Investigation note

Tomato spotted wilt orthotospovirus (TSWV) is not transmitted by tomato seed

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Abstract

To date, more than 231 viruses capable of being transmitted by seed are reported externally or internally, this transmission is one of the most important factors in the epidemiological development of diseases of viral origin of early infection. The spotted wilt virus of the tomato spot wilt orthotospovirus (TSWV), *Orthotospoviridae*) affects more than 900 plant families including Solanaceae, its main transmission is by vector insects such as thrips, however, little is known of its possible transmission through of seed. The objective of this work was to determine the possible transmission of TSWV by seed of infected tomato plants using serological techniques and RT-PCR in its detection. Tomato fruits were collected from plants naturally infected with TSWV previously analyzed by Das-Elisa and positive for the virus from four regions of the State of Mexico. Subsequently, the seeds of the diseased fruits were extracted, one group had the detection of the virus by Das-Elisa in embryo and testa and the other group of seeds were grown *in vitro* culture for 15 days. Once the seedlings were obtained, RNA extraction and RT-PCR were carried out, using nucleocapsid-specific oligos. *In vitro* embryo, seed and seedling analyzes with Das-Elisa and RT-PCR (respectively) were found to be negative for TSWV in the treated samples. Apparently, there is no virus infection in *in vitro* embryo, testa and tomato seedlings.

Keywords: *Solanum lycopersicum* L., *in vitro*, Das-Elisa, RT-PCR, seed, TSWV.

Reception date: July 2019
Acceptance date: August 2019

Plant viruses can be disseminated by mechanical transmission, vegetative propagation, vectors, parasitic plants, pollen or seed. Among these forms of transmission, those occurring by seed are of vital importance, since it constitutes one of the most determining factors in the epidemiological development of many diseases. The transmission of virus by seed is of great interest because it causes: 1) reduction in germination; 2) wide dissemination in the field; and 3) source of primary inoculum in the development of diseases in various agricultural crops (Sastry, 2013).

The transmission of virus by seed in plants and weeds has been studied since the 50's, in that period of time eight species of virus transmitted by seed were reported; to date, more than 231 viruses capable of being transmitted through seed are described externally where the viral particle is present in the seed cover or internally when it is inside the seed in the embryo or in the endosperm (Sastry, 2013), as detected using techniques with electron microscopy and serology with bean common mosaic virus (BCMV), pea seed-borne mosaic virus (PSbMV), tobacco ringspot virus (TRSV), lettucce mosaic virus) LMV, turnip yellow mosaic virus (TYMV) and cucumber mosaic virus (CMV) and with beet curly top virus (BCTV) and beet curly iran virus (BCTIV) in petunia seeds, in these the presence of the virus was confirmed by PCR and IC-PCR respectively (Anabestani *et al*., 2017).

However, due to the presence of some virus in seed, its prevalence declines as it matures, due to the drying that occurs during this process, as demonstrated by rice yellow mottle virus (RYMV) in rice seed. Similar non-transmission was reported in bean seed infected with Cowpea chlorotic mottle virus (CCMV) due to dehydration of the seed during its development. Jain *et al* (2006), report that some viruses transmitted through seed influence the virus-host interaction involved, the race of the virus involved and environmental conditions, as observed in the transmission of tomato streak virus (TSV); through, pumpkin seed (*Cucumis sativus*).

On the other hand, Prasada *et al*. (2009) did not obtain the same result with this same virus in peanut and sunflower. Regarding orthhotospovirus, tomato spotted wilt orthotospovirus (TSWV) was not transmitted in the test of mature peanut seeds but was detected in freshly harvested ones (Reddy *et al*., 1983). Likewise, TSWV was reported in *Jacobaea maritima* seed (senecio= cineraria); however, the serological tests were not completely reliable due to their low prevalence (1% infection) and especially because the virus was present in the testa and not in the embryo (Zitter, 1991; Sastry, 2013).

Pappu *et al*. (1999) showed that TSWV was located in pods and peanut testa through ELISA and PCR techniques in symptomatic plants to virus infection. TSWV was occasionally detected in the cotyledons by PCR while, in seedlings obtained from seeds previously evaluated by Elisa, no positive cases were identified, suggesting that the accumulation of TSWV in peanuts is in the shell and testa without passing to the progeny. Kritzman *et al*. (2007) reported that iris yellow spot orthotospovirus (IYSV) was not transmitted to onion seedlings (*Allium cepa*) of infected mother plants.

Hajimorad *et al*. (2015) mention the absence of transmission of the Soybean vein necrosis virus (SVNV) orthotopic virus in soybean (*Glycine max*). Authors such as Groves *et al*. (2016) reported for the first time the transmission of the Soybean vein necrosis virus (SVNV) up to 6%; through the analysis of germinated seedlings of soybeans (*Glycine max*) by PCR. It is well known that the

multiplicity of factors affects the transmission of virus by seeds, for example: the virus strain, the host species, the cultivar, the environment during the production of the seed and the inoculation time of the plant.

Such dissimilarities in the transmission of viruses make it necessary to confirm their transmission using molecular techniques that better clarify this phenomenon. The objective of the work was to determine the non-transmission of TSWV in seed of infected tomato plants through the use of Das-Elisa serological and molecular RT-PCR techniques.

During the months of June to September 2016, samples were directed to plants with characteristic symptoms of TSWV such as tanning and small rings on the leaves and fruits in the municipalities of Villa Guerrero, Villa Victoria, Chiltepec and Tlachaloya, State of Mexico, in plots commercial tomatoes grown under greenhouse. The method used for the detection of the TSWV virus in leaves of plants with symptoms was Das-Elisa following the protocol established by the commercial house Agdia®. The absorbance reading was carried out on a Thermo Scientific Multiskan FC model spectrophotometer, at a wavelength of 405 nm.

The tests were carried out in duplicate, those readings whose optical density was greater than or equal to three times the average of the negative control were considered positive (Cruz and Frías, 1997). Subsequently, the seed was extracted from the fruits of the plants sampled with the presence of the virus, separating the mesocarp, washed with sterile water and placed on absorbent paper to be stored in paper bags until processing *in vitro* culture. Six treatments were formed based on their origin, including the positive and negative controls.

The Das-Elisa technique was performed as the first intention to demonstrate the detection of the virus in the testa and embryo. 12 seeds were disinfested in a wash with soapy water for 3 min and a rinse with sterile distilled water, subsequently the seed was immersed in a solution of 15% sodium hypochlorite for a few seconds and again rinsed with sterile distilled water. Finally, the seed was immersed in 75% ethanol for 30 s with its subsequent rinse in water. Following the infestation, through a stereoscope and two sterile needles the embryo was removed from the seed, separating the testa to process the virus detection separately.

On the other hand, the obtaining of germinated seedlings of seeds from the fruits of infected plants was carried out with the *in vitro* culture technique of (Murashige and Skoog, 1962). Initially the seeds disinfested; through a washing in water with detergent (0.6 g of soap in 100 mL of water) for 3 min plus a rinse with distilled water, then 15% of commercial chlorine was immersed in solution for 5 min plus a rinse with distilled water.

Finally, the seeds were immersed in 75% alcohol-ethanol for 30 s and rinsed with distilled water to sow in the culture medium. Eight seeds were sown per bottle (with 25 mL of MS culture medium, 1X free of plant growth regulators) that were incubated in a culture room with a 15-day controlled environment (temperature of 25 °C, 2 000 lux intensity and photoperiod of 16 h light plus 8 h darkness). RNA extraction from seedlings and corns from seeds of plants infected with the virus was performed with Trizol reagent, (Ambion®).

Once the RNA of each treatment was obtained, cDNA synthesis was performed by the reverse transcription reaction (RT) with 5 µL of total RNA with the RevertAid First Strand cDNA Synthesis Kit® (Thermo Scientific) using the degenerated TOS-R15 primer (Uga and Tsuda, 2005). Following this, an end-point PCR was performed with the Kapa3G Plant PCR Kits® kit (Bio Systems) and 2 µL of cDNA tempering with the primers: TSWV-709 Forward: 5'-GTGTCATACTTCTTTGGGTC-3'; TOS-R15 Reverse: 5'-GGGAGAGCAATYGWGKYR-3', which amplify a 709 bp fragment of the capsid region of the virus (Uga and Tsuda, 2005).

The amplification was carried out in a PTC-100 model MJ Research Thermal® thermal cycler, with a program consisting of an initial step at 95 °C for 2 min, followed by 35 cycles with three steps: 95 °C for 20 s, 60 °C for 30 s and 72 °C for 1 min, plus a final extension at 72 °C for 5 min and a conservation at 7 °C for 7 min (all tests were done in triplicate). The visualization of the products was carried out by electrophoresis in a 1.5% non-denaturing agarose gel with 1 µL of ethidium bromide, in a Syngene® transilluminator, model GVM20.

The results in all tomato plants from the field of the different regions with symptoms of TSWV, were positive for the presence of the virus with the Das-Elisa test (Table 1). In the sampled plants, the symptoms of tanning, irregular brown spots and some ring patterns on the epidermis prevailed, while the fruits revealed patterns of irregular spots of brown coloring and cracked appearance on their surface (Figure 1). The symptoms caused by *Orthotospovirus* are very varied, since it depends on the age of the plant, species of virus and environmental factors, many plants respond to viral infection with systemic symptoms (European Food Safety Authority, EFSA, 2012).

Table 1. Results of the Das-Elisa test of leaves of plants infected with TSWV, testa and seed embryo.

Location	Sample	Absorbance	Result	Sample	Absorbance	Result
Villa Guerrero	Leaf	0.175	Positive	Testa	0.014	Negative
				Embryo	0.002	Negative
Villa Victoria	Leaf	0.189	Positive	Testa	0.015	Negative
				Embryo	0.003	Negative
Chiltepec	Leaf	0.214	Positive	Testa	0.007	Negative
				Embryo	0.003	Negative
Tlachaloya	Leaf	0.197	Positive	Testa	0.004	Negative
				Embryo	0.003	Negative
Positive control*		0.143		Testa	0.111	
				Embryo	0.171	
Negative control*		0.039		Testa	0.021	
				Embryo	0.019	

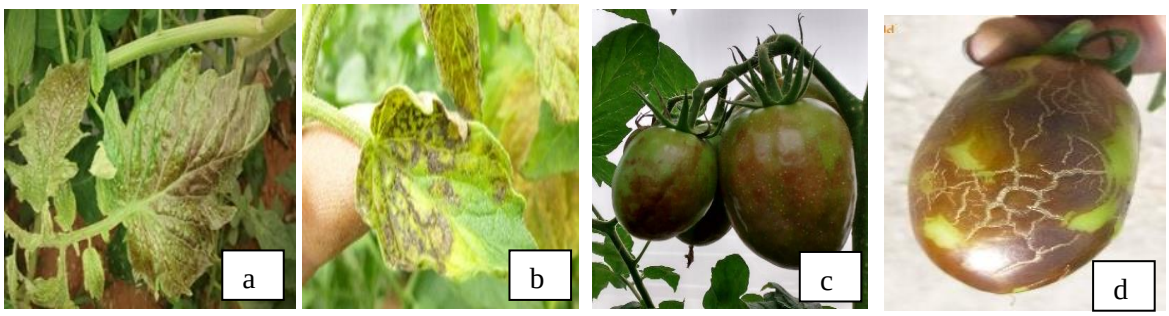

Figure 1. Symptoms of TSWV observed in foliage and fruits of tomato plants: a) foliage with tan; b) spots with concentric leaf rings; c) fruits with irregular brown spots; and d) fruit with dark and cracked spots.

The analysis by Das-Elisa in testa and embryo of seeds of the different regions were negative (Table 1), although the plants from which the seeds were extracted were positive in this test.

In this regard, Sastry (2013) points out that most viruses are unable to infect both stem cells of infected plants and developing embryos, due to the lack of a plasmodesmatal connection with the endosperm. Legumes are the family of plants with the highest frequency of virus transmission by seed, because in these, the connections are not interrupted during the development of the seed. Since 1969, Bennet mentions that for a virus to pass from one generation to another; through the seed embryo, a very determining factor is the ability of the virus to invade meristematic tissue and infect male or female gametophytes in early stages of development.

In addition to the gametophytic susceptibility to infection, which to date has been difficult to determine. Whitfield and German (2005) indicate that the *Orthotospovirus* are known for their exclusive transmission of thrips.

In vitro grown seedlings showed a normal appearance, without altering their development, color and height. In addition, callus development was observed only in the town of Villa Victoria, Mexico, (VVC). RNA extraction from these seedlings showed good concentration, quality and purity. The RNA was visualized on a 1% non-denaturing agarose gel and TAE buffer. Regarding the RT-PCR of the samples analyzed, no band amplification of the expected size was observed, as was the negative control (*in vitro* grown seedling from commercial seed).

On the contrary, the positive control (plant foliage infected with TSWV) showed the presence of a 709 bp amplicon. The products were observed on a 1.5% agarose gel. (Figure 2 B, C, D, E and F). The serological test like Elisa is very practical and reliable, but at present the high sensitivity and efficiency of the polymerase chain reaction (PCR) and its variants have proven to be the most efficient and safe for virus analysis (Eiras *et al*., 2001).

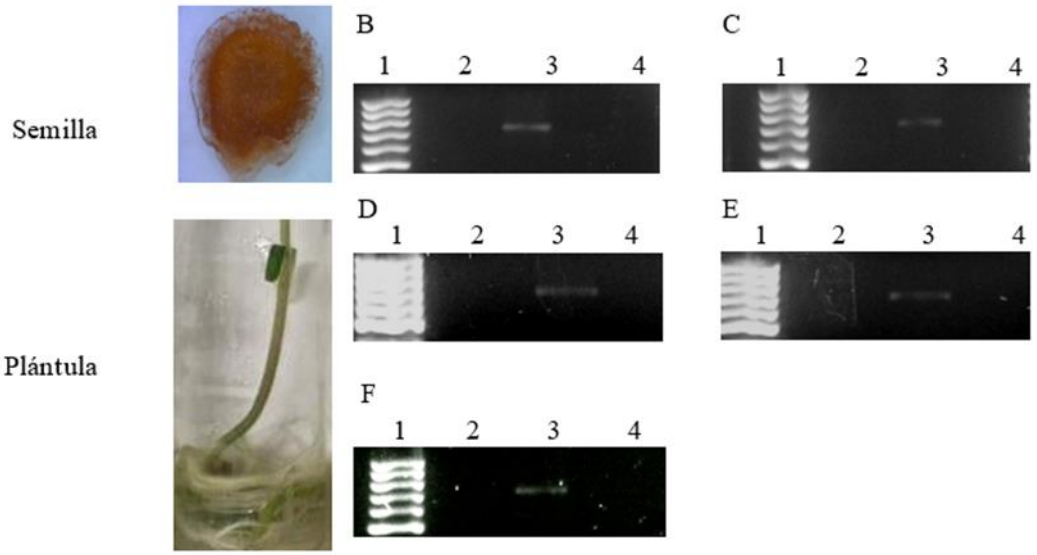

Figure 2. (A) Seed and germinated tomato seedling in vitro; (B) TSWV RT-PCR product from Villa Guerrero (VG) seedlings. Lane 1. Molecular marker (Mm) Thermo Scientfic (100 bp), lane 2. PCR product, lane 3. Positive control, lane 4. Negative control; (C) TSWV RT-PCR product of Villa Victoria seedlings; (D) Villa Victoria callus TSWV RT-PCR product; (E) Chiltepec seedling TSWV RT-PCR product; and (F) TSWV RT-PCR product of Tlachaloya seedling. 1.5% agarose gel, 65 volts, 60 min.

Conclusions

There is no transmission of TSWV virus in testa and in seed embryo from tomato-infected plants analyzed by the Das-Elisa technique, in the same way the transmission of the virus to seedlings from seed of infected plants analyzed by the RT-PCR technique was not detected.

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