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Filling the gaps in diagnostics of *Pepino mosaic virus* and *Potato spindle tuber viroid* in water and tomato seeds and leaves

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Waterborne and seedborne *Pepino mosaic virus* (PepMV) and *Potato spindle tuber viroid* (PSTVd) pose serious threats to tomato production due to seed transmission and mechanical transmission, coupled with their long-term stability outside the host plant. Therefore, rapid and sensitive diagnostic procedures are needed to prevent the spread of these quarantine pathogens. In particular, water and seed contamination are difficult to detect and confirm without efficient concentration methods. This study presents procedures that improve detection of PSTVd from tomato seeds and leaf tissue, and PepMV from water and tomato leaf tissue. For efficient concentration of PepMV from water samples, a procedure was optimized using convective interaction media monolithic chromatography columns, which provides concentration by three orders of magnitude. For concentration of PSTVd from seed extracts, an easy-to-use and efficient method was developed based on RNA binding to positively charged anion-exchange resin beads that provides up to 100-fold more sensitive detection in comparison with procedures without a concentration step. This thus allows confirmation of RT-qPCR results with sequencing of RT-PCR products in samples with low viroid levels. In addition, reverse-transcription loop-mediated isothermal amplification assays for detection of PSTVd and PepMV were optimized and adapted to both laboratory and on-site testing requirements. This allows rapid detection of these pathogens in crude leaf homogenates, in under 30 min. These procedures of concentration and detection are shown to be efficient and to fill the gaps in diagnostics of PepMV and PSTVd, especially when these pathogens are present at low levels in difficult matrices such as water and seeds.

Keywords: concentration, loop-mediated isothermal amplification, PepMV, PSTVd, seeds, water

Introduction

Pepino mosaic virus (PepMV) and *Potato spindle tuber viroid* (PSTVd) are serious threats to tomato production. Both are seedborne (Diener & Raymer, 1971; Hanssen *et al.*, 2010), very stable outside the host plant, and easily transmissible mechanically (van der Vlugt, 2009; Verhoeven *et al.*, 2010). Just one infected plant grown from a single infected tomato seed has the potential to rapidly spread the infection mechanically to neighbouring plants, resulting in a larger outbreak. Recently, it has also been shown that irrigation water can be a path for PepMV and PSTVd transmission between plants (Mehle *et al.*, 2014). Therefore, it is essential to monitor for these pathogens through all of the critical points in tomato production and pathogen transmission pathways.

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In irrigation waters and seed samples, viruses and viroids are usually present at levels below the detection limit using classical methods, so their detection requires an appropriate concentration step (Boben *et al.*, 2007). It has already been shown that monolithic chromatographic supports (i.e. convective interaction media; CIM) are appropriate for the concentration of PSTVd from water samples (Ruščić *et al.*, 2013). However, no efficient procedure for the concentration of PepMV from water samples has been described to date. Plant viruses and viroids are highly variable in terms of structure and surface charge, and therefore not all of them will be able to resist the shear forces generated when being flowed through a monolith. In addition, each virus/viroid will require particular conditions (CIM chemistry, buffer, pH, ionic strength) to optimally bind to the monolith. This work optimizes for the first time a CIM concentration method for a filamentous potexvirus (PepMV) which is tested on tap water and wastewater effluents at virus concentrations even lower than the limit of detection of real-time quantitative reverse-transcription PCR (RT-qPCR).

PepMV in tomato seeds can be revealed using RT-qPCR, which can detect as little as one naturally

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PepMV-infected seed among 5000 uninfected seeds (Gutiérrez-Aguirre *et al.*, 2009). Different RT-qPCR assays are used for the detection of PepMV, with the 'universal' assay that can detect all PepMV genotypes used for screening (Ling *et al.*, 2007). This can be followed by genotype-specific assays that serve as confirmatory tests (Gutiérrez-Aguirre *et al.*, 2009). Similarly, an RT-qPCR assay has been used as a screening method for PSTVd in tomato seed samples (Boonham *et al.*, 2004). However, this RT-qPCR also detects *Mexican papita viroid*, *Tomato chlorotic dwarf viroid* and *Tomato planta macho viroid*, and therefore a classic RT-PCR is required for confirmation by sequencing of the RT-PCR product (International Standards for Phytosanitary Measures, 2015). Typically, RT-PCR is less sensitive than RT-qPCR, and the confirmatory tests with RT-PCR can be limited and unsuccessful if the PSTVd levels are too low. To improve the sensitivity of PSTVd diagnostic methods, an efficient concentration method for PSTVd in seed samples is urgently called for.

In contrast to water and seed samples, the detection of PSTVd and PepMV in leaf tissue showing symptoms does not need a concentration step. For the detection of pathogens in leaf tissue, loop-mediated isothermal amplification (LAMP) procedures are being increasingly used. LAMP provides accurate, rapid detection of pathogens in the laboratory and on-site, because of its isothermal nature, speed, robustness and simplicity (Tomlinson *et al.*, 2010). In addition, LAMP has been shown to be less sensitive to inhibitors than real-time quantitative PCR (qPCR; Francois *et al.*, 2011). Consequently, some LAMP assays have been reported that can be used for direct testing of crude plant homogenates, without the need for nucleic acid extraction (Kogovšek *et al.*, 2015) and with minimal sample preparation, e.g. boiling a resuspended bacterial ooze sample from an infected plant for a few minutes (Lenarčič *et al.*, 2014). Reverse-transcription LAMP (RT-LAMP) assays for the detection of PSTVd (Tsutsumi *et al.*, 2010; Lenarčič *et al.*, 2013), and PepMV-specific RT-LAMP assays (Hasiów-Jaroszewska & Borodynko, 2013; Ling *et al.*, 2013) have already been developed. The present study adapts existing PepMV assays for single-tube fluorescent detection of all known PepMV genotypes, which allows high-throughput laboratory detection, prevents contamination during product handling, and enhances on-site detection. Moreover, this adapted RT-LAMP assay and RT-LAMP assay for the detection of PSTVd (Lenarčič *et al.*, 2013) are optimized for direct detection of PepMV and PSTVd without the need to extract the RNA.

This study focuses on two critical points within PSTVd and PepMV diagnostics where efficient methods for their concentration are needed: water samples (PepMV), and tomato seed extracts (PSTVd) (Fig. 1). Because proper diagnostics need not only efficient concentration methods but also fast, easy-to-use and on-site-deployable methods, the RT-LAMP assay has also been optimized for direct testing of both of these tomato pathogens in crude tomato leaf homogenates.

Materials and methods

Concentration of PepMV in water

Sample preparation

Samples of tap water and effluent from a wastewater treatment plant (1.2 L) were filtered through 0.2 μm (CA membrane) or 0.8 μm (GE Healthcare) filters. These were spiked with 60 μL of 1000-fold or 10 000-fold diluted homogenate from tomato leaf material infected with the Ch2 genotype of PepMV (isolate 20703230 from the Fera (UK) collection; internal reference number NIB V 121). For concentration of larger volumes for the large CIM quaternary amine (QA) monolithic column format (see below), 3.6 L tap water was filtered as indicated and spiked with 600 μL of the same 10 000-fold diluted homogenate.

Concentration of PepMV from water samples with CIM QA columns

Concentration of PepMV spiked into water was performed using positively charged CIM QA monolithic columns either in the small-disc format (bed volume, 0.34 mL) or in the large format (bed volume, 1 mL) (BIA Separations). Before each concentration run, the CIM monolithic column was conditioned following the manufacturer's instructions. The equilibration buffer was 50 mM MES (MES hydrate; Sigma), pH 6, and the elution buffer was 50 mM MES, 1 M NaCl, pH 6. After each concentration run, the CIM monolithic column was regenerated according to the

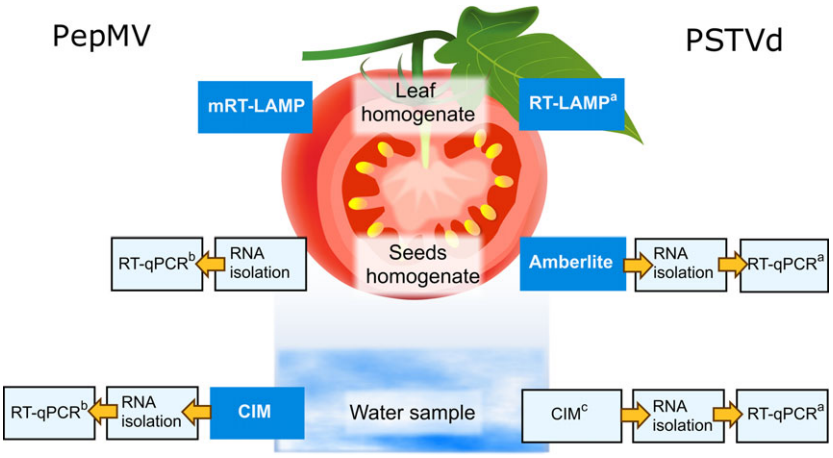

Figure 1 Overview of the detection procedures for PSTVd and PepMV in water, tomato seeds and leaves. The procedures described here are in framed boxes. [a]The specificity of RT-qPCR (Boonham *et al.*, 2004) and RT-LAMP (Lenarčič *et al.*, 2013) is not only for PSTVd, therefore the confirmation of PSTVd in the sample requires sequencing of RT-PCR product (International Standards for Phytosanitary Measures, 2015); [b]'universal' and/or genotype-specific RT-qPCR (Ling *et al.*, 2007; Gutiérrez-Aguirre *et al.*, 2009); [c]CIM for the concentration of PSTVd from water samples (Ruščić *et al.*, 2013). [Colour figure can be viewed at wileyonlinelibrary.com]

manufacturer's instructions. Chromatographic runs were carried out on an FPLC system (Purifier 100 ÄKTA system; GE Healthcare). To account for the different possible complexities of the water matrix, the procedure was applied to spiked tap water and spiked wastewater (as effluent from a wastewater treatment plant). The procedure was applied immediately after spiking.

Before loading the samples on the CIM monolithic columns, the small and large columns were equilibrated with 10 and 30 mL, respectively, equilibration buffer containing 0.4 mg mL^{-1} bovine serum albumin (BSA), followed by a 10 and 30 mL, respectively, wash with equilibration buffer without BSA. This BSA step (Table S1) was introduced to prevent non-specific binding of PepMV onto the CIM monolithic column observed at low virus concentrations (Table S2).

The spiked water samples were loaded onto the small CIM monolithic column at flow rates from 3.5 to 5 mL min^{-1} and at 16 mL min^{-1} for the large CIM monolithic column. The small CIM monolithic column was loaded with 1000 mL of the spiked tap water, and 580 mL of the spiked wastewater. The large CIM monolithic column was loaded with 3000 mL of the spiked tap water. Bound PepMV was eluted from the CIM monolithic columns in 1 mL elution buffer, at a flow rate of 1 mL min^{-1} for the small CIM monolithic column and 3 mL min^{-1} for the large CIM monolithic column. Aliquots of loading and elution samples were collected and stored at −20 °C, until RNA extraction and molecular analysis.

Concentration of PSTVd from tomato seeds

Sample preparation

This study used four tomato seed samples (D35/13, D19/14, D21/14, D25/14) of different PSTVd-contaminated seed lots from the National Institute of Biology, Slovenia, plus PSTVd-contaminated tomato seed lot ZZB-453 from Naktuinbouw (Netherlands) (internal reference number NIB V 276). In this study, a *Columnea latent viroid* (CLVd)-contaminated tomato seed lot from Fera (UK) (internal reference number NIB V 279) was also included with the aim of testing the procedure for concentration of other pospiviroids from tomato seed extracts. The initial analysis on 3000 tomato seeds divided into 12 subsamples confirmed the almost homogeneous distribution of PSTVd among the seed lot of sample D35/13, and demonstrated uneven distribution of PSTVd among the seed lots of samples D19/14, D21/14 and D25/14 (Table S3).

The PSTVd/CLVd-contaminated tomato seeds were mixed with healthy tomato seeds at different ratios, based on samples of 250 seeds: 250:0 (contaminated:healthy); 1:1 (125 + 125 seeds); 1:24 (10 + 240 seeds); 1:49 (5 + 245 seeds); and 1:249 (1 contaminated seed in a total of 250 seeds). For each sample, up to three subsamples were prepared and analysed on different days.

The samples of 250 tomato seeds were transferred into 15 mL centrifuge tubes that contained garnet matrix and five ¼-inch ceramic spheres (MP Biomedicals), to which was added 10 mL 0.1 M phosphate buffer (Na_2HPO_4/KH_2PO_4, pH 7.2). The seeds immersed in the buffer were incubated at 4 ± 2 °C overnight, and then ground (FastPrep homogenizer; MP Biomedicals) for 40 s at 5 m s^{-1}. After centrifugation at 10 000 ***g*** for 10 min at 4 °C, the samples were taken through different RNA extraction procedures, with or without prior concentration of the viroid.

Concentration of PSTVd from tomato seed extracts with positively charged resin beads

For concentration of the viroid, a procedure from Pérez-Méndez *et al.* (2014) for concentration of enteric viruses from tap water was adapted. Here, 4.5 mL of the supernatant from the homogenized samples was added to a 5 mL test tube with 0.5 g Amberlite IRA-900 anion-exchange resin (Polysciences). This was then mixed continuously at room temperature for 3 h, after which the test tube was centrifuged at 5000 ***g*** for 1 min, and all of the liquid was removed. The resin-adsorbed RNA was extracted as described below.

RT-LAMP optimization for PepMV

Sample preparation

For evaluation of the performance of the RT-LAMP assay for PepMV detection, different PepMV genotypes were analysed, both individually or as mixtures of up to three genotypes (Table S4). These included genotypes EU, Ch2 and US1 as isolates from the Scientia Terrae Research Institute (Belgium), Fera (UK) and Plant Research International (Netherlands). Tenfold dilutions of PepMV-positive RNA were prepared in water and in RNA isolated from healthy tomato.

RT-LAMP optimization for detection of all PepMV genotypes

For selection of the most appropriate RT-LAMP assay for a universal (all genotypes) detection of PepMV from among those already reported (Hasiów-Jaroszewska & Borodynko, 2013; Ling *et al.*, 2013), *in silico* analysis of primers was done. Previously reported primers targeting the triple gene block (TGB) were aligned with all available PepMV TGB sequences (62 in total) of different genotypes available in the National Centre for Biotechnology Information (NCBI) database using VectorNTI (ThermoFisher Scientific). Selected primers were synthesized by Integrated DNA Technologies.

To determine the ideal conditions for amplification, three different reverse transcriptases and different primer concentrations were tested in the RT-LAMP reactions. The conditions that gave the least time to generate the signal and were the most suitable for on-site use were selected for further testing, and are described hereinafter. The RT-LAMP reactions were performed in 8-well strips or 96-well plates in a 25 μL reaction volume, which contained 12.5 μL of 2× isothermal master mix (OptiGene), 5 μL of 5× Transcriptor RT buffer (Roche), and 5 U Transcriptor RT (Roche). The reaction included 0.2 μM F3 and B3 primers, 2 μM FIP and BIP primers, and 1 μM F-loop and B-loop primers, while in the single tube multiplex (m)RT-LAMP reaction, these PepMV genotype-specific primers were used at 0.1, 1 and 0.5 μM, respectively. Five microlitre samples of the 10-fold–diluted RNA, leaf homogenate, and CIM monolithic column eluate were tested. The RT-LAMP reactions were run at 65 °C for 30 min (GenieII, Optigene; or LC480, Roche devices). The melting temperatures (T_m) were determined for all of the amplified products. Fluorescence was detected in real-time (on the FAM channel for the Roche LC480 device). A sample was considered negative if it produced no fluorescence.

The analytical sensitivity of the mRT-LAMP assay for PepMV was evaluated by testing PepMV-positive RNA diluted in RNase-free water. Tenfold dilutions were analysed using the mRT-LAMP and RT-qPCR assays specific for PepMV (Ling *et al.*, 2007), using each of the PepMV genotypes individually (i.e. EU, Ch2, US1) and using a mix of all three genotypes, to mimic the multiple genotype infections that can occur in nature. The samples were analysed in triplicate with each method. In addition, this whole experiment was repeated with the mix of the three genotypes diluted 10-fold in RNA isolated from healthy tomato.

RT-LAMP evaluation of PepMV and PSTVd directly in crude tomato leaf homogenate

Sample preparation

For the direct application of the RT-LAMP assays, healthy tomato leaves (~1 g) were homogenized (Ultra-Turrax Tube Drive; IKA) in 5 mL ELISA buffer (Kogovšek *et al.*, 2015) for 30 s at maximum setting (6000 rpm). The homogenates were spiked with a similarly prepared homogenate from leaves of tomato plants infected with PepMV-Ch2/US2 (NIB V 121), PepMV-US1 (NIB V 133) (Table S4), or PSTVd (isolate from Fera [UK] collection; internal reference number: NIB V 223). A 10-fold dilution series was prepared with healthy tomato leaf homogenate.

RT-LAMP for direct detection of PepMV and PSTVd in crude tomato leaf homogenate and in concentrated water samples

The homogenates were tested for PepMV using mRT-LAMP (Table S5) and ELISA. For PSTVd detection, the LAMP reactions and the amplification conditions were as described by Lenarčič *et al.* (2013) (Table S5), with the exception of the reverse transcriptase used (i.e. Transcriptor RT, Roche, used in the present study). RNA was extracted from the same homogenates using RNeasy Plant minikits (QIAGEN) without the addition of the RLT buffer and tested with both RT-LAMP assays and RT-qPCR assays for comparison with the direct detection.

The CIM monolithic column elution fractions containing the concentrated PepMV were 10-fold diluted and tested directly in the mRT-LAMP assays. In addition, the inhibitory influence of the 1 M NaCl elution buffer on the mRT-LAMP amplification was evaluated via addition of 1 M NaCl to the reaction mix.

Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) was performed using NuncMaxisorp flat-bottomed microtitre plates and PepMV-specific antibodies (PRIME Diagnostics), following the manufacturer's instructions. The absorbance at 405 nm (A_{405}) was measured after 30 min, 1 h and 2 h of incubation with the substrate, using a microplate absorbance reader (Tecan Sunrise), with the Magellan software for the data analysis. The threshold for a positive reaction was defined as twice the mean of the healthy control value.

RNA extraction

Tomato seed and leaf RNA were isolated using RNeasy Plant minikits, according to the manufacturer's instructions, with some minor modifications. Briefly, 600 μL RLT buffer (without β-mercaptoethanol) was added to 600 μL supernatant of the homogenized samples and loaded onto RNeasy Mini Spin columns in two consecutive steps. The RNA elution from these RNeasy Mini Spin columns was performed in two consecutive applications of 50 μL of RNase-free warm water (65 °C).

The resin-adsorbed RNA (containing the viroid) was isolated by adding 560 μL AVL buffer (QIAmp Viral RNA minikits; QIAGEN) to the pelleted Amberlite beads, with carrier RNA (QIAmp Viral RNA minikits) and control luciferase RNA (Promega) (2 ng per sample; as external control), with an incubation for 10 min at room temperature, with occasional agitation. The samples were centrifuged at 5000 ***g*** for 1 min, and the supernatants (containing the nucleic acids) were transferred to 1.5 mL test tubes, and then processed according to the manufacturer's instructions. The RNA was eluted in 45 μL RNase-free water prewarmed to 65 °C. The same kits were also used for RNA extraction from non-concentrated tomato seed samples and the chromatographic fractions of the concentration of PepMV from water samples, following the manufacturer's recommendations. The RNA was eluted in 45 μL RNase-free warm water (65 °C).

RT-qPCR and RT-PCR

One-step RT-qPCR assays according to Boonham *et al.* (2004) and Ling *et al.* (2007) (Table S5) were used for PSTVd and PepMV detection, respectively, as described by Mehle *et al.* (2014). For detection of CLVd, the one-step RT-qPCR of Monger *et al.* (2010) was used (Table S5). A sample was considered positive if it produced an exponential amplification curve distinguishable from negative controls, and in such cases Cq values were calculated. If no exponential amplification curve was produced, a sample was considered negative. Plant cytochrome oxidase (*COX*)- and luciferase RNA (*LUC*)-specific primers and TaqMan probes (Weller *et al.*, 2000; Toplak *et al.*, 2004; Table S5) were used as controls, to evaluate the quality of the RNA in the extractions.

The viroid presence was confirmed using one-step RT-PCR assay kits (QIAGEN) and the primer sets described by Verhoeven *et al.* (2004): Pospi1-FW/RE and Vid-FW/RE (Table S5). To improve the sensitivity of the RT-PCR, the amplified products were used in additional amplification steps with RT-PCR (hereinafter indicated as two consecutive RT-PCR assays). Agarose gel (1%) electrophoresis and ethidium bromide were used to visualize the RT-PCR products.

Results

The main objective of this work was to fill some of the gaps that remain in the diagnostics of both PepMV and PSTVd. To do this, an efficient procedure was developed for the concentration of PepMV from water samples, and for the concentration of PSTVd from tomato seed extracts. In addition, the RT-LAMP assays were improved for rapid laboratory and on-site testing for both of these pathogens in tomato leaves, without the need for RNA extraction.

Concentration of PepMV from water samples with CIM monolithic columns

MES buffer at pH 6 was determined as the optimal buffer for the concentration of PepMV (also tested: 50 mM sodium acetate, pH 4.8; 10 mM trisodium citrate, 1 M urea, pH 7.4; data not shown). These conditions worked very efficiently at relatively high PepMV concentrations. However, at medium to low PepMV concentrations (i.e. RT-qPCR Cq > 21), PepMV bound efficiently to the CIM monolithic column, i.e. no signal detected in water flowing through the CIM monolithic column, but it could not be efficiently eluted (no or low decrease in RT-qPCR Cq values after the concentration step; Table S2), most probably due to irreversible adsorption of the PepMV to nonspecific sites on the CIM monolithic

column. A step in which BSA was loaded onto the CIM monolithic column before concentration of the virus from the water sample was included to successfully overcome this limitation (Table S1).

This optimized concentration method was tested with tap water (1 L) spiked with the low concentrations expected in contaminated irrigation water, in some cases lower than the limit of detection (LOD) of qPCR. Comparing the concentrated to non-concentrated samples with low levels of PepMV, the RT-qPCR Cq decreased by >9 cycles (i.e. a concentration factor of ~1000-fold). PepMV was also successfully detected after concentration of water samples spiked to a level below the LOD (Table 1).

As well as testing the concentration method with spiked tap water samples, the method was tested with spiked effluent from a wastewater treatment plant to determine the CIM monolithic column performance for more complex water samples. It is worth noting that wastewater effluent is reused as irrigation water in a number of arid countries. This concentration step was also successful, as the RT-qPCR Cq decreased by 7 cycles (Table 1).

To demonstrate that the concentration method for waterborne PepMV using these CIM monolithic columns can be scaled up and used for the concentration of greater sample volumes, 3 L tap water spiked with PepMV was also concentrated using a larger monolithic column that allows the use of higher flow rates, and therefore a more rapid concentration step. Using such a column, most of the PepMV was eluted in the same volume as with the small CIM disc-format monolithic column (i.e. 1 mL), which resulted in a decrease in the RT-qPCR Cq of 11 cycles (Table 1).

Concentration of PSTVd from tomato seeds

Using the concentration procedure based on binding PSTVd to positively charged resin beads (Amberlite), following RNA extraction by viral RNA minikits (QIAmp), the RT-qPCR Cq decreased on average by 4.8 ± 0.9 cycles (i.e. concentration factor up to 100-fold), compared to using only the RNeasy plant minikits, or 1.6 ± 0.9 cycles compared to using only viral RNA minikits (QIAmp) (Fig. 2). The amplification by RT-PCR with the Pospi1-FW and Pospi1-RE primers required for confirmatory purposes was more efficient using RNA isolated with the viral RNA minikit (QIAmp) than RNA isolated with the plant minikit (RNeasy) [D35/13 (1:1)a, Fig. 3], and, most importantly, in one of the tested samples [D35/13 (1:24)a], the amplification by RT-PCR succeeded only when the concentration step was included before the RNA isolation (Fig. 3). This concentration procedure is easy to implement and improves the diagnostics of the PSTVd procedure in this complex seed matrix.

Single-tube multiplex RT-LAMP for detection of all PepMV genotypes

Using *in silico* analysis, three RT-LAMP assays (Table 2) were determined to have the greatest probability for the detection of all of the currently sequenced PepMV genotypes available in the NCBI database and simultaneously distinguish between the three main PepMV groups (i.e. EU, CH2 & US2, and US1). The performance of each of these assays was first tested in the singleplex format (data not shown) and then in the single-tube mRT-LAMP format, where all three assays were mixed in the same reaction mixture (Table S4). The time to a positive result (t_{pos}) was between 5 and 34 min, with a mean t_{pos} of 13 min. The T_m of the amplified products was genotype specific, which thus allowed identification of the PepMV genotypes in the samples (Table S6). However, in artificially mixed samples, where two or three PepMV genotypes were mixed, the T_m of the amplified product corresponded to one of the genotypes present in the reaction only (Table S4). The T_m for the PepMV-US2/Ch2

Table 1 RT-qPCR measurements of PepMV from spiked water samples

	PepMV spiked input level					
	Small CIM QA column					Large CIM QA column
	Tap water				Wastewater	Tap water
Sample	Low[a]		Below LOD[ab]		Low	Low
Volume loaded (mL)	1000	1000	1000	1000	580	3000
Non-concentrated sample (Cq)	37.4	37.1	n.d.	n.d.	34.3	38.2
Concentrated sample (Cq)	27.6	28.0	34.7	29.3	27.1	26.8
Signal increase[c] (Cq)	9.8	9.1	n.a.	n.a.	7.2	11.4

RT-qPCR measurements of each sample were made in triplicate, with mean Cq shown. Luciferase control RNA was used to allow for any deviations due to RNA isolation and RT-qPCR.

n.d., not determined.

n.a., not applicable.

[a]Two duplicate runs were analysed.

[b]Limit of detection.

[c]Difference in Cq between non-concentrated and concentrated sample.

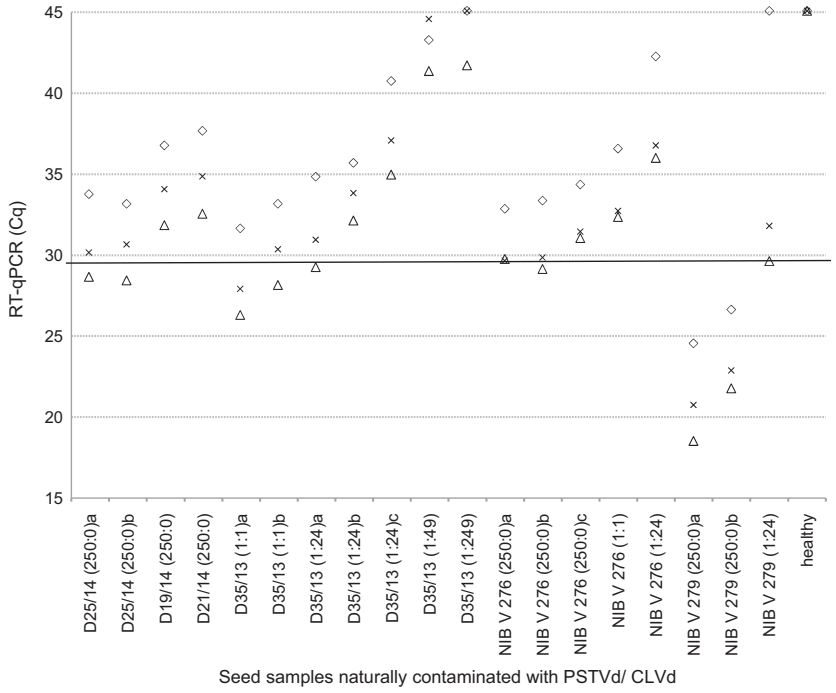

Figure 2 Comparison of one-step RT-qPCR (Cq as mean of two parallels tested; no amplification given as Cq 45) for PSTVd-contaminated (D25/14, D19/14, D21/14, D35/13 and NIB V 276), CLVd-contaminated (NIB V 279) and healthy tomato seed samples isolated with (◇) RNeasy Plant minikits (RNeasy); (×) QIAmp Viral RNA minikits (QIAmp); (△) QIAmp Viral RNA minikits after concentration of PSTVd using Amberlite IRA-900 (Amberlite + QIAmp). The contaminated tomato seeds were mixed with healthy tomato seeds at different ratios (250:0, 1:1, 1:24, 1:49 and 1:249). For each sample, up to three subsamples (a–c) were prepared and analysed. Dots above horizontal line at Cq ~30 corresponds to low titre of PSTVd, which was below the limit of detection of conventional RT-PCR assay with Pospi1-FW and Pospi1-RE primers (not applicable for tomato seed samples contaminated with CLVd [NIB V 279]).

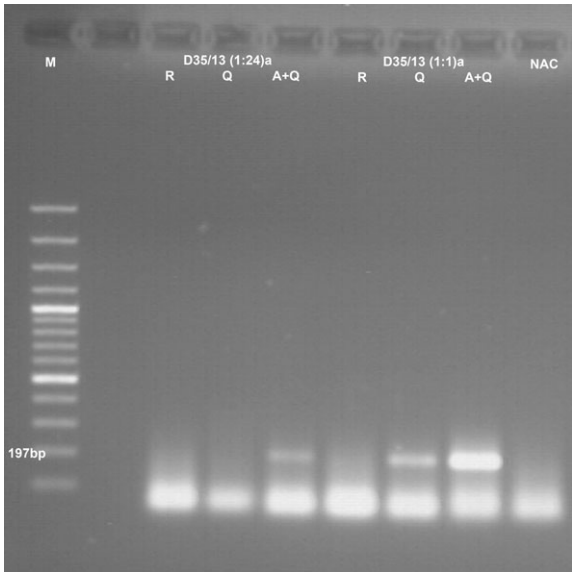

Figure 3 Agarose gel (1%) showing amplicons yielded in two consecutive RT-PCR assays with the Pospi1-FW and Pospi1-RE primers. M, Gene Ruler 100-bp DNA ladder (Fermentas); R, RNA isolated with RNeasy Plant minikits (QIAGEN); Q, RNA isolated with QIAmp Viral RNA minikits (QIAGEN); A+Q, RNA isolated with QIAmp Viral RNA minikits after concentration of PSTVd using Amberlite IRA-900 (Polysciences); NAC, negative amplification control.

genotype was 85.3 ± 0.4 °C, and for PepMV-US1, 87.0 ± 0.3 °C, regardless of the singleplex (data not shown) or single-tube mRT-LAMP assay format (Table S6) and of the device used for the amplification. The PepMV-EU genotype-specific assay was an exception, where the T_m of the product was 88.4 ± 0.3 °C for the Genie II device and at 89.5 ± 0.2 °C for Roche LC480 assisted amplification.

The RT-qPCR and mRT-LAMP for detection of PepMV were compared, and their sensitivities were determined (Table 3). On extracted RNA, the sensitivity of mRT-LAMP in comparison to RT-qPCR ranged from equal (EU genotype) to 1000-fold lower (CH2 genotype), where the difference in the sensitivity was PepMV genotype-specific.

RT-LAMP for direct detection of PepMV and PSTVd in crude tomato leaf homogenate and in concentrated water samples

Direct testing of crude leaf homogenates is important as it is rapid and facilitates the on-site applicability of RT-LAMP. For this sample type, mRT-LAMP in comparison to ELISA showed equal (CH2/US2; Table S7) or 10-fold better (US1; Table 4) sensitivity for PepMV detection when the same homogenates were tested.

The RT-LAMP for PSTVd (Lenarčič *et al.*, 2013) was evaluated on crude tomato leaf homogenates. Here, the sensitivity of RT-LAMP in comparison to RT-qPCR was 1000-fold lower (Table 5). Because the sensitivity of RT-PCR has also been shown to be 1000-fold lower compared to the sensitivity of RT-qPCR (Lenarčič *et al.*, 2013), RT-LAMP can be used instead of RT-PCR for rapid laboratory or on-site screening of tomato leaf samples with symptoms where high titre of PSTVd is expected.

Chromatographic fractions containing PepMV that were concentrated using the CIM monolithic column and

Table 2 Selected RT-LAMP assays for the detection of PepMV genotypes

RT-LAMP assay	Representative target sequences	PepMV genotypes[a]	Reference
US2 + CH2	DQ000985, AY509927, FJ263361	PepMV-US2, PepMV-Ch2	Hasiów-Jaroszewska & Borodynko (2013)
US1	AY509926, DQ00984, FJ940225	PepMV-US1, PepMV-Ch1	Ling *et al.* (2013)
EU	AJ438767, AJ606361, FJ940223	PepMV-EU, PepMV-LP	Ling *et al.* (2013)

[a]Genotypes described by Hanssen & Thomma (2010) are included in the table.

elution buffer alone were also directly tested with mRT-LAMP, without RNA extraction, to determine the on-site applicability of the concentration step and LAMP. These results showed that the high concentration of salts present in the elution buffer and the concentrated fractions inhibited the amplification (data not shown), and therefore 10-fold dilution of the sample prior to the mRT-LAMP reaction was needed to obtain successful amplification.

Discussion

The present study has developed new tools (i.e. CIM monolithic column, Amberlite beads) and optimized existing ones (i.e. RT-LAMP) to improve the diagnostics of PepMV and PSTVd, with particular focus on critical points within the diagnostic procedures, such as water samples (CIM monolithic column), tomato seed extracts (Amberlite), and on-site requirements (RT-LAMP). These developed/optimized methods now represent an important part of the diagnostic procedures for monitoring of PSTVd and PepMV in water, tomato seeds and leaf materials (Fig. 1).

Mehle *et al.* (2014) have previously shown that transmission of PepMV through water can greatly contribute to the spread of the virus through fields and greenhouses. The monolithic concentration method developed here for waterborne PepMV is the first concentration method that has been dedicated to improved detection of PepMV in water. This study has demonstrated that the monolithic chromatography-based method successfully concentrated PepMV in tap water samples with virus concentrations close to and below the LOD of RT-qPCR. This is very important, as the levels of PepMV in environmental water samples are expected to be low (Mehle & Ravnikar, 2012). Pretreatment of the CIM monolithic column with BSA also increased the efficiency of the concentration step by reducing the nonspecific binding of PepMV to the CIM monolithic column. As the complexity of the water samples can vary, the effluent from a wastewater treatment plant was also used with the concentration

Table 3 Sensitivity of the RT-qPCR analysis and mRT-LAMP assays for detection of PepMV in extracted RNA

	RNA diluted in water												RNA diluted in plant RNA			
	CH2 (NIB V 131)			US1 (NIB V 133)			EU (NIB V 135)			CH2 + US1 + EU			CH2 + US1 + EU			
	RT-qPCR	mRT-LAMP		RT-qPCR	mRT-LAMP		RT-qPCR	mRT-LAMP		RT-qPCR	mRT-LAMP		RT-qPCR	mRT-LAMP		
Fold-dilution	Cq ± SD	t_{pos} ± SD	T_m ± SD	Cq ± SD	t_{pos} ± SD	T_m ± SD	Cq ± SD	t_{pos} ± SD	T_m ± SD	Cq ± SD	t_{pos} ± SD	T_m ± SD	Cq ± SD	t_{pos} ± SD	T_m ± SD
10	10.2 ± 0.2	11.8 ± 0.1	86.3 ± 0.1	9.0 ± 0.1	8.5 ± 0.1	87.7 ± 0.1	10.6 ± 0.1	9.8 ± 0.1	89.3 ± 0.0	n.t.	n.t.	n.t.	13.6 ± 0.0	10.8 ± 0.4	88.0 ± 0.1
10^2	13.6 ± 0.1	13.1 ± 0.1	86.1 ± 0.1	11.8 ± 0.0	9.6 ± 0.1	87.6 ± 0.1	14.0 ± 0.0	10.9 ± 0.3	89.2 ± 0.1	13.7 ± 0.1	10.6 ± 0.3	88.1 ± 0.2	17.1 ± 0.0	13.4 ± 0.4	87.9 ± 0.4
10^3	17.1 ± 0.2	15.4 ± 0.0	86.0 ± 0.0	15.4 ± 0.1	10.6 ± 0.2	87.5 ± 0.0	17.6 ± 0.1	12.9 ± 0.2	88.9 ± 0.0	16.6 ± 0.1	12.8 ± 0.2	87.8 ± 0.1	20.6 ± 0.1	17.8 ± 3.7	87.2 ± 0.3
10^4	20.7 ± 0.1	18.5 ± 0.6	86.0 ± 0.0	19.0 ± 0.1	12.7 ± 0.2	87.5 ± 0.1	21.3 ± 0.1	15.8 ± 1.6	88.9 ± 0.0	20.0 ± 0.0	15.0 ± 0.5	87.5 ± 0.0	24.0 ± 0.0	29.9 ± 1.2	89.1 ± 0.0
10^5	24.2 ± 0.0	28.9 ± 11.9	86.2 ± 0.1	22.7 ± 0.0	15.1 ± 1.0	87.8 ± 0.1	25.0 ± 0.1	26.9 ± 2.1	89.4 ± 0.0	23.4 ± 0.1	17.3 ± 1.3	87.5 ± 0.1	27.5 ± 0.1	33.4 ± 1.4	89.3 ± 0.0
10^6	28.0 ± 0.2	–	n.t.	26.5 ± 0.1	30.9 ± 9.4	87.6 ± 0.1	28.8 ± 0.1	37.8 ± 7.2	88.9 ± 0.3	26.8 ± 0.2	30.9 ± 6.9	89.0 ± 0.0	30.6 ± 0.1	–	n.t.
10^7	31.7 ± 0.3	–	n.t.	31.2 ± 0.4	–	n.t.	33.5 ± 0.4	42.9 ± 3.5	88.9 ± 0.0	30.1 ± 0.2	–	n.t.	32.6 ± 0.0	–	n.t.
10^8	33.7 ± 0.1	–	n.t.	–	–	n.t.	–	–	n.t.	32.0 ± 0.3	–	n.t.	–	–	n.t.
10^9	–	–	n.t.	–	–	n.t.	–	–	n.t.	–	–	n.t.	–	–	n.t.
Healthy material	–	–	n.t.	–	–	n.t.	–	–	n.t.	–	–	n.t.	–	–	n.t.

RT-qPCR and mRT-LAMP measurements of each sample were made in triplicate, with mean Cq, t_{pos} and T_m shown.
SD, standard deviation; –, negative result; n.t., not tested.

Table 4 Sensitivity of the mRT-LAMP assays for detection of PepMV (NIB V133 = US1) in crude tomato leaf homogenates compared to RT-qPCR analysis (on extracted total RNA) and ELISA (on homogenates). Total RNA extracted from PepMV-infected material was also tested using mRT-LAMP

	PepMV detection					
	Total RNA			Homogenate		
	RT-qPCR	mRT-LAMP		mRT-LAMP		ELISA
Fold-dilution	Cq ± SD	t_{pos} ± SD	T_m ± SD	t_{pos} ± SD	T_m ± SD	(A_{405})
10	20.0 ± 0.0	9.2 ± 0.6	87.1 ± 0.0	6.0 ± 0.0	87.2 ± 0.0	3.3
10^2	23.0 ± 0.0	11.0 ± 0.7	87.0 ± 0.1	6.7 ± 0.6	87.0 ± 0.0	1.1
10^3	27.6 ± 0.0	15.2 ± 0.7	86.8 ± 0.0	8.0 ± 0.5	87.0 ± 0.0	0.3
10^4	30.2 ± 0.2	18.5 ± 2.8	86.8 ± 0.0	9.0 ± 4.3	87.0 ± 0.0	0.2
10^5	32.2 ± 0.2	26.2 ± 7.8	87.1 ± 0.1	15.7 ± 2.2	87.2 ± 0.0	– (0.1)
10^6	33.5 ± 0.1	–	n.t.	–	n.t.	– (0.1)
10^7	–	–	n.t.	–	n.t.	– (0.1)
10^8	–	–	n.t.	–	n.t.	– (0.1)
Healthy material	–	–	n.t.	–	n.t.	– (0.1)

RT-qPCR and mRT-LAMP measurements of each sample were made in triplicate, with mean Cq, t_{pos} and T_m shown.
SD, standard deviation; A_{405}, mean of two absorbance replicates measured (2 h incubation with substrate); –, negative result; n.t., not tested.

Table 5 Sensitivity of the RT-LAMP assay for detection of PSTVd in crude tomato leaf homogenates compared to RT-qPCR analysis (on extracted total RNA)

	RT-qPCR	RT-LAMP	
Dilution	Cp ± SD	t_{pos} ± SD	T_m ± SD
2	19.6 ± 0.1	14.0 ± 0.7	90.9 ± 0.1
2×10^{-1}	22.5 ± 0.2	20.0 ± 0.6	91.4 ± 0.1
2×10^{-2}	26.1 ± 0.2	21.8 ± 0.6	91.5 ± 0.0
2×10^{-3}	29.2 ± 0.0	22.2 ± 0.8	91.5 ± 0.2
2×10^{-4}	32.5 ± 0.1	–	n.a
2×10^{-5}	35.5 ± 0.1	–	n.a.
2×10^{-6}	37.2 ± 1.2	–	n.a.
2×10^{-7}	–	–	n.a.
Healthy material	–	–	n.a.

RT-qPCR and RT-LAMP measurements of each sample were made in triplicate, with mean Cq, t_{pos} and T_m shown.
SD, standard deviation; –, negative result; n.a, not applicable.

method as an example of a more complex water sample. This method provided satisfactory concentration of these samples, as the RT-qPCR Cq dropped by 7 cycles after the concentration step. Many arid countries reuse wastewater for irrigation and therefore being able to concentrate PepMV from such water could be of help in preventing or identifying a potential infection source.

The present study has also demonstrated that the concentration of water samples can be scaled up. Using a large 1 mL CIM monolithic column, a 3 L tap water sample was concentrated. Most of the PepMV was also eluted in the same volume of buffer as for the small CIM monolithic column size, and therefore this was the most successful concentration step tested, decreasing the RT-qPCR Cq by >11 cycles, increasing the signal more than 1000-fold. Given that the manufacturer also offers these CIM monolithic columns at 8 and 80 mL, this method could be scaled up even more to process greater and more representative sample volumes. Even if the results using spiked samples show high sensitivity, testing of field water samples where PepMV occurs naturally should be performed as a definitive confirmation of the usefulness of CIM monolithic columns in such applications.

CIM monolithic columns have great potential for adaptation to be used on-site, as already demonstrated for waterborne rotavirus (Gutiérrez-Aguirre *et al.*, 2011). This would eliminate the need for transportation of several litres of each irrigation water sample to the laboratory. The concentrated samples could be analysed rapidly and on-site using the mRT-LAMP optimized in this work. The need for 10-fold dilution of the sample would be compensated by the 1000-fold signal increase achieved by the CIM concentration.

Viroid concentration and the level of contamination may vary greatly between tomato seeds, therefore it is very difficult to recommend a sample size and number of seeds for a single test (International Standards for Phytosanitary Measures, 2015). In this laboratory, the 3000 tomato seeds were divided into 12 subsamples, which went through grinding and centrifugation, with individual and pooled samples going through RNA isolation and RT-qPCR assays (Fig. 4). In preliminary studies, it was also confirmed that the efficiency of RNA extraction is the same whether the tomato seed samples are prepared as described here or if they are ground in a mill after freezing in liquid nitrogen (data not shown).

In 2013 and 2014, five of 29 official tomato seed samples from the imported seeds were weakly positive using the Boonham *et al.* (2004) RT-qPCR assay. Here, the RNA was re-extracted separately from all subsamples (600 μL supernatant per subsample for extraction; as described above). The subsample with the highest titre of PSTVd (i.e. the one with the lowest Cq) was then chosen for further confirmation of PSTVd in the sample by sequencing of the conventional RT-PCR product.

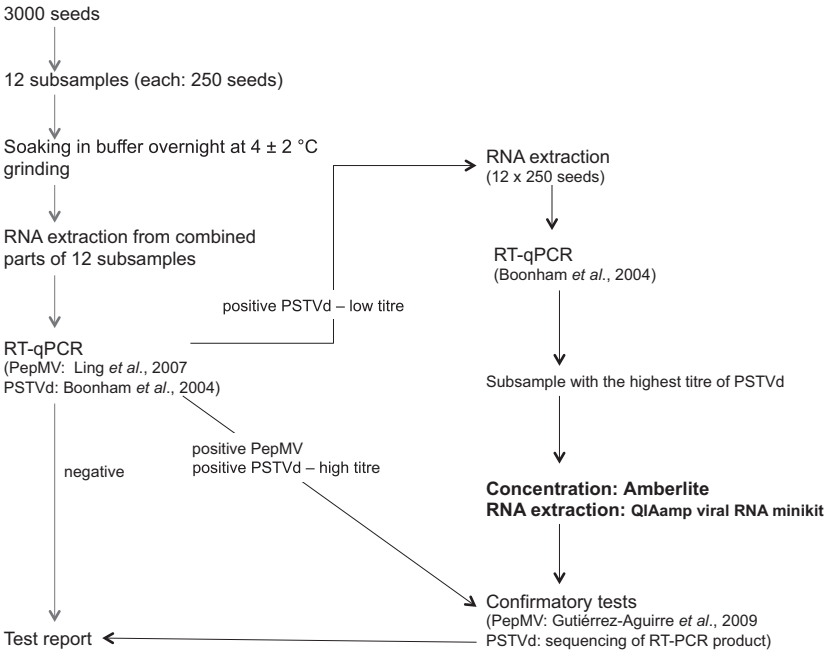

Figure 4 The proposed diagnostic procedure for detection and confirmation of PepMV and PSTVd in tomato seeds.

However, the concentration of PSTVd in some subsamples with the highest titre of PSTVd was still below the LOD of conventional RT-PCR (i.e. Cq > 30), which is required for reliable confirmation of PSTVd. Therefore, a procedure for concentrating PSTVd from the tomato seed extract based on binding to positively charged beads was also developed here to allow confirmation of PSTVd in these tomato seeds (Fig. 4).

Using the concentration procedure for PSTVd from tomato seed extracts developed in this study resulted in the greatest success for the required 197 bp amplicon using universal pospiviroid primers Pospi1-FW/RE, although this was not confirmed for the 359 bp amplicon using the Vid-FW/RE primers (data not shown). During the analysis of standard tomato seed samples, the sensitivity of the RT-PCR using the Vid-FW and Vid-RE primers was lower compared to the Pospi1-FW and Pospi1-RE primers (data not shown).

The concentration procedure based on binding to positively charged beads has the potential to be used for concentration of other pospiviroids from tomato seed extracts, as it was efficient also for CLVd. Additionally, pepper seeds have been reported to be contaminated with PSTVd (Lebas *et al.*, 2005), and therefore the method developed here should be useful for testing other related plants species in the future. In preliminary experiments, 3 h of mixing of the anion exchange resin with the supernatant of tomato seed extracts was more efficient for viroid concentration than 90 min (data not shown).

In the preliminary studies for concentrating PSTVd from the tomato seed extracts, the CIM monolithic chromatography supports were used, which have already been shown to be efficient for the concentration of highly diluted PSTVd in water samples (Ruščić *et al.*, 2013). However, concentration of viroid RNA from tomato seed extracts was not possible here using the CIM monolithic column because polysaccharides disturb the flow through the CIM monolithic column, and these are present at high concentrations in seeds.

Initially, the concentration step with positively charged beads was performed with artificially contaminated tomato seeds (data not shown). Here, when the tomato seeds were artificially contaminated with an extract from infected leaves, this concentration step was much less efficient than that seen in other studies where the tomato seeds were artificially contaminated with isolated RNA of PSTVd, or when naturally contaminated tomato seeds were used. It appears that residues of the leaf tissue can disrupt the binding of PSTVd to the positively charged beads, which would also be expected for the residues of tomato fruit tissue. Although tomato seeds from the Naktuinbouw collection (NIB V 276) were treated as commercial tomato seeds, there were some fruit tissue residues that might account for the less efficient concentration with the positively charged beads. Residues from tomato fruit tissue may also be attached to the surface of commercial tomato seeds. In such cases, it is possible that the concentration step will be less efficient. However, for all of the tomato seed samples, the isolation of RNA with the viral RNA minikits (QIAmp) was shown to be more efficient than isolation with plant minikits (RNeasy), which are one of the recommended kits in the International Standards for Phytosanitary Measures (2015).

The applicability of the RT-LAMP assay for rapid detection has been demonstrated here, and its potential use on-site for detection of PepMV and PSTVd in leaf tissue with symptoms, which are expected to have higher virus/viroid titres than water and tomato seeds. The RT-LAMP assay for PSTVd developed previously (Lenarčič *et al.*, 2013) was evaluated in this study on crude tomato

leaf homogenates. The sensitivity of this assay was lower than RT-qPCR, but similar to the sensitivity of conventional RT-PCR with primer sets described by Verhoeven *et al.* (2004) (Fig. 2; Lenarčič *et al.*, 2013). The RT-LAMP is not specific for PSTVd, but can also detect some other pospiviroids, e.g. *Tomato chlorotic dwarf viroid* (Lenarčič *et al.*, 2013), and therefore the confirmation of PSTVd in the sample requires sequencing of the RT-PCR product (International Standards for Phytosanitary Measures, 2015). Nevertheless, this RT-LAMP assay may be used on-site for fast screening of tomato leaves with symptoms.

The mRT-LAMP assay represented a combined assay from Hasiów-Jaroszewska & Borodynko (2013) and Ling *et al.* (2013), and this allowed detection of all of the described PepMV genotypes in the crude tomato leaf homogenate. In addition, melting curve analysis (T_m) of the amplified products provided the identification of the genotype present in these samples. However, in samples with two or three PepMV genotypes, only one of the PepMV isolates was amplified to give its specific T_m. The sensitivity of the optimized assay was equal to or lower than RT-qPCR and equal to or higher than the ELISA assay. Interestingly, when mRT-LAMP was carried out on crude tomato leaf homogenate or isolated RNA, no differences were seen in sensitivity. This emphasizes the appropriateness of this assay for rapid on-site testing of crude leaf homogenates without the risk of losing sensitivity, as already observed in a previous study on grapevine (Kogovšek *et al.*, 2015). With direct homogenate testing, higher levels of RNA can be retrieved in comparison to the RNA extraction procedure, which involves dilution and loss of RNA, leading to lower levels of target RNA in the final sample. This might be the reason for the observed lower sensitivity in comparison to RT-qPCR.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. Effects of different BSA treatments to mitigate non-specific binding of spiked PepMV to the CIM column. Concentration of the samples was performed as described in Table S2, at 3 mL min^{-1}. The RT-qPCR (Ling *et al.*, 2007) for each sample was in triplicate, with mean threshold cycle (Cq) shown.

Table S2. PepMV for the CIM column was spiked at various virus titres. RT-qPCR (Ling *et al.*, 2007) for each sample was in triplicate, with mean threshold cycle (Cq) shown.

Table S3. Results of the analysis performed on tomato seeds to determine the distribution of PSTVd-contaminated seeds in the seed lots. Joint RNA extracts from the individual subsample extractions (250 seeds each) and for the pooled 12 subsamples (total 3000 seeds). The RNA was isolated using RNeasy Plant mini-kits (QIAGEN) according to the manufacturer's instructions, with some minor modifications as described in the Materials and methods section. For PSTVd detection, the one-step RT-qPCR assay by Boonham *et al.* (2004) was used and performed as described by Mehle *et al.* (2014). A cytochrome oxidase (*COX*) gene-specific real-time RT-PCR assay was used as the endogenous control (Weller *et al.*, 2000). The RT-qPCR of each sample was in triplicate, with mean threshold cycle (Cq) shown. The identity of PSTVd was determined by sequencing of the RT-PCR product using Pospi1-FW/RE primer set (Verhoeven *et al.*, 2004) for at least one subsample per sample (in most cases this was possible only after using an optimized RNA extraction procedure, e.g. by including the concentration step).

Table S4. Specificity of the mRT-LAMP assay for PepMV, with mean t_{pos} and T_m given. To determine the mRT-LAMP performance in mixed infections, PepMV isolates were mixed and tested.

Table S5. Primers and probes for RT-LAMP, RT-qPCR and RT-PCR assays used in this study.

Table S6. T_m values of the mRT-LAMP amplified products for different PepMV isolates using two different devices (GenieII, Roche LC 480).

Table S7. Sensitivity of the mRT-LAMP assays for detection of PepMV (NIB V121 = CH2/US2) in crude tomato leaf homogenates compared to RT-qPCR (on extracted total RNA) and ELISA (on homogenates).