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Duplex RT-PCR assay for simultaneous detection of TSWV and CSVd in chrysanthemum

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ABSTRACT

A novel duplex RT-PCR assay for simultaneous detection of TSWV and CSVd in chrysanthemums was developed. Previous reported primers for amplification of TSWV and CSVd were used and a novel pair of primers for CSVd was designed to improve duplex amplification compatibility. Sensitivity and efficiency of the previous reported and novel primers for CSVd were assessed. Then, the sensitivity of the combined primers to amplify both TSWV and CSVd cDNA were also evaluated. Both TSWV and CSVd were detected in preparations diluted up to 10^{-4} and 10^{-5} respectively, from total RNA extracts. This duplex RT-PCR method showed an estimated diagnostic sensitivity (DSe) of 97% and diagnostic specificity (DSp) of 99%. For combination of the primers TSWV L1/ L2 and CSVd UCO-1 F/ UCO-1R, the protocol could detect pathogen RNA from naturally infected plants until 0.1 ng and 1 ng respectively. This novel protocol for detection of TSWV/CSVd represents a useful diagnostic tool without the need of expensive probes and less extensive laboratory work. This method could be helpful to assist the selection and further propagation of healthy chrysanthemums on the field as well as to understand the dynamics and the interaction of this virus and viroid within farms.

1. Introduction

Diagnosis of plant pathogens is the most important component in current integrated disease management since it avoids the entry of new pathogens in a farm or a region, allows the prevention of dissemination when the pathogen is already present and serves to monitor the effectiveness of the control measures that growers adopt (Hull, 2014; Kovalskaya and Hammond, 2014).

Tomato spotted wilt virus (TSWV) and *Chrysanthemum stunt viroid* (CSVd) are among the most important pathogens that affect chrysanthemum production in the world, since they cause decreases in productivity, quality and profitability (Horst and Nelson, 1997). For instance, in Colombia losses caused by TSWV and CSVd may reach US $ 2.7 million/year with incidences of 1%. This is particularly true in highly susceptible varieties such as Vyball, Zembla, Orinoco, Athos and Champagne (Ministerio de comercio industria y turismo de Colombia, 2016). In nursery areas the incidences can be as high as 3–4%, which is critical due its relevance in the production process (Unpublished data).

Classified as a member of the family *Bunyaviridae* and the genus *Tospovirus*, TSWV is an enveloped virus, which genome is composed by three single-stranded linear segments of RNA containing two glycosylated membrane proteins, a putative RNA-dependent RNA polymerase and a nucleocapsid protein (de Haan et al., 1991). Symptoms caused by infection with TSWV in chrysanthemum include chlorotic and necrotic spots on leaves and stems, as well as stunting and reduced number of flowers on the stem (Horst and Nelson, 1997). TSWV affects more than 1000 plant species and it is known to be transmitted by several species from the genera *Frankliniella* sp. and *Thrips* sp., in a circulative-propagative manner (Goldbach and Peters, 1994; Pappu et al., 2009; Parrella et al., 2003; Wijkamp et al., 1993).

CSVd is a viroid member of the family *Pospiviroidae*, genus *Pospiviroid*, consisting of a naked single-stranded circular RNA genome with no protein-coding sequences (Matsushita, 2013; Palukaitis and Symons, 1980). Symptoms induced by infection of CSVd vary among chrysanthemum cultivars, but in general they are plant stunting, young leaves become light green, the leaves and flowers become small and rooting ability decreases (Hosokawa et al., 2007). In comparison to TSWV, the host range of CSVd is smaller, including nine plant species (Cho et al., 2013; Di Serio and Flores, 2008; OEPP/EPPO, 1990).

For detection of TSWV some procedures are available which include tissue blot immunoassay (TBIA), ELISA (Cho et al., 1988; Louro, 1995; Whitfield et al., 2003), RT-PCR and real time RT-PCR (Munford et al., 1996; Roberts et al., 2000) and more recently mass spectrometry (Guy et al., 2015). For CSVd the list of available methods for detection is made up of RT-PCR, real time RT-PCR and hybridization (Gucek et al., 2017; Kim et al., 2015; Zhao et al., 2015). These methods may vary in

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Table 1
Primers used in this study.

Virus/Viroid	Primer name	Direction	Sequence (5'-3')	Expected size (bp)	[a]Ta (°C)	Reference
TSWV	TOSR5	Forward	GGGAGAGCAATYGWGKYR	709	55	Uga and Tsuda (2005)
	TSWV 709	Reverse	GTGTCATACTTCTTTGGGTC			
	TSWV L1	Forward	ATCAGTCGAAATGGTCGGCA	273	60	Mumford et al (1996)
	TSWV L2	Reverse	AATTGCCTTGCAACCAATTC			
CSVd	EPPO-CS1	Forward	CTTCAGTTGTTTCCACCGGGTAG	262	60	OEPP/EPPO
	EPPO-CS2	Reverse	TTCCTGTGGTGCACTCCTGACC			Bulletin 32, 2002
	UCO-1 F	Forward	GAGCTTAGGACCCCACTCCT	207	55	This work
	UCO-1R	Reverse	TTCCACGGGCTTACTCCCTA			

[a] Ta: Annealing temperature.

usefulness because of their sensitivity and their specificity (Boonham et al., 2002; Shcherbakova, 2007).

Multiple RT-PCR protocols for detection of TSWV with other viruses have been developed, in some cases for several tospoviruses (Munford et al., 1996; Uga and Tsuda, 2005) and for several viruses that share a host, like tomato, peanut, potato and ornamentals (Aparicio et al., 2009; Dobhal et al., 2015; Panno et al., 2012). In the case of CSVd detection, multiple RT-PCR has been developed for diagnosis of several pospiviroids (Botermans et al., 2013; Hosokawa et al., 2007; Olivier et al., 2014; Van Brunschot et al., 2014; Yanagisawa et al., 2017) as well as detection of CSVd with some viruses, different to TSWV (Liu et al., 2014; Song et al., 2013; Zhao et al., 2015).

Although dual infections of TSWV and CSVd can be found in the field, very few tests have been developed to evaluate them together. Besides this work, only Asano et al. (2015) has developed a method to detect both pathogens and *Dahlia mosaic virus,* in dahlias. As far as we know, there is not a duplex test to detect both pathogens that has been tested in naturally infected chrysanthemum before.

In this work, a diagnostic test for simultaneous detection of TSWV and CSVd in naturally infected chrysanthemums by means of RT-PCR was obtained. For this, experiments were carried out in two stages. The first stage was the standardization of the method using four combinations of primers in order to select the best one and the determination of its sensitivity by estimating the lowest amount of RNA that can be detected. The second stage was the comparison of ELISA and hybridization against this new duplex RT-PCR for the simultaneous detection of TSWV and CSVd, using naturally infected and healthy chrysanthemum plants.

TSWV and CSVd were detectable in preparations diluted up to 10^{-4} and 10^{-5} respectively, from total RNA extracts of naturally infected plants. This duplex RT-PCR method showed an estimated diagnostic sensitivity (DSe) of 97% and diagnostic specificity (DSp) of 99%. This novel protocol for detection of TSWV and CSVd represents a useful diagnostic tool without the need of expensive probes and extensive laboratory work. This method could be used to assist the selection and further propagation of healthy chrysanthemums on the field and also to understand the dynamics and the interaction of this virus and viroid within farms.

2. Materials and methods

2.1. Source of plant material, ELISA and hybridization assay

Chrysanthemum plants showing symptoms of TSWV or CSVd were obtained from commercial crops in Rionegro, Antioquia, Colombia. Presence of TSWV in symptomatic plants was confirmed by double-antibody sandwich DAS-ELISA assay (Agdia®, USA), following the protocol of the manufacturer. CSVd symptomatic plants were tested using a dot-blot hybridization assay (Agdia®, USA). The membrane was sent to Agdia® in USA to be developed. Plant breeding material from healthy plants was used as negative controls. For validation purposes, a total of forty-five positive samples for TSWV or CSVd and twenty-six negative samples were selected.

2.2. RNA extraction and quantitation

Total RNA was isolated from plant material by using 100 mg of foliar symptomatic tissue and a GeneJet RNA Purification kit (Thermo Scientific, USA). The RNA isolation process was made according to the manufacturer's protocol (Thermo Scientific, USA). Total RNA isolations were quantified using a Qubit® RNA HS (high sensitivity) assay kit (Thermo Scientific, USA), which is highly selective for RNA over DNA, protein or free nucleotides. All the measures were obtained using a Qubit® 3.0 fluorometer (Life technologies, USA).

2.3. Primers selection and design

Two sets of primers for TSWV and two for CSVd were evaluated. The two sets of TSWV primers were selected according to the EPPO/bulletin protocols for detection of this virus. One set of the CSVd primers was selected from EPPO/bulletins and the other one was designed using the Primer-blast designing tool from the NCBI (Altschul et al., 1990) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). To achieve this, sequences previously obtained in our lab were used from local isolates of CSVd amplified with the CSVd 1R/ CSVd 1 F primers, published by Chung et al., 2001 (Genbank accession N° AJ 001850). The set conditions for the design of the specific primers were: size of amplicon from 70 bp to 1000 bp, melting temperatures between 50–60 °C, at least 2 of total mismatches to unintended targets, including at least 2 mismatches within the last 5 bp at the 3' end. Ten pairs of predicted primers were selected. These were evaluated again in the Primer-blast tool in order to look for potential cross-amplification of TSWV. We chose one pair of primers that show no coincidence with TSWV sequences from Gene Bank at the last 5 bp at the 3' end of the CSVd primers. The four pairs of primers were sent to Integrated DNA Technologies to be synthetized (IDT, USA) (Table 1).

2.4. RT-PCR

According to the manufacturer's protocol, a reverse transcription was performed with a commercial kit for first strand cDNA synthesis using 100 ng of total RNA, random hexamers and superscript® III reverse transcriptase (Invitrogen, USA). PCR assay was optimized using 2µl of obtained cDNA, 200 µM of dNTPs (Thermo Scientific, USA), 2 mM of $MgCl_2$, 1X NH_4 reaction buffer (16 mM (NH4) 2SO4, 67 mM Tris – HCl (pH 8.8 at 25 °C and stabilizer) (Bioline, USA), 1 U of Biolase™DNA polymerase (Bioline, USA) and different combination of primers. Concentrations for each reverse and forward primer set were 0.5 µM for: EPPO-CS1/EPP- CS2, UCO-1R/UCO-1F, TOSR5/TSWV709 and 0.4 µM for TSWV L1/ TSWV L2. PCR was performed under the following conditions: initial denaturalization at 5 min at 94 °C followed by thirty-five cycles of melting for 30 s at 94 °C, annealing for 30 s at 55 °C and extension for 30 s at 72 °C. Final extension was performed for 5 min at 72 °C. For duplex PCR, the same conditions described above

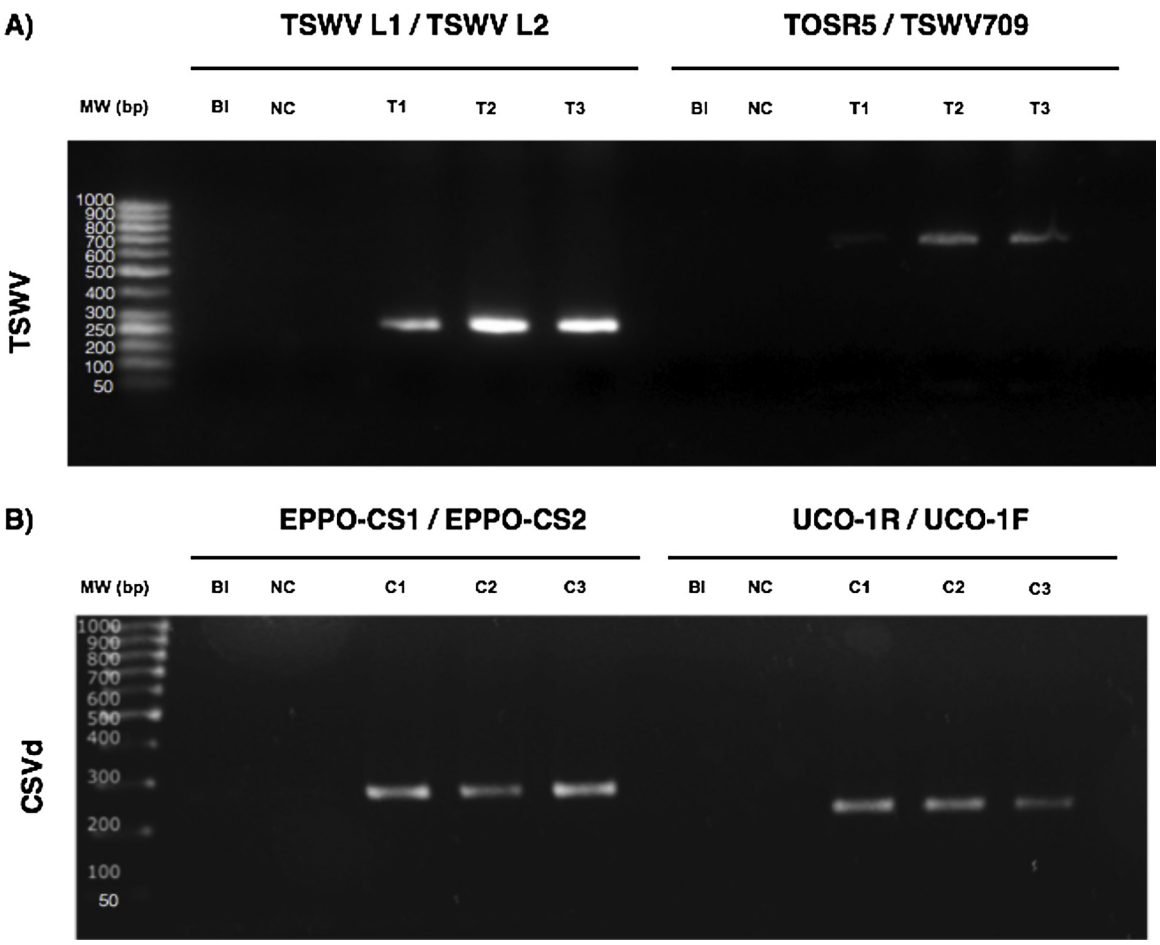


Fig. 1. Comparison of two sets of primers for amplifying TSWV and CSVd nucleic acids by RT-PCR in naturally infected chrysanthemums. A) RNA preparations from chrysanthemums naturally infected with TSWV from commercial crops (T1, T2 and T3) were tested by RT-PCR using two sets of specific primers (TOSR5/TSWV709 and TSWV L1/ TSWV L2). B) RNA preparations from chrysanthemums naturally infected with CSVd also from commercial crops (C1, C2 and C3) were tested by RT-PCR. Lane MW, molecular marker in bp. Lane Bl, blank with no DNA. Lane NC, negative control consisted in cDNA from non-infected plants.

were used using two sets of primers. PCR products were separated and visualized after electrophoresis on 1.7% agarose by staining with EZ-VISION™ DNA dye (Amresco, USA) and comparing band sizes with a GeneRuler 50 bp DNA ladder (Thermo Scientific, USA).

2.5. Sensitivity of single and duplex RT-PCR for TSWV and CSVd

For limit detection assays, 100 ng of RNA from TSWV or CSVd samples were serially tenfold diluted (10°–10^{-6}) in DEPC-treated water. The reverse transcription and PCR amplification was performed with the same conditions mentioned above. dsDNA from PCR products were quantified using a Qubit dsDNA HS assay kit, which is highly selective for dsDNA over RNA. For sensitivity of duplex RT-PCR a mixture of 100 ng of RNA from TSWV and CSVd infected plants were prepared. All the measures were obtained using a Qubit 3.0 fluorometer (Life technologies, USA). Individual and duplex PCR were carried out simultaneously to compare the products obtained by the dilutions. All the procedures were made by triplicate.

2.6. Sequence confirmation

To confirm that the products came from the original virus and viroid, the amplified PCR products were sent directly to Macrogen (Seoul, Korea) for capillary sequencing. These products were sequenced in two senses and by duplicate. Obtained sequences were compared to Gene Bank database through BLAST analysis.

2.7. Comparison between RT-PCR and two traditional commercial methods

In order to compare the efficiency of the RT-PCR protocol with the traditional methods we evaluated the sensitivity of DAS-ELISA for TSWV and a nucleic acid hybridization membrane for CSVd (Agdia®, USA). First, we took 100 mg of tissue from TSWV infected plants, which were ground in ELISA extraction buffer and then serially diluted tenfold (10°–10^{-6}) in the same buffer (Agdia®, USA). DAS ELISA was performed according to the Agdia® protocol for TSWV detection employing an anti-TSWV capture antibody (Agdia®, USA). 100 mg of CSVd infected tissue were ground in Ames extraction buffer (3% SDS, 20%Etanol, 1 M NaCl, 0.5 M Sodium acetate, 10 mM $MgCl_2$ and pH 6.0). The resulting extract were also serially diluted tenfold (10°–10^{-6}) and transferred to a 1.5 mL centrifuge tube and incubated for 15 min at 37 °C. Following the incubation, equal volume of chloroform was added. Finally, the membrane was spotted with the different samples following the manufacturer's protocol. The membrane was sent to Agdia® to be developed. For RT-PCR, 100 mg of symptomatic tissue was used for RNA extraction as it was described in section *2.3*, then the total RNA was serially diluted tenfold (10°-10^{-6}) and RT-PCR was performed with primer pairs described in Table 1.

2.8. Statistical analysis

Validation analysis and comparison between commercial protocols for TSWV and CSVd with PCR diagnosis results were conducted with a 2×2 contingency table. All the statistical analyses were performed

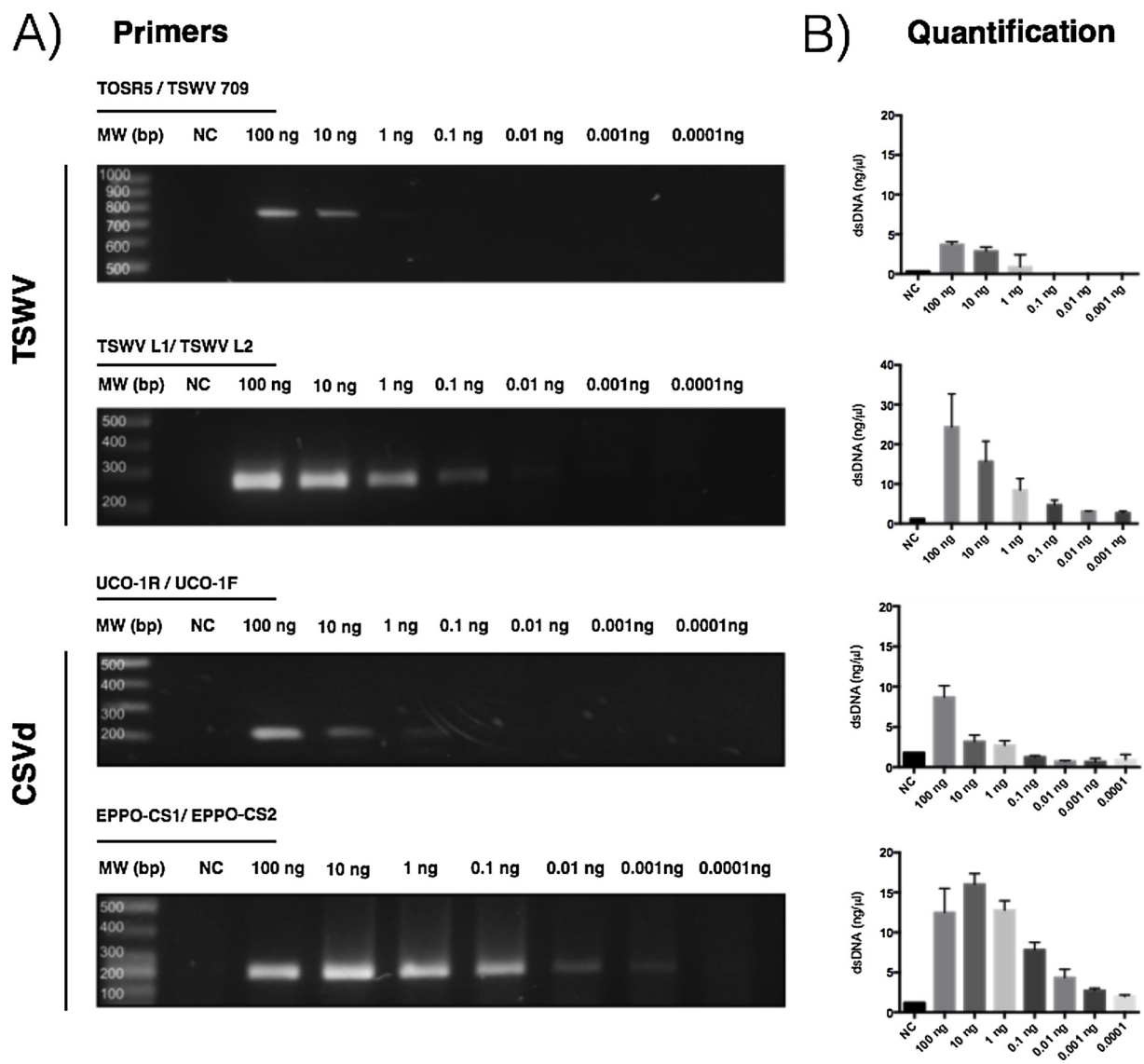


Fig. 2. Sensitivity assay of single RT-PCR for TSWV and CSVd. A) Amplification of target fragments from serially diluted RNA samples from TSWV and CSVd naturally infected plants. 100 ng of purified RNA were serially diluted in DEPC treated water and then amplified by RT-PCR using two different sets of primers for each pathogen as indicated. B) Amplification products from PCR reactions were quantified by fluorometry using Qubit assay for dsDNA. NC, negative control consisted in cDNA from non-infected plants.

using the statistical software package Graph-Pad Prism version 5.0 (*Graphpad Software Inc* 2007). Diagnostic sensitivity (DSe) and Diagnostic specificity (DSp) were calculated according to the manual of OIE for PCR diagnosis (World Organisation for Animal Health, 2008). Forty-five positive controls for each of the two pathogens and twenty-six negative controls were used to compare the commercial procedures with our PCR protocol. The confidence intervals were obtained by calculating the Receiver Operating Characteristic (ROC) curves using the infected and healthy plant samples. Statistical analyses were conducted with an interval of 95% of confidence.

3. Results

3.1. Evaluation of two pair of primers for each pathogen

The amplification with all tested primers was optimum. For the set of primers TOSR5/TSWV 709 and TSWV L1/TSWV L2 the obtained bands were of 709 and 273 bp respectively. The length of the obtained bands corresponded to the expected for each pairs of primers. Efficiency during the amplification of TSWV samples with the primers TSWV L1/TSWV L2 was better than TOSR5/TSWV 709 since by comparison of the intensity of amplicons, the second one was of lesser intensity (Fig. 1A). Efficiency during the amplification of CSVd samples of primers EPPO-CS1/EPPO-CS2 was similar to the amplification with the primers UCO-1R/UCO-1F by comparing the intensity of both bands. However, the C3 sample showed more intense bands with the EPPO-CS1/EPPO-CS2 set of primers (Fig. 1B).

Efficiency was further evaluated through a sensitivity assay. TSWV and CSVd positive samples that were serially diluted were tested by RT-PCR comparing and quantifying the amplicons obtained with different set of primers. The set of primers TOSR5/TSWV 709 showed an amplification of up to 1 ng of initial RNA while the TSWV L1/TSWV L2 showed an amplification up to 0.01 ng (Fig. 2A). The set of primers UCO-1R/ UCO-1 F showed amplification up to 1 ng while EPPO-CS1/ EPPO-CS2 primers showed amplification up to 0.001 ng (Fig. 2A). The quantification of the TSWV PCR products was higher with the TSWV L1/TSWV L2 set of primers and the quantification of CSVd PCR products was higher with the EPPO-CS1 / EPPO-CS2 set (Fig. 2B).

Same amounts of tissue from the same samples earlier employed were processed by conventional methods (ELISA for TSWV and

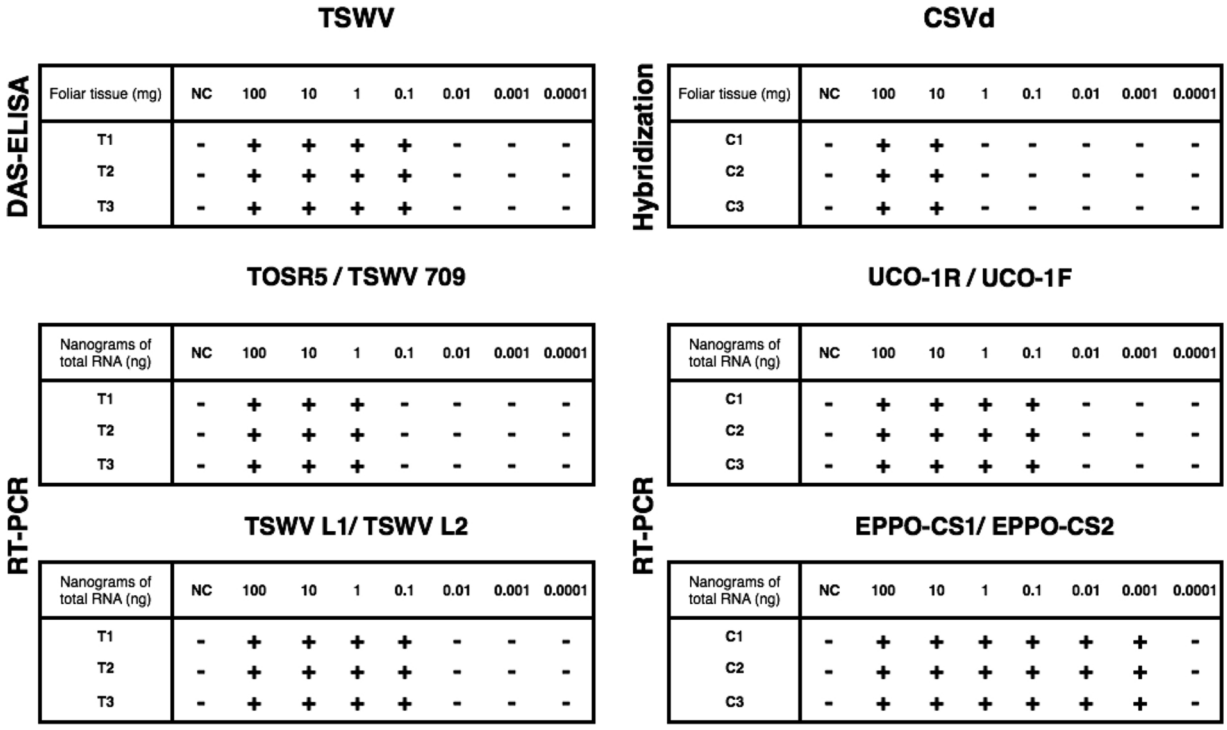


Fig. 3. Comparison of conventional and molecular methods for diagnosis of TSWV and CSVd. Foliar of tissue samples (100 mg) from naturally infected plants were used for testing each pathogen. For ELISA and hybridization assays, grinded and lysed tissue were ten-fold serially diluted and tested by ELISA or hybridization. For RT-PCR assays RNA was isolated and 100 ng of RNA was then ten-fold serially diluted. NC, negative control consisted in cDNA from non-infected plants.

Hybridization for CSVd) simultaneously and their sensitivity was estimated. The TSWV ELISA allowed us to detect a positive result up to 0.1 mg, whereas for CSVd detection by hybridization, the minimal amount that could be detected was 10 mg. Previous results of RT-PCR test with the tested set of primers and conventional methods were summarized and compared (Fig. 3).

3.2. Development of a duplex RT-PCR for TSWV and CSVd detection

In order to achieve a duplex PCR detection of pathogens different combinations of set of primers were tested. Initially the primers with the higher sensitivity were selected (TSWV L1 / TSWV L2 and EPPO-CS1/ EPPO-CS2). However, the amplification of CSVd was not achieved with this combination, since EPPO-CS1 / EPPO-CS2 align with TSWV sequences and this amplification is preferred. Eleven nucleotides at the 5' end of the primer EPPO CS1 aligned with L segment of multiple isolates of TSWV (data not shown). Therefore, the combination of TSWV L1/ TSWV L2 and UCO-1R/ UCO-1 F were selected to develop the duplex RT-PCR. Three samples of 100 ng of RNAs from TSWV infected plants and CSVd infected plants were mixed and tested by duplex RT-PCR (Fig. 4A). The band of 273 bp corresponds to the TSWV amplified fragment and the 207 bp band corresponds to CSVd. Further analysis of sensitivity shows that CSVd band was observable up to 1 ng of initial RNA and TSWV band was observable up to 0.1 ng of initial RNA (Fig. 4B and C).

All the obtained amplicons for TSWV and CSVd with the four sets of primers were sent to Macrogen (Soul, Korea) for capillary sequencing. Identities of both pathogens were confirmed by BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences matched with 99% of identity in all cases with multiple isolations of TSWV or CSVd (Data not shown).

3.3. Statistical validation of RT-PCR diagnostic test for TSWV and CSVd

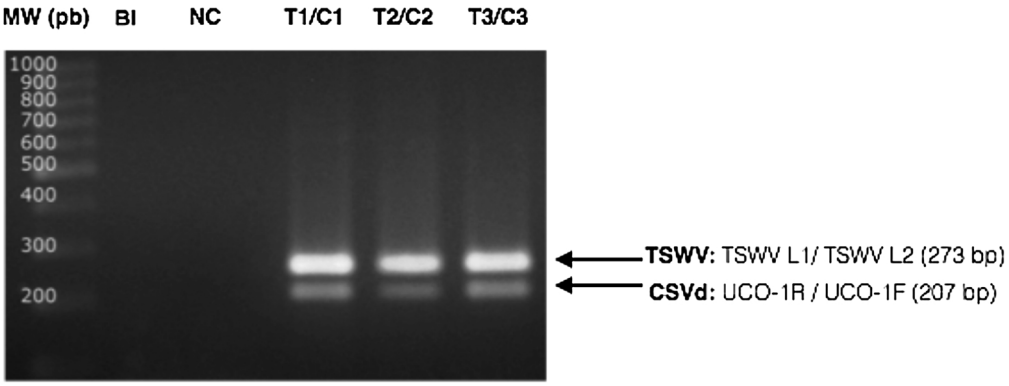
To validate the test developed in this work, a ROC curve was obtained based on calculated DSp and DSe. These values were calculated using forty-five known positive samples and twenty-six known negative samples according to the recommendations of OIE for terrestrial manual for a confidence interval of 95% (World Organisation for Animal Health, 2008). All known positive samples resulted as true positives and all known negative samples resulted as true negatives. No sample resulted as false positive or false negative. The obtained DSp was 99% with a CI of 0.3383 to 0.6617 and DSe was 97%, with a CI of 0.3800 to 0.6200. These results show that this test is sensitive and specific with a 95% of confidence (Table 2).

4. Discussion

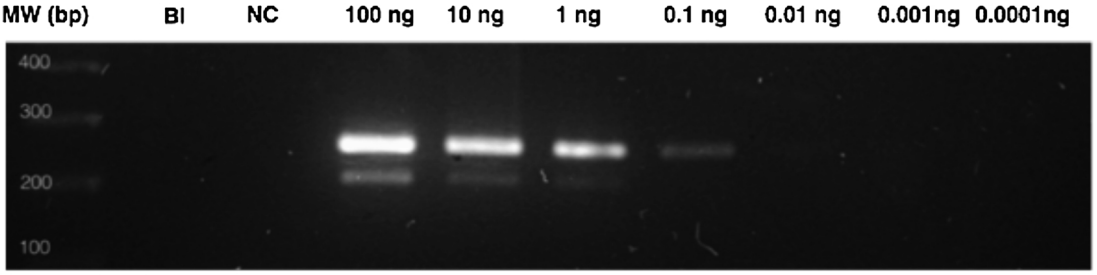
Serological kits for detection of TSWV are commonly used in most diagnostic laboratories and two formats are currently available: DAS-ELISA and immunostrip (Agdia®). For detection of CSVd, Agdia offers a northern blot hybridization method commercially®. However, many RT-PCR methods for single detection of these pathogens have been published but not a duplex protocol for TSWV/CSVd in chrysanthemum (Debreczeni et al., 2011; Gobatto et al., 2014; Kim et al., 2015; Mason et al., 2003; Seepiban et al., 2015). A recent investigation has developed the simultaneous detection of TSWV, *Dahlia mosaic virus* (DMV) and CSVd in dahlia plants, but not in chrysanthemum (Asano et al., 2015). Although ELISA and immunostrip are still easy and cheap methods to evaluate many samples of TSWV on the field, PCR is becoming more accessible and less demanding every year. Particularly, duplex RT-PCR assay has become a convenient, rapid and cost-effective tool for the identification of different pathogens (Elnifro et al., 2000; Markoulatos et al., 2002).

This work aimed to develop a rapid and sensitive method to detect simultaneously TSWV and CSVd in naturally infected chrysanthemums. Therefore, four primer set were evaluated and the best combination of two pairs were selected. The developed assay showed approximately ten times higher analytical sensitivity than DAS-ELISA for TSWV detection and hybridization for CSVd. Both TSWV and CSVd were

A)



B)



C)

Nanograms of mixed RNA (ng)	NC	100	10	1	0.1	0.01	0.001	0.0001
TSWV	-	+	+	+	+	-	-	-
CSVd	-	+	+	+	-	-	-	-

Fig. 4. Duplex RT-PCR for TSWV and CVSd detection and determination of its sensitivity. A) Duplex RT-PCR assay performed using three samples of mixed TSWV/ CSVd RNA from naturally infected plants. B) Ten-fold serial dilution assayed by RT-PCR using 100 ng of mixed RNA from naturally infected plants with TSWV and CSVd. C) Table showing the positive amplification for the limiting dilution for each pathogen in the duplex RT-PCR. Lane Bl, blank with no DNA. Lane NC, negative control consisted in cDNA from non-infected plants.

Table 2
Validation parameters for the RT-PCR test for single detection of each pathogen.

	Number of samples	
	Known positive samples (45)	Known positive samples (26)
TSWV RT-PCR	DSe of 97%	DSp of 99%
RT-PCR	TSWV TP (45)	TSWV FP (0)
Positives	CSVd TP (45)	CSVd FP (0)
RT-PCR	TSWV FN (0)	TSWV TN (26)
Negatives	CSVd FN (0)	CSVd TN (26)
	DSe (Diagnostic sensitivity)	DSp (Diagnostic specificity)
	Estimated DSe of 97 %	Estimated DSp of 99 %
	[a]CI = 0.3800 to 0.6200	[a]CI = 0.3383 to 0.6617

TP, true positives. FP, false positives. TN, true negatives. FN, false negatives.
[a] Confidence interval (CI) was calculated with a ROC curve for a 95% of confidence for estimation of DSe and DSp.

detected in preparations diluted up to 10^{-4} and 10^{-5} respectively, for total RNA extracts. This multiple RT-PCR method showed an estimated diagnostic sensitivity (DSe) of 97% and diagnostic specificity of 99%. We found that by combining the primers TSWV L1/ TSWV L2 and CSVd-1 F/ CSVd-1R, we could detect up to 0.1 ng and 1 ng of RNA dilution endpoint respectively. Although this compared to the single detection reduced its sensitivity for CSVd by tenfold, the TSWV detection was not affected.

Nevertheless, a more refined and better design of primers could overcome this issue. If primers are designed from signature regions of the pathogen's genome, molecular methods based on nucleic acid-detection are more reliable, sensitive and rapid than most serological methods (Arif et al., 2014; Dai et al., 2013). Previously, the detection of multiple infections in a single host demanded individual diagnostic assays. Lately, multiplex RT-PCR has become a popular test even over single RT-PCR in routine diagnostics due to its reduced cost and efficiency (Chen et al., 2013). In addition, low cost tests with capacity to detect multiple targets in a single reaction encourage the evaluation of a higher amount of plant material, especially in early stages of the crop,

for example stock plants in propagation area, which makes it a very useful strategy in integrated management.

Other methods capable of detecting several pathogens are currently available, such as real time PCR, Luminex xTAG, microarrays and even robotics (Bald-Blume et al., 2017; Botermans et al., 2013; Choi et al., 2015; Gucek et al., 2017; Kim et al., 2015; Osman et al., 2017; Schor et al., 2016; Yanagisawa et al., 2017; Zhao et al., 2015) but they require more sophisticated and expensive equipment, which is often not available in some laboratories. The advantage of the method developed in this work relies in a good diagnostic sensitivity and specificity without the need of expensive probes and less extensive laboratory work. This method could be a helpful tool to assist with the selection and further propagation of healthy chrysanthemums, in the surveillance of TSWV and CSVd and to understand the dynamics and the interaction of this virus and viroid within farms.

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Competing interests

The authors declare that they have no competing interests.

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