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Serological Detection and Symptomology of *Tomato Spotted Wilt Virus* in Tehran Province, Iran

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ABSTRACT

Tomato spotted wilt virus **(TSWV) was detected in tomato leaf and fruit samples collected from Varamin region in Tehran province using indicator test plants including *Petunia hybrida, Nicotiana glutinosa, N. tabacum* cv. Samsun NN, *N. clevelandii* and N. *benthamiana* and serological tests. Small browinsh local necrotic lesions appeared on *P. hybrida* leaves 2-4 days post-inoculation. Systemic symptoms included concentric ring spots on leaves, stem necrosis, wilting and tissue collapse of plants 7-10 days following the inoculation. Among 145 tomato samples collected from Ghazvin, Hashtgerd, Karaj, Malard, Shahriar and Varamin in Tehran province, only Varamin samples were infected with TSWV using ELISA, DIBA and SSEM. TSWV host range specificity and symptom expression were tested on *Capsicum annuum* L., *Chenopodium amaranticolor* L., *Citrullus vulgaris* L., *Cucumis melo* var. *inodorus, C. melo* var. *reticulatus, C. sativus* L., *Lycopersicon esculentum* Mill., *Phaseolus vulgaris* L., *Solanum melongena* L. and *S. tuberosum.* Typical symptoms on these plants included concentric ring spots, chlorosis, vein clearing, tissue necrosis, stunting and local lesion formation. Antiserum prepared against a partially purified TSWV preparation cross-reacted with TSWV-infected tomato samples.**

Keywords: DAS-DIBA , DAS-ELISA , SSEM , *Tomato Spotted Wilt Virus* , Tomato

INTRODUCTION

Tomato spotted wilt virus (TSWV) was first discovered on tomato plants in Australia in 1915 (Brittlebank, 1919). Samuel *et al.* (1930) studied the disease etiology and showed the causal agent to be a virus. Today, TSWV occurs in tropical, subtropical and temperate regions worldwide. Although TSWV causes severe and lethal infections and has been known for a long time, its physico-chemical properties have remained obscure due to difficulties in its purification and lack of stability. Recently, TSWV has been spreading rapidly in the Northern hemisphere, Western Europe and Asia through association with its major insect vector, the Western flower thrips (*Frankliniella occidentalis* Perg.) (Allen *et al.* 1986; Peters *et al.* 1991). Because of recent advances in molecular techniques and the production of some highly sensitive antibodies, TSWV detection has drawn a considerable attention as to its distribution.

TSWV was first reported in Varamin region of Iran by Bananej *et al.* (1996 and 1998). The economic importance of TSWV lies in the fact that it has a wide host range infecting more than 900 plant species among monocots

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and dicots including 85 families that are mainly ornamentals and vegetables. There are more than 180 host species susceptible to TSWV that belong to Solanaceae and 230 species to Compositae (D. Peters, 1998, personal communication, Peters *et al.* 1991). Yield losses due to TSWV infection can vary from 50 to 90% (Cho *et al.* 1986; German *et al.* 1992).

Recent molecular studies have revealed that TSWV is taxonomically related to the family of animal viruses known as *Bunyaviridae*. Therefore, the genus *Tospovirus* falls in *Bunyaviridae* and TSWV was designated as the type member of this new genus. Other viruses that are similar to TSWV but possess serologically-distinct nucleocapsid proteins include *impatiens necrotic yellow spot virus watermelon silver mottle virus, peanat bud necrosis virus* and *peanut yellow spot virus* (German *et al.* 1992).

Tospoviruses cause necrosis on various parts of the plant in addition to chlorosis, concentric ring spots, silver mottling, mosaic, stunting and local lesions. Symptoms may vary depending on virus, host and environmental conditions (German *et al.* 1992 and Ie, 1970). Host plants that are susceptible to *Tospoviruses* include ageratum, begonia, chrysanthemum, impatiens, lettuce, papaya, peanut, pepper, potato, tobacco and tomato. Symptoms induced by TSWV on tomato plants appear as brown spots on leaves, vessel browning, stem necrosis, brownish to black necrosis on leaf tissue, mosaic and leaf mottling, development of light green concentric ring spots with black centers on immature fruits, yellow spots discoloration on ripe fruits and subsequent wilting and complete collapse of plants (Allen *et al.* 1989 and McHugh, 1991). These symptoms appear on tomato plants 8 days after the inoculation in the greenhouse and the virus infectivity remains high for 3-4 days. *Tospovirus* can spread systemically in the host plant and is disseminated by different species of thrips (German *et al.* 1992 and Wijkamp *et al.* 1995).

Petunia hybrida seems to be one of the best indicator test plants for TSWV identification since typical small brownish to black local lesions appear on leaves as early as 2-3 days following inoculation (Selman and Milne, 1961). Among various serological methods available, ELISA (enzyme-linked immunosorbent assay) and DIBA (dot blot immunobinding assay) have been proven to be sensitive and reliable techniques for the detection of TSWV in infected plant sap as well as thrips (Cho *et al.* 1988, Gonsalves and Trujillo, 1986; Hsu and Lawson, 1991 and Peters *et al.* 1991). The use of riboprobes and cDNA probes recommended for the detection and identification of TSWV has been limited (Peters *et al.* 1991).

The main objectives in this research were to detect TSWV in tomato fields in Tehran province using serological techniques and to characterize symptom development and determine host-range specificity.

MATERIALS AND METHODS

Sample Collection and Preservation

Young tomato leaf and fruit samples suspected of TSWV infection were collected from various tomato fields in Tehran province during 1996-97, placed in plastic bags and kept in an ice box before preserving at -15°C in the lab.

Plant Growth Conditions

Seeds for indicator test plants were sown in a mixture of pasteurized compost, sand and soil in pots and irrigated regularly in a greenhouse with a temperature set between 20 and 25°C.

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Virus Inoculation on Indicator Test Plants

Indicator plants that were used in the inoculation tests were *Nicotiana benthamiana, N. clevelandii, N. glutinosa, N. tabacum* cv. Samsun NN and Petunia hybrida. (German *et al.* 1992; Ie, 1970). Virus-infected tomato leaf tissue was homogenized in 0.5-1 ml ice cold extraction buffer (phosphate buffer-Tween 80-PVP (10 g per liter) (pH 7.4)) in a mortar. The inoculum mixed with carborundum was rubbed on the leaf surface. The inoculated plants were subsequently sprayed with distilled water and then kept in the greenhouse. Prior to the inoculation, indicator test plants were kept in dark for 2 days to increase their sensitivity (Peters *et al.* 1991). Each inoculation test was repeated several times independently using three replicates in each experiment.

Serological Tests

DAS-ELISA-Plantest-ELISA kit manufactured by Sanofi-Pasteur Co. in France was used to monitor samples from suspected plants for the presence of TSWV. DAS (direct double antibody sandwich)-ELISA was performed according to the procedures provided by the manufacturer. Tomato leaf tissues were homogenized in the extraction buffer, centrifuged in Eppendorf tubes using an MSE microcentrifuge (1300 rpm, 5 min) at 4°C. Anti-TSWV polyclonal antibody was diluted in the coating buffer at $\frac{1}{1000}$ and 200 μl was pipetted into each well in a microtiter plate. The plate was incubated at 37°C for 2 hrs, washed three times with the washing buffer (PBS-Tween-20, pH 7.4). This was followed by the addition of 200 μl tomato extract, incubation at 37°C for 2-3 hrs and washing as above. Alkaline phosphatase (AP)-conjugated anti-TSWV antibody diluted at $\frac{1}{1000}$ in the conjugate buffer (PBS, Tween-20 and albumin, pH 7.4) was pipetted into each microplate well, incubated at 37°C for 2 hrs, followed by rinsing for three times. Para-Nitrophenyl phosphate, the substrate for AP, was dissolved in the substrate buffer (diethanolamine, pH 9.8) and added to each well. Yellow color development, indicative of positive reaction, was observed between 0.5-2 hrs. Healthy tomato samples were used as negative control.

DAS-DIBA (dot blot immunobinding assay)-Nitrocellulose membrane (Bio-Rad, Rich- mond, CA) (0.42 μm in diameter) was cut into strips (1.5x10.5 cm), soaked in TBS buffer (2.42g Tris-HCl, 29.24 g NaCl per liter, pH 7.5) for 15 min and then dried on a Watman filter paper. Two μl anti-TSWV antiserum was spotted in the center of each square (1.5x1.5 cm), after being completely dried, the strips were placed in a blocking solution (3% gelatin in TBS) for 30 min. Each square was spotted with 2 μl tomato crude extract in the center, and after 1 hr, the immunoblots were washed in TBS and TTBS (0.5 ml Tween-20 per liter of TBS), 5 min each. The blots were then incubated in TBS containing AP-conjugated anti-TSWV antiserum for 1 hr. This was followed by washing as above and incubating in the substrate solution (10 mg nitroblue tetrazolium (NBT) and 2.5 mg, 5-bromo-4-chloro-3-indolyl phosphate (BCIP) per 20 ml AP buffer). After color development, strips were soaked in a fixative solution (Tris-Na_2 EDTA, pH 7.5) for 5 min.

SSEM-(serologically specific electron microscopy). The virus morphology was studied using the method of Hampton *et al.* (1990). Electron microscope grids were treated with 0.5% Formvar in ethylene dichloride and dried in the open air. Grids were carbon-coated under vacuum in a sputter coater, treated with anti-TSWV antiserum for 30 min. and then washed with Tris buffer. Grids covered with TSWV antiserum were placed in virus-infected tomato sap for 1-4 hrs at

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4°C and subsequently washed in distilled water. Grids were floated over 2% uranyl acetate solution for 10 min., washed in distilled water and checked using Zeiss transmission electron microscope.

Symptomology and Host-range Determination

Plants used in studying symptom expression and determining host range specificity were tomato (*Lycopersicon esculentum* Mill), bell pepper (*Capsicum annuum* L.), eggplant (*Solanum tuberosum* L. watermelon (*Citrullus vulgaris* L.), cucumber (*Cucumis sativus*), melon (*C. melo* var. *inodorus*), cantaloupe (*C. melo* var. *reticulantus*), *Chenopodium amaranticolor* L., bean (*Phaseolus vulgaris* L.). Inoculation was done mechanically and negative and positive control plants were used as provided in the Sanofi kit.

Virus Partial Purification

TSWV purification was carried out at 4°C according to the method of Adam *et al.* (1995). Hundred grams of TSWV systemically-infected *N. glutinosa* leaf tissue was homogenized in 300 ml extraction buffer (100 mM K-phosphate buffer, 10 mM Na sulfite, pH 7.0) in a blender for 10 seconds. The homogenate was centrifuged at 10,000 xg in a refrigerated Beckman centrifuge for 10 min. The pellet was resuspended in 100 ml of resuspension buffer (10 mM K-phosphate buffer, 10 mM sodium sulfite, pH 7.0) and stirred very slowly for 30 min. This suspension was centrifuged at 1000 xg for 15 min. The supernatant was transferred into R28 Beckman tubes and centrifuged at 70,000 xg for 30 min. The pellet was resuspended in 2.5 ml resuspension buffer.

Antibody Preparation

One ml of partially purified virus preparation was administered intramuscularly into a New Zealand white rabbit using a syringe with 40 gauge needle (Hampton *et al.* 1990). Bleeding (10 ml) from the posterior vein on the ear was done once a week after the last injection. Following blood coagulation at room temperature, antiserum was separated at 4°C by centrifugation at 2000 rpm for 5 min. In order to remove non-specific antibodies, the antiserum was cross-absorbed with an equal volume of the crude extract from *N. glutinosa* healthy leaf tissue in a tube and incubated at 32°C for 2 hrs and occassionally shaked. The antiserum was then centrifuged at 7,000 rpm for 5 min. The antiserum was tested for cross-reactivity using ELISA with antibody from the first bleeding diluted at 1/2 , 1/4 , 1/8 and 1/16 for coating the microtiter plate as recommended by the Sanofi kit.

RESULTS

Indicator Test Plants' Reaction to TSWV

Petunia hybrida-Brownish small local necrotic lesions appeared on the leaves 2-4 days after inoculation (figure 1a).

Nicotiana glutinosa, N. benthamiana, N. Clevelandii, N. tabacum cv. *Samsun NN*-Local necrotic lesions appeared on the leaves 7-10 days after inoculation. Symptoms due to systemic infection were concentric ring spots on the leaves, stem necrosis and wilting followed by plant death (Figure 1b).

Host-range Determination and Symptom Development

Plants inoculated with TSWV exhibited various symptoms in the greenhouse as follows:

Tomato-Necrotic brown spots appeared on the leaves 7 to 10 days after the inoculation. The spots gradually enlarged, coalesced and caused a complete leaf necrosis. Virus became

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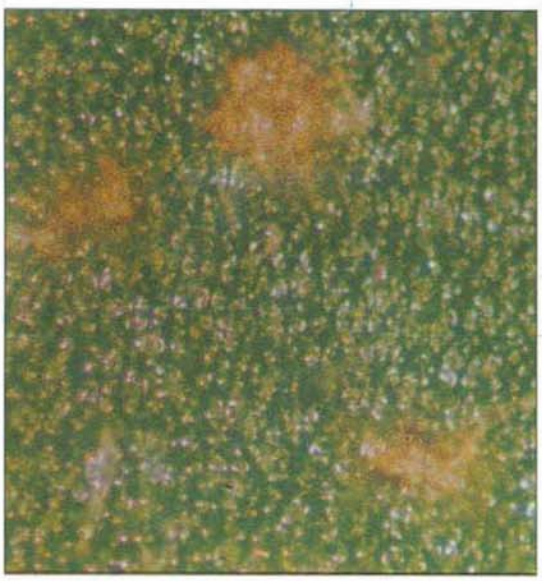

(a)

(b)

Figure 1. Symptom development on indicator test plants: (a) *P. hybrida* leaf showing small brown necrotic local lesions, 10x; (b) *Nicotiana glutinosa* exhibiting concentric ring spots on the leaf.

systemic 2 weeks after the inoculation and the stem tissue developed brown necrosis. Yellow concentric ring spots appeared on reddish fruits followed by wilting and complete collapse of the entire plant (figures 2a-d).

Bell pepper-Symptoms appeared as vein clearing in the minor veins and interveinal region 10-18 days after the inoculation and while the infection progressed, they turned into a mosaic and the tissue collapsed (figure 2e).

Eggplant-Light green to yellow mosaic patterns were observed in the interveinal region 10 to 18 days following inoculation. Chlorotic spots, thus formed, coalesced and turned into tissue necrosis. Plants showed severe wilting and collapsed (figure 2f).

Potato-Brown necrotic spots appeared on leaves 7-10 days-post inoculation and following their coalescence, leaves became completely necrotic. Necrosis was then spread to stem tissue which led to the collapse of the entire plant (figures 2g & h).

Watermelon-Brown spots along with ring spots appeared on the leaves 10-18 days after inoculation. These spots gradually enlarged and coalesced leading to leaf necrosis and collapse (figure 2i).

Melon, cantaloupe and cucumber-symptoms due to TSWV infection were similar in these plants. Chlorotic spots appeared on the leaves 1-2 weeks following the inoculation. These spots gradually coalesced leading to tissue necrosis in the leaf and stem, and plant death (figures 2j & k).

Chenopodium amaranticolor-Concentric ring spots appeared on the leaves 7 to 10 days after inoculation and following the systemic infection. Tissue necrosis, wilting and collapse of inoculated plants were observed.

Bean-Chlorotic spots appeared on the leaves 7-10 days post inoculation. These spots gradually coalesced resulting in tissue necrosis, wilting and plant death.

Serological Tests

DAS-ELISA- Among 145 tomato samples

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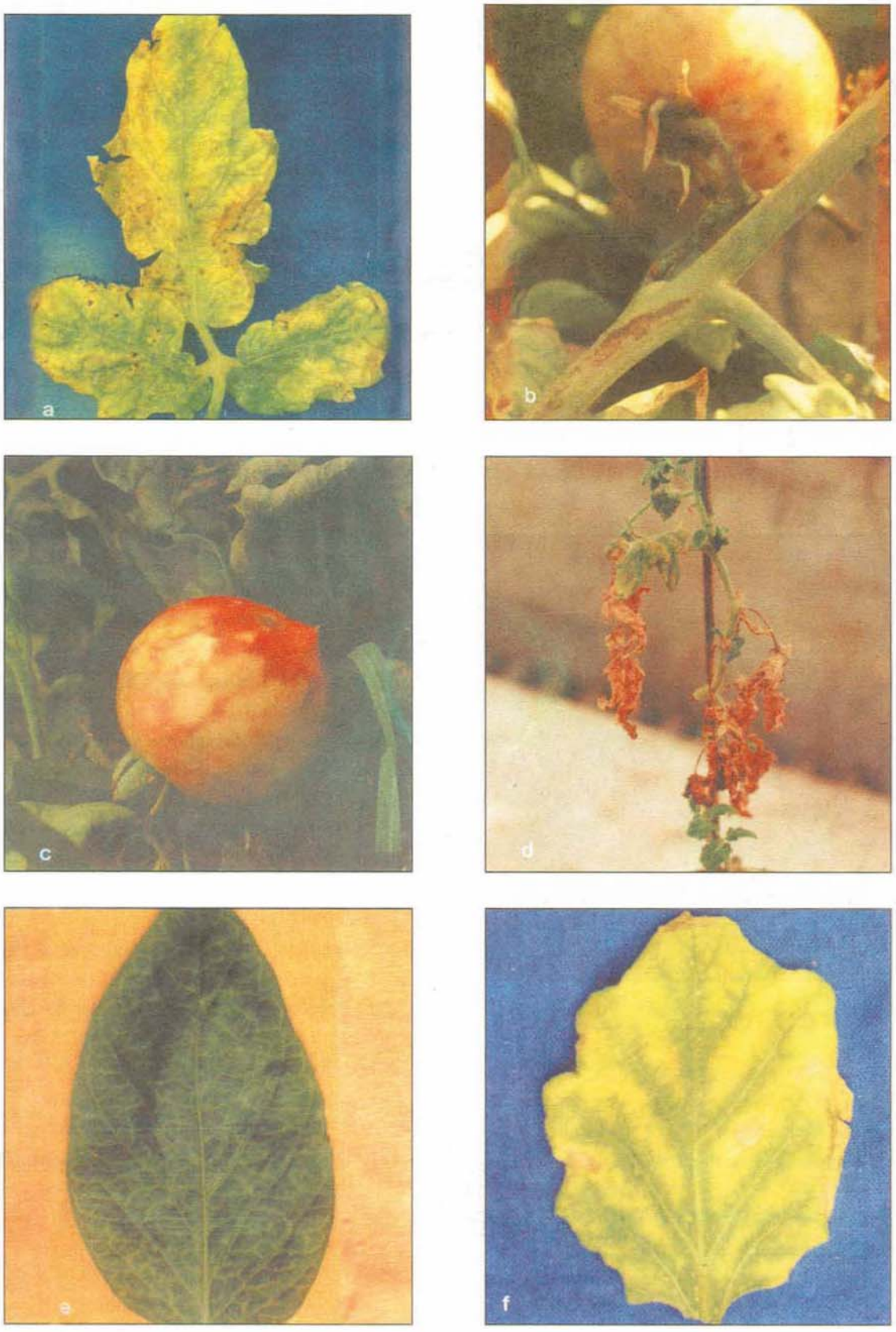

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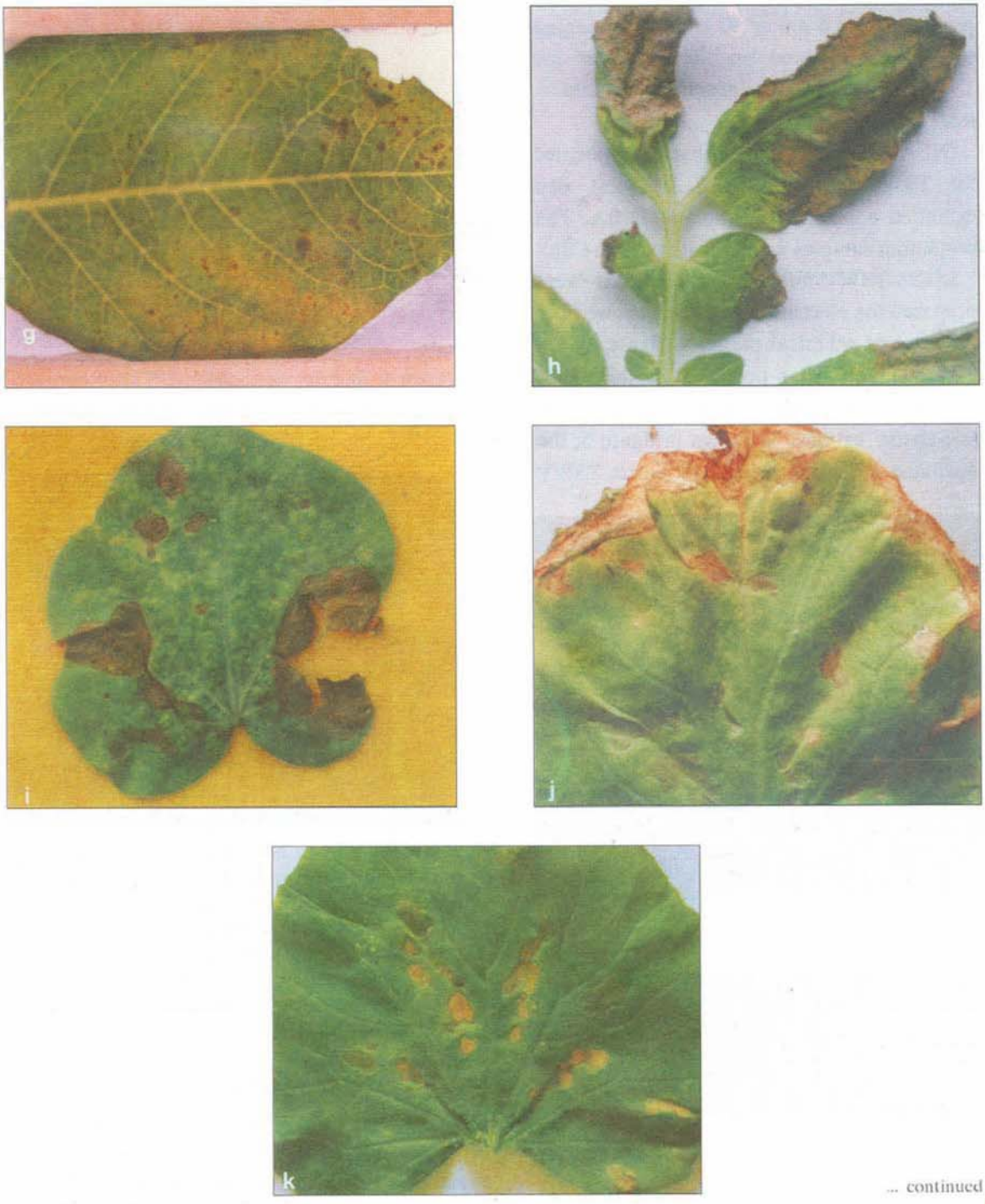

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Figure 2. Host-range specificity test and symptom expression. Tomato, (a) brown necrotic spots with chlorosis on the leaves; (b) brown stem necrosis; (c) yellow circular spots on fruits and (d) wilting and tissue collapse. Bell pepper, vein clearing on the leaf (e). Eggplant, greenish-yellow mosaic pattern and yellow chlorotic spots on the leaf (f). Potato, brown necrotic spots on the leaf (g) and leaf necrosis (h). Watermelon, brown concentric ring spots on the leaf (i). Melon, leaf chlorotic and necrotic spots (j). Cantaloupe, chlorotic spots on the leaf (k).

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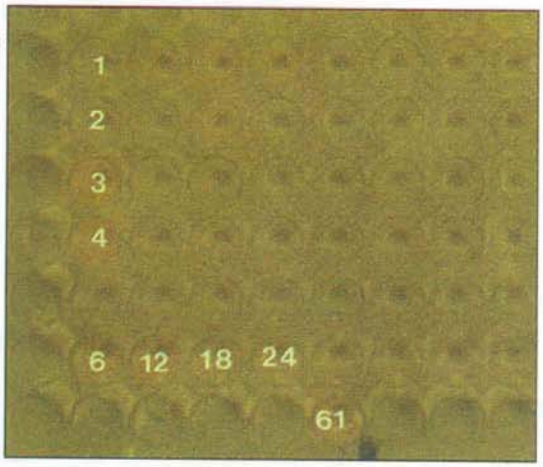
collected from various regions in Tehran province, only 6 out of 60 samples belonging to Varamin showed positive reactions for TSWV (figure 3a). The remaining tomato samples were negative.

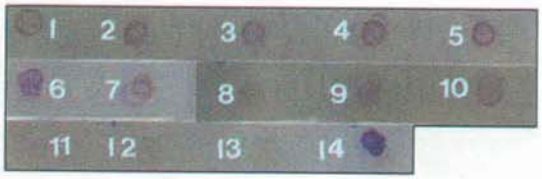
DIBA-Varamin samples that were positive for TSWV infection in ELISA test also exhibited a positive reaction in DIBA. The remaining samples were negative (figure 3b).

SSEM-TSWV-infected samples that were prepared for electron microscopy showed the presence of spherical particles (figure 4).

TSWV antiserum prepared in this study was tested against TSWV-infected *N. glutinosa* leaf tissue extract. As shown in figure 5, the undiluted antiserum could only detect TSWV



(a)



(b)

Figure 3. TSWV serological detection tests: (a) DAS-ELISA microtiter plate, Wells 1 & 2 are negative controls; 3 & 4 are positive controls; wells 6, 12, 18, 24 and 61 are TSWV-infected tomato samples and the remaining show negative reaction; (b) DIBA immunoblot, squares 1 & 8 are negative controls; 7 & 14 are positive controls; 2-6 and 9-10 are TSWV-infected tomato samples and 11-13 show a negative reaction.

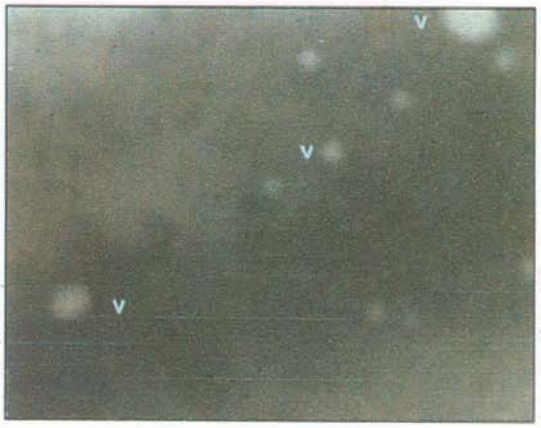


Figure 4. Electron micrograph of TSWV particles showing spherical and circular structures. 40,000 x.

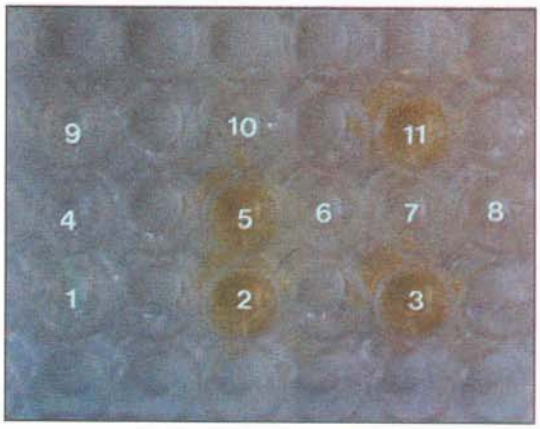


Figure 5. DAS-ELISA microtiter plate showing cross-reactivity between the polyclonal antibody prepared against TSWV and virus-infected leaf samples. Wells 1 and 4, negative controls; 2 and 3, positive controls; 5 and 11, TSWV-infected *N. glutinosa* reaction with an undiluted antiserum; 6-9, TSWV-infected *N. glutinosa* reaction with antiserum diluted at 1/2, 1/4, 1/8 and 1/16.

in infected leaf sample wells 5 and 11. Antiserum dilutions at 1/2, 1/4, 1/8 and 1/16, were unable to detect TSWV in the infected samples indicating the antiserum's low titer.

DISCUSSION

Although members of *Tospovirus* group are exclusively thrips transmitted, their transmission efficiencies by different thrips species, serological and biological properties may vary

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significantly. In spite of such differences, they may induce similar symptoms on indicator test plants. Therefore, parallel mechanical inoculation tests and serology are necessary to distinguish different viral species.

TSWV was detected in tomato samples from Varamin region using several indicator test plants and serological techniques. Symtoms on *P. hybrida* leaves were small brownish local necrotic lesions, whereas systemic symptoms including concentric ring spots on leaves, stem necrosis, wilting and tissue collapse were observed on tobacco leaves. Similar symptoms have previously been reported by Selman *et al.* (1961), Ie (1970), Peters *et al.* (1991) and German *et al.* (1992). More recently, Bananej *et al.* (1998) observed the development of necrotic local lesions on *P. hybrida, N. glutinosa, V. unguiculata* and *G. globosa,* chlorotic local lesions on *C. sativus* and systemic infection on *N. rustica, N. tabacum* cv. Samsum and *N. benthamiana*. Typical symptoms on tomato plants appeared as brownish local lesions on stem and leaves, concentric ring spots, tissue necrosis, vein clearing, yellow discoloration on fruits, wilting, stunting and plant collapse. These observations are in agreement with those reported earlier by Samuel *et al.* (1930), Allen *et al.* (1989) and McHugh (1991). Bananej *et al.* (1998) also demonstrated typical fruit deformation with large yellow rings on tomatoes from Varamin.

In this study, TSWV was identified in tomato samples using DAS-ELISA, DAS- DIBA and SSEM. The former techniques have been shown to be sensitive and reliable in the detection of TSWV in infected plant sap as well as thrips vectors (D. Peters, 1998, personal communication). Out of 145 tomato samples collected from various regions in Tehran province, only 6 samples belonging to Varamin exhibited positive reactions in DAS-ELISA (figure 3a). Negative controls provided in the Sanofi kit showed no reaction. Bananej *et al.* (1998) also reported the presence of TSWV in tomato samples collected from Varamin fields using DAS-ELISA and DIBA techniques.

Gonsalves *et al.* (1986) detected TSWV in infected papaya plants using ELISA. Hsu *et al.* (1991) used both ELISA and DIBA in the identification of TSWV in *Nicotiana rustica* L. and showed that the latter is even more sensitive than the former. Immunoblot analysis revealed that Varamin samples that were positive for TSWV infection in ELISA test also exhibited positive reaction in DAS-DIBA (figure 3b). The latter techique is easy, reliable and utilizes a minute amount of biological material which seems to be much less than that used in ELISA indicating a greater sensitivity of DIBA.

In SSEM study, round and spherical particles were observed indicative of TSWV varions which appear to be consistent with the results of Peters *et al.* (1991) and Bananej *et al.* (1998). This technique seems to be suitable for morphological study of TSWV. Antiserum prepared in this investigation was of low titer due to partial purification of the virus and its physical instability. Antiserum dilutions at 1/2, 1/4, 1/8 and 1/6 could not detect TSWV antigen in the infected samples (figure 5).

The results obtained in this study suggest that Varamin tomato fields seemed to be infected with TSWV. However, only 10% of the symptomatic tomato plants showed TSWV infection. It is possible that the remaining samples were infected with other *Tospoviruses* or other viral or bacterial agents. Since the climatic conditions in Varamin are conducive and TSWV has a wide host range, it is probable that the virus could be disseminated through various thrips species in the region.

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شناسایی سرولوژیک و علائم‌شناسی بیماری ویروس پژمردگی لکه‌ای گوجه‌فرنگی در استان تهران

چکیده

بیماری ویروس پژمردگی لکه‌ای گوجه‌فرنگی (TSWV) در نمونه‌های آلودهٔ برگ و میوه گوجه‌فرنگی در منطقه ورامین در استان تهران با استفاده از گیاهان محک، مانند گل اطلسی *(Petunia hybrida)* و گونه‌های مختلف توتون و آزمون‌های سرولوژیک شناسایی گردید. زخم‌های نکروتیک موضعی کوچکی به رنگ قهوه‌ای ۲ تا ۴ روز پس از مایه‌کوبی بر روی برگ‌های اطلسی پدیدار گشت. علائم سیستامیک بیماری شامل لکه‌های گرد متحدالمرکز بر روی برگ‌ها، نکروز ساقه، پژمردگی و اضمحلال بافت گیاهان ۷ تا ۱۰ روز بعد از تلقیح بود. در میان ۱۴۵ نمونه گوجه‌فرنگی جمع‌آوری شده از قزوین، هشتگرد، کرج، ملارد، شهریار و ورامین در استان تهران، تنها نمونه‌های ورامین براساس آزمون‌های الایزا (ELISA) ، دیبا (DIBA) و میکروسکوپ الکترونی (SSEM) به ویروس TSWV آلوده بودند. اختصاصی بودن دامنه میزبانی و ابراز علائم بر روی گوجه‌فرنگی، فلفل دلمه‌ای، بادنجان، سیب‌زمینی، هندوانه، خیار، خربزه، طالبی، لوبیا و سلمک مورد آزمایش قرار گرفت. علائم تیپیک بر روی این گیاهان شامل لکه‌های گرد متحدالمرکز، کلروز، رنگ پریدگی رگبرگها، نکروز بافت، کوتولگی و تشکیل زخم‌های موضعی بود. آنتی‌سرم تهیه شده علیه نمونه نسبتاً خالص TSWV توانست نمونه‌های گوجه‌فرنگی آلوده به این ویروس را شناسایی کرده و واکنش متقابل نشان دهد.

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