Biological and Molecular Characterization of Tomato Spotted Wilt Virus in Israel

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Symptoms resembling tomato spotted wilt virus (TSWV) infections were documented among ornamental and vegetable crops in commercial greenhouses and open fields in Israel. Plants exhibiting these symptoms were collected from January 1992 to December 1996. Among cultivated plants analyzed for TSWV by enzyme-linked immunosorbent assay (ELISA), 19 species representing five families were found to be infected; natural infection was also recorded in six plant species of weeds. Virus identity was characterized by host range, serology and electron microscopy. Serological reaction with the isolates, found in Israel, using antisera from different sources as well as the sequence analysis of the nucleocapsid gene, demonstrated that the Israeli isolates of TSWV are a member of tospovirus serogroup I, type I (BR-01 strain). No virus transmission was found in seeds collected from virus-infected vegetable and ornamental crops. A non-radioactive molecular probe derived from the cloned nucleocapsid isolate enables specific detection of the virus in crude sap from infected plants. The detection of TSWV in Israel constitutes a severe potential threat to the ornamental and vegetable industry.

KEY WORDS: Tomato spotted wilt virus; tospovirus; serology; host range; biology; riboprobe; sequence; seed transmission; ELISA.

INTRODUCTION

Tomato spotted wilt virus (TSWV) belongs to the tospovirus genus within the Bunyaviridae, a large family of mainly arthropod-borne viruses (10). Tospoviruses are rated among the ten most devastating plant viruses (12,19). The impact of tospoviruses is enormous, mainly due to their extremely broad host range, which exceeds 650 different plant species in more than 70 distinct botanical families (13), and their worldwide distribution. The host range includes ornamentals such as *Aster, Vinca, Impatiens, Ranunculus* and *Zinnia*, and vegetable crops such as *Capsicum annuum, Lycopersicon esculentum, Lactuca sativa* and *Solanum tuberosum,* as well as *Arachis hypogaea*. At least five species have been distinguished within the genus *Tospovirus*, based on serological properties and nucleotide sequence. The established species are TSWV (type species), impatiens necrotic spot virus (INSV), tomato chlorotic spot virus (TCSV), groundnut ringspot virus (GRSV) and watermelon silver mottle virus (WMSMV) (13,18).

TSWV is transmitted by a limited number of thrips species in a persistent manner (12,28). The western flower thrips (WFT), *Frankliniella occidentalis*, is the most efficient

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vector of the virus (22,23). WFT was identified for the first time in Israel in 1991 on a wide variety of plant hosts in the open field and in greenhouses (3) and TSWV was identified in 1992 (2,11). The latter virus contains four structural proteins: a nucleocapsid protein N (29 kDa), two glycoproteins, G1 (78 kDa) and G2 (58 kDa), and a large protein L of approximately 200 kDa (12,17). The viral genome consists of three single-stranded RNA molecules, denoted S RNA (2916 nucleotides, nts), M RNA (4821 nts) and L RNA (8897 nts) (8,9,18).

This study was initiated to identify plant species that may serve as hosts for TSWV in major ornamental and vegetable production areas of Israel. Here we report the occurrence of TSWV in several cultivated and weed crops, the characterization of the Israeli isolate of TSWV and a procedure for its diagnosis.

MATERIALS AND METHODS

Plant material and virus culture

Disease surveys were conducted from January 1992 through December 1996 in major flower- and vegetable-growing regions in Israel. Samples of cultivated plant species were selected according to symptoms. To reveal new potential TSWV-reservoir hosts within wild plants, additional plant species were selected at random. Sap from plants to be tested for the TSWV presence was rubbed onto carborundum-dusted leaves of test plants grown from seed in an insect-proof greenhouse and sprayed periodically with insecticides. A single lesion isolate of the virus was excised from systemically infected leaves of *Nicotiana glutinosa* and used for further inoculations. A list of host plants used for this study is summarized in Table 2.

The virus was purified from either *Datura stramonium* or *Nicotiana benthamiana*-infected leaves by the method described by Mohammed *et al.* (17).

Enzyme-linked immunosorbent assay (ELISA)

TSWV infection in plants was determined by ELISA using commercial kits (Loewe Biochemica, Germany). Plant samples were prepared by grinding leaf, flower, root or fruit material of each sample. The serological identity of the Israeli isolate was studied further by using monoclonal antibodies produced by Adam *et al.* (1).

Electron microscopy

Crude plant extracts in 0.1 M phosphate buffer or virus preparations were negatively stained with 1% uranyl acetate and examined for virus in a JEOL 100CX II electron microscope.

Seed transmission

Mature seeds were collected from infected tomato and pepper fruits in the field, and *Celosia*, *Vinca* and *Petunia* grown in greenhouses. Seeds were washed with either water or 10% Na_3PO_4 (TSP) prior to ELISA or germination in sterilized soil. Seedlings as well as ungerminated seed from the same stock were tested for TSWV by ELISA as described previously (1,7).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots

Samples from semi-purified TSWV (density-gradient centrifugation in sucrose gradient) (17) and from healthy plants were analyzed in SDS-12% polyacrylamide (75 $\times$ 5 mm) gel electrophoresis (15). Phosphorylase b (97.4 kDa), bovine serum albumin (66.3 kDa), aldolase (42.4 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and lysozyme (14.4 kDa) were used as size markers. The gels were either stained with 0.1% Coomassie Brilliant Blue, or electroblotted to nitrocellulose (21) and probed with different antisera.

RNA isolation and cloning

Total plant RNA was isolated from TSWV-infected tobacco plants according to Verwoerd *et al.* (27). cDNA was synthesized using total plant RNA as a template and M-MLV reverse transcriptase (Promega) and a specific primer CP5-Bam: (5'-

TABLE 1. **Clinical Superiority**

	PULSATILLA 1000: based on transcranial electrostimulation	TENS: based on transcutaneous electrical nerve stimulation
• Point of contact	**Brain**	Peripheral nerves
• Electrode placement	**Fixed on the head**	Various parts of the body
• Efficacy	**Stand-alone treatment**	Part of multi-modal therapy
• Drawbacks	**None – Raises pain threshold *without* blocking pain signals to the brain.**	Blocks pain signals to the brain.

ACAACTTTTAGGATCCTCATGTCTAAGGTT- 3') corresponding to nucleotides 2783–2755 of the S RNA with a BamHI restriction site. The cDNA was amplified by PCR, using the above primer and primer CP3-Pst (5'- AACCTGCAGCTGCTTTCAAGCAAGTTC-3', corresponding to nucleotides 1972–1995 with a PstI site) (16). After amplification, the expected DNA fragment was digested with BamHI and PstI and ligated into similarly digested pBluescript (Stratagene, USA). The nucleotide sequence was determined by the dideoxy chain termination using dsDNA as a template. Sequence comparisons were performed using the DNAStar program.

Preparation of a molecular probe

The plasmid containing the nucleocapsid (N) gene of TSWV was linearized by BamHI for *in vitro* transcription by T7 RNA polymerase. cRNA transcripts were labeled with digoxigenin according to the manufacturer's instructions (Boehringer GmbH, Germany).

Dot blot hybridization

Samples from TSWV-infected plants were prepared and spotted onto Zeta-Probe GT nylon membrane (Bio-Rad, Hercules, CA, USA) according to Crosslin *et al.* (4). The membrane was hybridized overnight at 50°C with a digoxigenin-labeled probe (DIG Luminescent detection kit, Boehringer GmbH) at a concentration of $\sim$1.40 μg/ml, and then processed and washed according to the manufacturer's instructions.

RESULTS

Symptoms

Diseased plants showing vein clearing, stem necrosis, curling, necrotic spots and rings on the leaves were occasionally found in the field. The plants were small and stunted, compared with healthy plants. Among cultivated plants 20 species representing five families were selected at several places in Israel. They were *Asclepias tuberosa, Aster, Brassica oleracea* (cabbage), *C. annuum* (pepper), *Celosia* sp., *Cestrum* sp., *Cucumis sativus, Cucurbita pepo, Eustoma ruselliannum* (lisianthus), *Gerbera* sp., *Gloxinia* sp., New Guinea *impatiens, L. sativa* (lettuce), *L. esculentum* (tomato), *Ocimum* sp., *Solanum melongena* (eggplant), *S. tuberosum* (potato), *Vigna sinensis, Vinca* sp. and *Zinnia* sp.

Fig. 1. Symptoms of tomato spotted wilt virus on New Guinea *impatiens* (a), *Asclepias tuberosa* (b), *Eustoma rusellianum* (c) and *Gloxinia* (d).

The following symptoms were discerned: on New Guinea *impatiens*, chlorotic or black ring spot and line patterns in leaves (Fig. 1a), black lesions on stems, stunted plants, malformed leaves and terminal dieback; on *A. tuberosa*, severe leaf vein necrosis, necrotic ring spots in leaves (Fig. 1b), severe necrosis on stem and terminal dieback; on *Eustoma*, chlorotic or necrotic tan leaf spots, stem necrosis, necrotic terminals, color break and flower distortion (Fig. 1c); on *Zinnia*, mild mosaic and ring spots were seen on leaves; and on *Gloxinia*, reddish spots and/or concentric brown rings on the leaves and malformed flowers (Fig. 1d).

TABLE 2. Plant species found in the major ornamental and vegetable growing regions of Israel and found by ELISA to be infected with tomato spotted wilt virus

Species	Symptoms
Sonchus olearaceus	RS, SM
Solanum nigrum	RS
Conyza bonariensis	LD
Portulaca oleracea	LL, RS
Silybum marianum	RS, TN
Cichorium pumilum	LL

LD, leaf distortion; LL, local lesion; RS, ringspot; SM, systemic mosaic; TN, tip necrosis.

Fig. 2. SDS-polyacrylamide gel electrophoresis of the Israeli isolate of tomato spotted wilt virus (TSWV) (a) and Western blots with the following antisera: groundnut ringspot virus (b), tomato chlorotic spot virus (c), TSWV (BR-01) (d), and impatiens necrotic spot virus (e) (Loewe Biochemica GmbH). Lanes 1a and 2a are molecular weight markers and the structural proteins (N, G1, G2), respectively. Lanes b1-e1 represent virus mock preparations from healthy plants; lanes b2-e2 represent purified preparations of TSWV; lanes b3-e3 are prestained molecular weight markers.

Infected tomatoes were dwarfed and evinced chlorosis, narrowing and curling on younger leaves, followed by severe mosaic, necrosis and stunting; symptoms on mature fruit were blotchiness of color and rugosity of the fruit surface. In pepper (cv. 'Mazurka'), chlorotic and necrotic lesions, vein chlorosis and rugosity followed by leaf chlorosis,

severe growth reduction, and stem necrosis were observed. On lettuce, the disease generally started as necrotic brown leaf spots on one side of the plant, becoming systemic and extending to the heart leaves; the outer leaves wilt and the whole head collapses. Symptoms resulting from mechanical inoculation were similar in all respects to those observed in naturally infected plants.

Wild plants

The list of wild plants that were tested as hosts for TSWV includes 128 plant species representing 16 botanical families. The plants were collected at several locations in the north and center of Israel. Natural infections were detected in six plant species (Table 1). TSWV was not detected in the following weeds common to the ornamental and vegetable growing areas (numbers in parentheses represent total number of plants tested): *Amaranthus gracilis* (17), *Heliotropium rotundifolium* (4), *Lolium temulentum* (4), *Malva nicaensis* (9), *Panicum* sp. (2), *Prosopis farcta* (4), *Raphanus raphanistrum* (4), and *Solanum eleagnifolium* (4). In addition, the following wild hosts were mechanically inoculated in the laboratory with TSWV (denominator, number of plants used; nominator, number of plants infected): *Beta vulgaris* (4/10), *Calendula arvensis* (4/12), *Chrysanthemum coronarium* (3/7), *Conyza canadiensis* (2/10), *Notobasis syriaca* (2/8), *Silybum marianum* (2/8) and *Tolpis virgata* (3/17).

Host plants infected by mechanical inoculation

Using mechanical inoculation, 11 plant species representing four families were virus-infected (Table 2). Infected plants showed chlorotic and/or necrotic spots and rings on inoculated leaves, followed by systemic veinal mottle or necrosis. The virus infected systemically many solanaceous species, including *D. stramonium, N. rustica, N. glutinosa, N. tabacum* cvs. 'Samsun' and 'Xanthii'. These species reacted with local lesions or rings on inoculated leaves followed by mosaic or systemic necrosis. Mechanical inoculation of *N. benthamiana* and *N. clevelandii* plants resulted in necrotic and chlorotic local lesions on the inoculated leaves followed by vein necrosis, mosaic or mottling, and leaf deformation on fully-grown leaves. Local chlorotic and necrotic lesions followed by leaf wilting were observed in *Chenopodium amaranticolor, C. quinoa* and *C. sativus. Petunia hybrida* showed necrotic lesions in inoculated leaves. These plant species were not infected systemically.

Electron microscopy

Electron microscopic examination of TSWV-infected plant tissue demonstrated the presence of spherical enveloped particles which were 80–120 nm in diameter. In ultrathin sections of cells from infected plants, numerous spherical particles, similar in structure and size to those found in dip preparations, were always observed within membrane-bound cavities (data not shown).

ELISA

Presence of TSWV in the plants was determined by DAS-ELISA using commercial kits (Loewe, Otterfing, Germany). Antisera against the following isolates were used: of TSWV, BR-01 (Serogroup I, Type I); GRSV (A5) (Serogroup II, Type III), found in South Africa and Brazil; TCSV (B3) (Serogroup II, type II), found in Brazil; and INSV

TABLE 3. Host plant reactions to the Israeli isolate of tomato spotted wilt virus (TSWV)

Host plant	Reaction to TSWV	
	Local	Systemic
Amaranthaceae		
Gomphrena globosa	+	NS, LD
Chenopodiaceae		
Chenopodium quinoa	+	-
C. amaranticolor	+	-
Solanaceae		
Datura stramonium	+	CS, M, LS
Lycopersicon esculentum	-	M, LD, S
Nicotiana benthamiana	+	M, NS
N. tabacum	+	LD, NS
N. glutinosa	+	LD, NS
N. rustica	+	LD, CS
Petunia hybrida	+	-
Cucurbitaceae		
Cucumis sativus	+	-

Reactions to TSWV: +, local lesion; -, no symptoms; CS, chlorotic spots; LD, leaf distortion; M, mosaic; NS, necrotic spots; S, stunting.

(H7) (Serogroup III). Samples from field-infected pepper, tomato and lettuce plants were tested for their reaction with antisera against different strains of TSWV. Antigen (as crude sap from infected pepper) was diluted 1:30 before being applied to the plates coated with the different antisera. No reactions were obtained in ELISA using heterologous antiserum combinations of GRSV, TCSV or INSV (H7) except with TSWV-BR-01, which reacted positively (data not shown). The serological identity of the Israeli isolate was studied further using polyclonal and monoclonal antibodies developed by Adam *et al.* (1). The Israeli isolate reacted in DAS-ELISA only when the plates were coated with TSWV polyclonal antibodies. No reaction was found when plates were coated with INSV antiserum instead (data not shown).

TABLE 4. Effect of Na_3PO_4 treatment on the survival of tomato spotted wilt virus (TSWV) in seeds collected from infected pepper fruits [ELISA values are readings of single seeds using TSWV (BR-01) antiserum. Average of eight experiments and two replicates ± S.E.]

	$O.D._{405}$
Non-infested seed	0.065 ± 0.007a
Infested seed, untreated	1.900 ± 0.270b
Infested seed, treated with Na_3PO_4	0.070 ±0.007a

Values followed by the same letter do not differ significantly ($P<0.05$), according to a non-parametric one-factor ANOVA.

SDS-PAGE and immunoblotting

Purified virion proteins, when denatured with SDS and analyzed by PAGE, yielded three sharp bands, which, by analogy with TSWV, were G1 (78 kDa), G2 (58 kDa) and N (29 kDa) proteins, respectively (Fig. 2a). Immunoblots with the antiserum against the

TSWV BR-01 isolate gave a clear, specific and strong reaction with N protein and a weaker band with G2, as identified by their molecular weight and in comparison with healthy extracts (Fig. 2d, lane 2). Weak signals were obtained with N, and G2 proteins when these proteins were reacted with antiserum against GRSV (Fig. 2b, lane 2), and TCSV (Fig. 2c, lane 2). No signal was obtained when antiserum against INSV was applied (Fig. 2e, lane 2). The relatively small proportion of G1 in the viral preparations (Fig. 2a, lane 2) did not allow us to compare the reactivity of the antisera with this protein.

Cloning and sequencing of S RNA from TSWV

The gene coding for the nucleocapsid of TSWV was amplified using RT-PCR and cloned into the plasmid pBluescript. The N gene contains 777 nts which codes for a protein of 259 amino acids with a Mr of 28900. A nucleic acid comparison with the published sequence for the S RNA of the Brazilian TSWV isolate, BR-01 (8,16), revealed almost complete identity (97.4%), and the amino acid sequence was almost identical (98%) (Fig. 3). Only five amino acids differed out of 259.

Use of a molecular probe to detect TSWV in crude sap

To test the possibility of using a nonradioactive molecular probe to detect TSWV in plant extracts, crude plant sap extracts were prepared, as described in Materials and Methods. The samples were spotted onto membranes and hybridized with a digoxygenin-labeled riboprobe derived from the cloned virus N gene. As seen in Figure 4, a strong and specific reaction was obtained with sap from mechanically inoculated tomato and tobacco and a weak reaction was obtained with sap from mechanically infected pepper. The limit of detection by the probe was 0.1 ng of total RNA from infected *D. stramonium*. Spotting of 40 μl of diluted leaf extract of infected *D. stramonium* enabled detection at a dilution of 1:1000 (wt/vol). The probe gave no signals with extracts from mechanically inoculated eggplant or with healthy controls.

Seed transmission

Seeds were harvested from fruits of hot pepper (var. 'Sultan') showing severe necrotic symptoms. The presence of TSWV in fruit pericarp was confirmed by DAS-ELISA prior to seed collection. Seeds from non-infected plants were collected and served as controls. A portion of the seeds that were collected from infected fruits was stirred in a solution of 10% TSP for 2 h at room temperature. Seeds from the same batch were stirred under the same conditions in distilled water. After treatment, both groups were washed in running tap water and dried at room temperature. Each seed sample consisted of two seeds, homogenized separately with a mortar and pestle in the presence of 0.6 ml sample buffer, and tested by DAS-ELISA. The efficiency of seed treatment with 10% TSP was tested by ELISA. The values for seeds derived from infected TSWV plants were similar to those of seeds from non-infected plants (Table 3), indicating that the viral antigen was eliminated by the treatment. Similar results were obtained when seeds from infected tomatoes were tested (data not shown).

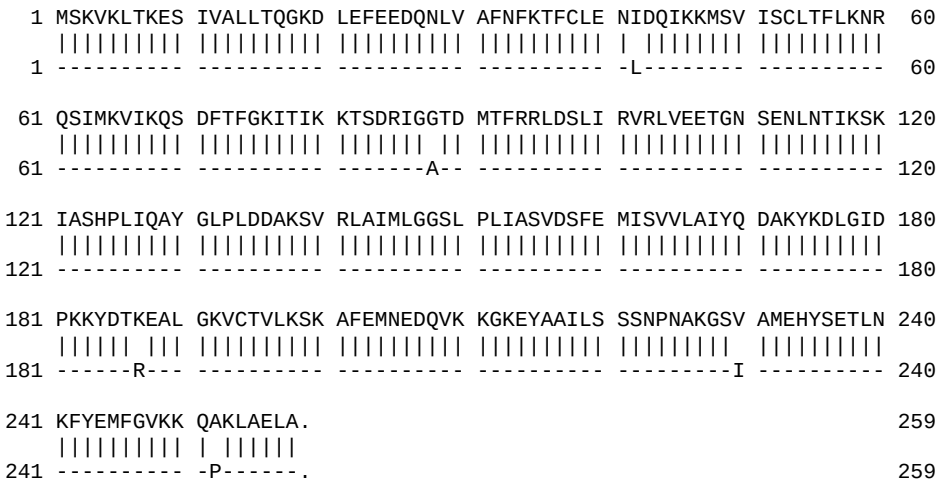

Fig. 3. The nucleocapsid deduced amino acid sequences of the Israeli tomato spotted wilt virus (TSWV) isolate (lower sequence) in comparison with TSWV- BR-01 (upper sequence). Vertical bars mark identical amino acids.

Fig. 4. Detection of tomato spotted wilt virus by dot blot hybridization using a digoxigenin-labeled riboprobe. Crude sap from mechanically inoculated tomato, 1 a, b; pepper, 2 a, b; eggplant, 3 a, b; tobacco, 4 a, b; field-infected tomato, 5 a, b; pepper, 5 c, d; healthy controls, 1 c, d - 4 c, d.

Seedlings produced from seeds of infected pepper (var. 'Maor') and tomato (var. 'Marmande') fruits were germinated in Speedling® trays and transplanted later to plastic pots. Seedlings were grown for 6 weeks before being tested for TSWV. Screening for TSWV started at the three-leaf stage. Plants were tested weekly for TSWV symptoms.

No visible symptoms were observed in Marmande tomato (0/300), Maor pepper (0/300), *Petunia* (0/185) or *Celosia* (0/115), up to 2 months after germination. All samples from these seedlings were also negative in ELISA. These results indicate that the virus is not seedborne.

DISCUSSION

This study describes symptoms associated with infection by TSWV among ornamentals, vegetables and weeds grown under various conditions in commercial greenhouses and open fields. Symptoms were variable and consisted of chlorotic spots, foliar necrosis, bronzing, rings and line patterns, stem cankers, internal necrosis and stunting that are characteristics of infection by TSWV. The symptoms on ornamental crops resemble those in the Netherlands (24,25). The virus also has been isolated from plants with mild symptoms as well as from symptomless plants. The latter are particularly important, as they may serve as a virus reservoir for other plants. Host plants mechanically inoculated with the Israeli isolate of TSWV produced symptoms that resemble those caused by the common isolate found in Europe (26). The outbreak of TSWV in Israel is associated with large populations of the western flower thrips, *Frankliniella occidentalis*. However, despite the wide distribution of this insect in the natural vegetation and in different crops (23), outbreaks of the disease are sporadic and limited. The detection of TSWV in Israel may have an impact on the plastichouse ornamental and vegetable industry due, in part, to the extremely large host range of TSWV. It is advisable, therefore, to maintain vegetable and ornamental plants separately.

All the Israeli isolates of TSWV obtained from either vegetable or ornamental plants were serologically identical and, on the basis of our results, can be clearly assigned to TSWV. Comparison of structural proteins among the Bunyaviridae viruses indicates that one or more of the three major structural proteins (N, G1, G2) is serologically related (17,18,19). In the present study a very close serological relationship was found between the N protein of the Israeli isolate and that of the type strain (BR-01). Sequence analysis of the N gene showed the Israeli TSWV isolate to be closely related to BR-01. Further characterization of the virus, based on detection of viral-specific nucleic acids, is useful for diagnostic purposes and can alleviate problems associated with a nonspecific background and with detection of serologically distinct isolates. Riboprobes and cDNA probes are available for diagnostic purposes (12,20). However, the disadvantage of these procedures is the need for special facilities when using radioisotopes, which restricts their use. Our riboprobe hybridized strongly and specifically to TSWV-infected samples with a detection limit of 0.1 ng of total RNA. No background reactions were observed when the riboprobe was tested with extracts from healthy plants. The development of a nonradioactive molecular probe and its successful use in detection of the virus in plant tissues, may further the adoption of viral-specific nucleic acids procedures for diagnostics.

The question whether TSWV can be transmitted through seeds has been raised often. The virus was reported to be seedborne by Jones (14), who determined 96% transmission in seeds of *Cineraria* sp. and tomato, whereas Crowley (5) found only 1% infection. In view of these conflicting reports, it was assumed that the virus is apparently carried in the testa, but not in the embryo. Our results show that although TSWV antigens were readily detected by ELISA on the surface of seeds harvested from naturally infected vegetables and ornamentals, we failed to prove virus transmission. Our inability to detect the virus by

ELISA in TSP-treated seeds indicates that the seeds are externally contaminated with the virus. Thus, it may be concluded that seeds from TSWV-infected plants are not able to pass the virus to the progeny plants. No visible symptoms were observed in tomato, pepper, *Petunia* sp. or *Celosia* sp. that derived from seeds harvested from TSWV-infected plants, up to 2 months after germination. These results indicate that the virus is not seedborne in the host plants used for seed transmission tests. However, it is well known that a multitude of factors affect seed transmission [*e.g.* virus strain, host species and cultivar, environment during seed production, and time of plant inoculation (6)]; this may explain the contradictory results obtained by different workers.

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