVA tables for the three series of experiments

Experiment series	Source of variation	df	*F* value	Prob > *F*
Dried droplet experiments	Disinfectant	6	2.516	0.1431
	Experiment	1	5.112	0.0645
First series of in aqueous solution experiments	Disinfectant	6	10.045	1.41E-05
	Experiment	4	3.283	0.0279
Contact time recommended by manufacturer				
Second series of in aqueous solution experiments	Disinfectant	6	11.087	6.26E-06
	Experiment	4	2.858	0.0454
Contact time 15 min for each product				

Table 13: Means and standard deviations of the percentage of infected plants (untransformed data) for each of the three series of experiments and for each tested product. For each experiment, the statistical group to which a product belongs according to Dunnett's test is mentioned in parentheses.

	Dried droplet experiments			Aqueous solution experiments Contact time recommended by manufacturer			Aqueous solution experiments Contact time 15 min for each product		
	Mean (%)		SD	Mean (%)		SD	Mean (%)		SD
Bleach	8.3	(a)	11.8	8	(a)	17.8	8	(a)	11
Hyprelva™ SL	58.3	(a)	35.4	20	(a)	44.7	4	(a)	8.9
Jet 5®	91.7	(a)	11.8	20	(a)	44.7	28	(a)	30.3
Menno® clean	75	(a)	11.8	68	(b)	26.8	44	(b)	43.4
Virkon®	50	(a)	70.7	9	(a)	12.4	0	(a)	0
Virocid™	33.3	(a)	47.1	5	(a)	11.2	0	(a)	0
Water	100	(a)	0	96	(b)	8.9	80	(b)	28.3

Discussion

Despite the fact that the experiments on dried droplets were carried out in the same laboratory and with the same isolates, which should have reduced the variability compared to in aqueous solution experiments which were performed in five laboratories and with different PSTVd isolates, a lack of reproducibility between the two experiments was observed especially for Virkon® treatment (Table 13). In this series of experiments, a general lack of effectiveness was also observed for all commercial disinfectants compared with the equivalent experiments in aqueous solutions. This variability and this lack of effectiveness might be attributed to thick halos of sap which formed in the periphery of dried droplets and which would have allowed the PSTVd particles to escape disinfectant. Another explanation might be that the disinfectant droplets did not always cover perfectly the dried sap spot. This suggests that, in practical situation, the correct application and coverage of the infected areas with disinfectant is crucial for the disinfection efficacy. Quick application on infested surfaces as well as prolonged contact and even a rubbing in case of dried sap contamination should be advised.

The recommended concentrations, but more importantly, the recommended contact times of the five disinfectants tested here can vary a lot depending upon the application and sometimes upon the information source. For instance, the contact time recommended by the manufacturer for MENNO® clean varies from 3 min for knife/equipment disinfection to 16 h for hard surface disinfection. Hence, in order to assess the efficacy of products, in the first series of experiments in aqueous solution, the minimum recommended contact time of each disinfectant was chosen. These experiments showed that all disinfectants but MENNO® clean were not significantly different from the bleach control when applied at their recommended concentration and minimum contact time and that MENNO® clean was not significantly different from the water control. It should, however, be stressed that MENNO® clean is approved for disinfection of PSTVd infested surfaces at a contact time of 16 h.

The second series of experiments in aqueous solution where all products were compared at 15 min of contact time showed that Virocid™, Hyprelva™ SL, Virkon® and Jet 5® still grouped with the bleach control. Hyprelva™ SL thus kept its effectiveness even with 15 min less than the recommended contact time. MENNO® clean however was again not significantly different from the water control even when the contact time was increased from 3 to 15 min.

The significant differences for the experiment factor observed in both series of experiments in aqueous solution (Table 12) could be explained by two main sources of variation. The first was the different strains which have been used in each laboratory (see Section 2.3.) although a previous study (Singh *et al.*, 1989) showed that no difference between severe and mild strains was observed in a similar comparative test of disinfectants. The second main source of variation is the unknown concentration of PSTVd in the plant used to produce the inoculum. No sign of phytotoxicity was observed on the plantlets inoculated with healthy sap whatever the disinfectant used. It should, however, be stressed that plantlets were rinsed with water shortly after the inoculation, thereby reducing the potential phytotoxic effect of virucids.

In conclusion, at the recommended concentrations and at the same contact time (15 min), Virocid™, Hyprelva™ SL, Virkon® and Jet 5 ® were not significantly different from a solution of 0.8 % household bleach. MENNO® clean, although approved for viroid disinfection in several European countries, was not found to be significantly different from the water control at 3 or 15 min of contact time for the minimum recommended concentration of 1%. A reduced effectiveness, which can lead to a total ineffectiveness for Virkon® and Jet 5®, was observed when the five tested commercial products were applied on dried infected sap. This highlights the fact that an appropriate contact between viroid particles and disinfectant active compounds is crucial for disinfection efficacy.

References

Allen, M. L., Walker, W. B. (2012) Saliva of Lygus lineolaris digests double stranded ribonucleic acids. *Journal of Insect Physiology*, 58, 391–396.

ANSES. (2013). Détection des Pospiviroïdes sur feuilles de plantes hôtes par RT-PCR en point final. MOA 034 version 1a.

Antignus, Y., Lachman, O., Pearlsman, M. (2007). Spread of Tomato apical stunt viroid (TASVd) in greenhouse tomato crops is associated with seed transmission and bumble bee activity. *Plant Disease*, 91, 47-50.

Antignus, Y., Lachman, O., Pearlsman, M., Gofman, R., Bar Joseph, M. (2002). A new disease of greenhouse tomatoes in Israel caused by a distinct strain of Tomato apical stunt viroid (TASVd). *Phytoparasitica*, 30, 502-510.

Bates, D., Maechler, M., Bolker, B., & Walker, S. (2013). lme4: Linear mixed-effects models using Eigen and S4. R package version 1.0-4. http://CRAN.R-project.org/package=lme4.

Boonham, N., Gonzalez Perez, L., Mendez, M. S., Lilia Peralta, E., Blockley, A., Walsh, K., Barker, I., Mumford, R. A. (2004). Development of a real-time RT-PCR assay for the detection of Potato spindle tuber viroid. *Journal of Virological Methods*, 116, 139-146.

Botermans, M., van de Vossenberg, B.T.L.H., Verhoeven, J.Th.J., Roenhorst, J.W., Hooftman, M., Dekter, R., Meekes, E.T.M. (2013). Development and validation of a real-time RT-PCR assay for generic detection of pospiviroids. *Journal of Virological Methods*, 187, 43–50

Bradley, R. H. E., Harris, K. F. (1972). Aphids can inoculate plants with tobacco mosaic virus by clawing. *Virology*, 50, 615-618.

Candresse, T., Marais, A., Tassus, X., Suhard, P., Renaudin, I., Leguay, A., Poliakoff, F., Blancard, D. (2010). First report of tomato chlorotic dwarf viroid in tomato in France. *Plant Disease*, 94, 633.

Christiaens, O., Swevers, L., Smagghe, G. (2014) DsRNA degradation in the pea aphid (Acyrthosiphon pisum) associated with lack of response in RNAi feeding and injection assay. *Peptides*, 53, 307-314.

Chung, B.N., Pak, H.S. (2008). Seed transmission of Chrysanthemum stunt viroid in Chrysanthemum. *The Plant Pathology Journal*, 24, 31-35.

De Bokx, J. A. & Piron, P. G. M. (1981). Transmission of Potato spindle tuber viroid by aphids. *Netherlands Journal of Plant Pathology*, 87, 31-34.

Di Serio, F. (2007). Identification and characterization of Potato spindle tuber viroid infecting Solanum jasminoides and S. rantonnetii in Italy. *Journal of Plant Pathology*, 89, 297-300.

EFSA Panel on Plant Health (2011). Scientific Opinion on the assessment of the risk of solanaceous pospiviroids for the EU territory and the identification and evaluation of risk management options. *EFSA Journal*, 9, 2330.

EPPO (2002). PM 7/6 (1) Diagnostic protocols for regulated pests. Chrysanthemum stunt pospiviroid. EPPO Bulletin 32, 245-253.

EPPO. (2010). PM 7/76 (2): Use of EPPO diagnostic protocols. *EPPO Bulletin*, *40*, 350–352.

EPPO. (2014). PM 7/98 (2): Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity. *EPPO Bulletin*, *44*, 117–147.

EUPHRESCO (EUPHRESCO Phytosanitary ERA-NET) (2011). Detection and epidemiology of pospiviroids (DEP) final report, pp. 70. Pilot project report of the virtual common pot. Published on 7/01/2011.

Faggioli, F., Costantini, E., Di Serio, F., Luison, D., Luigi, M., Navarro, B., Silletti, M.R., Tomassoli, L., Torchetti, E., Trisciuzzi, N., 2013. Protocollo diagnostico per PSTVd. *Petria*, 23, 395-424.

Fleiss, J.L., Levin, B., Paik, & M.C. (2003). Statistical Methods for Rates and Proportions, 3rd Edition. New York: John Wiley & Sons.

Gamer, M., Lemon, J., Singh, P., & Fellows, I. (2012). irr: Various Coefficients of Interrater Reliability and Agreement. Retrieved from http://CRAN.R-project.org/package=irr

Garnsey, S.M., Jones, J.W., 1967. Mechanical transmission of exocortis virus with contaminated budding tools. *Plant Disease*, 51, 410.

Garnsey, S.M., Whidden, R., 1971. Decontamination treatments to reduce the spread of Citrus exocortis virus (CEV) by contaminated tools. *Proceedings of the Florida State Horticultural Society*. 84, 63-67.

Goulson, D. (2010) Bumblebees: behaviour, ecology, and conservation: Oxford University Press)

Hanssen, I. M., Mumford, R., Blystad, D.-R., Cortez, I., Hasiow-Jaroszewska, B., Hristova, D., Pagan, I., Pereira, A.-M., Peters, J., Pospieszny, H., Ravnikar, M., Stijger, I., Tomassoli, L., Varveri, C., van der Vlugt, R., Nielsen, S. L. (2010). Seed transmission of Pepino mosaic virus in tomato. *European Journal of Plant Pathology*, 126, 145-152.

Harris, P. S., Browning, I. A. (1980) The effects of temperature and light on the symptom expression and viroid concentration in tomato of a severe strain of potato spindle tuber viroid. *Potato Research*, 23, 85-93.

Hothorn, T., Bretz, F., & Westfall, P. (2008). Simultaneous Inference in General Parametric Models. *Biometrical Journal*, *50*, 346-363.

Koenraadt, H., Jodlowska, A., van Vliet, A., Verhoeven K. (2009). Detection of TCDVd and PSTVd in seeds of tomato. *Phytopathology*, 99, S66.

Kryczyński, S., Paduch-Cichal, E., & Skrzeczkowski, L.J. (1988). Transmission of three viroids through seed and pollen of tomato plants. *Journal of Phytopathology, 121*, 51-57.

Li, J. L., Cornman, R. S., Evans, J. D., Pettis, J. S., Zhao, Y., Murphy, C., Peng, W. J., Wu, J., Hamilton, M., Boncristiani, H. F., Zhou, L., Hammond, J., & Chen, Y.P. (2014) Systemic Spread and Propagation of a Plant-Pathogenic Virus in European Honeybees, Apis mellifera. *Mbio*, 5 (1):e00898-13. doi:10.1128/mBio.00898-13.

Luigi, M., Costantini, E., Luison, D., Mangiaracina, P., Tomassoli, L., & Faggioli, F. (2014). A diagnostic method for the simultaneous detection and identification of pospiviroids. *Journal of Plant Pathology*, *96*, 151-158.

Mackie, A., McKirdy, S., Rodoni, B., & Kumar, S. (2002) Potato spindle tuber viroid eradicated in Western Australia. *Australasian Plant Pathology*, 31, 311–312.

Matsushita, Y., Usugi, T., Tsuda, S. (2011). Distribution of *tomato chlorotic dwarf viroid* in floral organs of tomato. *European Journal of Plant Pathology*, 130, 441-447.

Matsuura, S., Matsushita, Y., Kozuka, R., Shimizu, S., & Tsuda, S. (2010) Transmission of Tomato chlorotic dwarf viroid by bumblebees (Bombus ignitus) in tomato plants. *European Journal of Plant Pathology*, 126, 111-115.

Mitchell, P. L. (2004) Heteroptera as vectors of plant pathogens. *Neotropical Entomology*, 33, 519-545.

Mommaerts V., Put K., Smagghe G. (2011) Bombus terrestris as pollinator-and-vector to suppress Botrytis cinerea in greenhouse strawberry. *Pest Managment Science*, 67, 1069-75.

Monger, W., Tomlinson, J., Boonham, N., Marn, M.V., Plesko, I.M., Molinero-Demilly, V., Tassus, X., Meekes, E., Toonen, M., Papayiannis, L., Perez-Egusquiza, Z., Mehle, N., Jansen, C., & Nielsen, S.L. (2010). Development and inter-laboratory evaluation of real-

time PCR assays for the detection of pospiviroids. *Journal of Virological Methods, 169*, 207–210.
Olivier, T., Demonty, E., Fauche, F., & Steyer, S. (2014). Generic detection and identification of pospiviroids. *Archives of Virology, 159*, 2097-2102.
R Core Team. (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.
Schumann, G. L., Tingey, W. M., & Thurston, H. D. (1980) Evaluation of 6 Insect Pests for Transmission of Potato spindle tuber viroid. *American Potato Journal*, 57, 205-211.
Shipp, J. L., Buitenhuis, R., Stobbs, L., Wang, K., Kim, W. S., & Ferguson, G. (2008) Vectoring of Pepino mosaic virus by bumble-bees in tomato greenhouses. *Annals of Applied Biology*, 153, 149-155.
Singh, R.P., Boucher, A., Somerville, T.H., 1989. Evaluation of chemicals for disinfection of laboratory equipment exposed to Potato spindle tuber viroid. *American Potato Journal*, 66, 239-245.
Singh, R.P., & Dilworth, A.D. (2009). *Tomato chlorotic dwarf viroid* in the ornamental plant *Vinca minor* and its transmission through tomato seed. *European Journal of Plant Pathology, 123*, 111-116.
Singh, R. P., Nie, X., Singh, M. (1999). Tomato chlorotic dwarf viroid: an evolutionary link in the origin of pospiviroids. *Journal of General Virology*, 80, 2823-2828.
Spieker, R.L. (1996). A viroid from *Brunfelsia undulate* closely related to the *Columnea* latent viroid. *Archives of Virology, 141*, 1823–1832.
Timmermann, C., Mühlbach, H.P., Bandte, M., Büttner, C., 2001. Control of Mechanical Viroid Transmission by the Disinfection of Tables and Tools. *Mededelingen Faculteit Landbouwwetenschappen Universiteit Gent*, 66, pp. 151-156.
Torchetti, E.M., Navarro, B., & Di Serio, F. (2012). A single polyprobe for detecting simultaneously eight pospiviroids infecting ornamentals and vegetables. *Journal of Virological Methods, 186*, 141–146.
Uzest, M., Gargani, D., Drucker, M., Hébrard, E., Garzo, E., Candresse, T., Fereres, A., Blanc, S. (2007) A protein key to plant virus transmission at the tip of the insect vector stylet. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 17959-17964.
Van Bogaert, N., De Jonghe, K., Van Damme, E. J. M., Maes, M., & Smagghe, G. (2015) Quantitation and localization of pospiviroids in aphids. *Journal of Virological Methods*, 211, 51-54.
Vandekerkhove, B. & De Clercq, P. (2010) Pollen as an alternative or supplementary food for the mirid predator Macrolophus pygmaeus. *Biological Control*, 53, 238-242.
Verhoeven, J. T. J., Huner, L., Marn, M. V., Plesko, I. M., & Roenhorst, J. W. (2010) Mechanical transmission of Potato spindle tuber viroid between plants of Brugmansia suaveoles, Solanum jasminoides and potatoes and tomatoes. *European Journal of Plant Pathology*, 128, 417-421.
Verhoeven, J.Th.J., Jansen, C.C.C., Willemen, T.M., Kox, L.F.F., Owens, R.A., & Roenhorst, J.W. (2004). Natural infections of tomato by Citrus exocortis viroid, Columnea latent viroid, Potato spindle tuber viroid and Tomato chlorotic dwarf viroid. *European Journal of Plant Pathology*, 110, 823–831.
Verhoeven, J. T. J., Jansen, C. C. C., Roenhorst, J. W., Steyer, S., Michelante, D. (2007) First Report of Potato spindle tuber viroid in Tomato in Belgium. *Plant Disease*, 91, 1055.

Van Brunschot, S. L., Verhoeven, J. T. J., Persley, D. M., Geering, A. D. W., Drenth, A., Thomas, J. E. (2014) An outbreak of Potato spindle tuber viroid in tomato is linked to imported seed. *European Journal of Plant Pathology*, 139, 1-7.

Verhoeven, J. T. J., Jansen, C. C. C., Roenhorst, J. W., Flores, R., de la Peña, M. (2009) Pepper chat fruit viroid: biological and molecular properties of a proposed new species of the genus Pospiviroid. *Virus Research*, 144, 209-214.