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Seed and Pollen Transmission of Tomato spotted wilt orthotospovirus (TSWV) in Pepper

Yunus Emre USLU[1*] Sevdiye YORGANCI[1]

[1]Aydın Adnan Menderes University, Agriculture Faculty, Department of Plant Protection, Aydın
***Corresponding author's email:** yunusemreuslu75@gmail.com

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Abstract: Orthotospovirus tomatomaculae also known by Tomato spotted wilt orthotospovirus (TSWV) is a harmful pathogen that significantly reduces crop production and quality. TSWV can infect approximately 1,000 plant species in 84 different plant families, such as tomato, pepper, lettuce, tobacco, and various types of wild plants, belonging to the Solanaceae family. TSWV is a member of the *Orthotospovirus* genus and is an isometric-enveloped particle. TSWV can be transmitted mechanically, seed-borne, and by nine species of thrips belonging to the genera Thrips, Scirtothrips,Frankliniella.In this study, it was determined that TSWV could be transmitted by seeds, but no transmission by pollen was detected. RT-PCR and visual assessment data demonstrate the seed transmission ability of TSWV in peppers. TSWV was detected in infected pepper seeds (seed and seed coat). Additionally, TSWV was detected in different organs of the pepper plant. TSWV was detected in the endocarp, mesocarp, exocarp, pedicel, and placenta of pepper fruit and sepal, petal, pistil, and stamen of pepper flower. After the germination of infected pepper seeds, TSWV was detected with specific primers by RT-PCR in the cotyledons and true leaves, but no symptoms were observed. These data provided information about the localization of TSWV in pepper plants and provided further evidence of TSWV seed transmission ability.

Keywords: Pepper plant, tomato spotted wilt orthotospovirus, pollen transmission, seed transmission, localization of virus.

Biberde Domates Lekeli Solgunluk Virüsünün Tohum ve Polen ile Taşınması

Öz: Orthotospovirus tomatomaculae olarakta bilinen Domates lekeli solgunluk virüsü (TSWV), ürün verimini ve kalitesini önemli ölçüde azaltan zararlı bir patojendir. TSWV, domates, biber, marul, tütün ve Solanaceae familyasına ait çeşitli yabani bitkiler gibi 84 farklı bitki familyasındaki yaklaşık 1.000 bitki türünü enfekte edebilir. TSWV, Orthotospovirüs cinsinin bir üyesi olup izometrik zarflı bir partiküldür. TSWV, mekanik olarak, tohum yoluyla ve Thrips, Scirtothrips ve Frankliniella cinslerine ait dokuz trips türü tarafından bulaşabilir. Bu çalışmada, TSWV'nin tohumlarla taşınabildiği, ancak polen yoluyla taşınma kabiliyetinin olmadığı belirlenmiştir. RT-PCR ve görsel değerlendirme verileri, TSWV'nin biberlerde tohumla taşınma kabiliyetini göstermektedir. Enfekteli biberlerden toplanan tohumlarda (tohum ve tohum kabuğu) TSWV tespit edilmiştir. Ek olarak, enfekteli, biber meyvesinin endokarp, mezokarp, ekzokarp, pedicel ve plasentasında ve biber çiçeğinin çanak yaprağı, taç yaprağı, pistili ve erkek organında TSWV saptanmıştır. Enfekteli biber tohumlarının çimlenmesinden sonra, TSWV kotiledonlarda ve gerçek yapraklarda spesifik primerler kullanılarak RT-PCR ile tespit edilmiş, ancak hiçbir simptom gözlemlenmemiştir. Bu veriler, TSWV'nin biber bitkisinde ki lokalizasyonu hakkında bilgi sağlamış ve TSWV tohum ile taşınabildiğine dair daha fazla kanıt sağlamıştır.

Anahtar kelimeler: Biber bitkisi, domates lekeli solgunluk virüsü, polen taşınması, tohum taşınması, virüs lokalizasyonu.

1. Introduction

Orthotospovirus tomatomaculae (TSWV) is a plant virus belonging to the *Bunyaviridae* family. It is characterized by isometric enveloped particles and possesses a tripartite genome consisting of three RNA segments: 8.9 kb L-RNA, 5.4 kb M-RNA, and 2.9 kb S-RNA (de Haan et al., 1990). Among these, one RNA is negative-sense while the other two are positive-sense (Usta et al., 2023). TSWV is notorious for its detrimental impact on nearly 1000 plant species across 84 families, including important crops like tomato, pepper, squash, and others within the Solanaceae family (Gordillo et al., 2008). Transmission of TSWV primarily occurs through mechanical transmission and via thrips vectors. Nine species of thrips from the *Thrips* sp., *Scirtothrips* sp., and *Frankliniella* sp., genera can transmit the virus in a circulative and propagative manner. Notably, *Frankliniella occidentalis* is particularly effective in transmitting TSWV (Coutts & Jones, 2005).

Symptoms of TSWV infection in plants are distinct, including leaf bronzing, black spots on leaves and stems, fruit wilting, and deformations (Güldür et al., 1995). Fruits exhibiting these symptoms are unsuitable

for fresh consumption and lose economic value, particularly affecting industrial tomato production (Turhan & Korkmaz, 2006).

Apart from mechanical and vector transmission, several studies have also demonstrated seed transmission of Tomato spotted wilt virus (TSWV). The initial study on *Senecio cruentu* reported a 96% efficiency in TSWV seed transmission (Jones, 1944). Another study indicated a 1% transmission rate of TSWV through tomato seeds (Crowley, 1957). Furthermore, RT-PCR and electron microscope analyses confirmed the transmission of TSWV through pepper seeds in a different study (Wang et al., 2022). Additionally, Groundnut bud necrosis orthotospovirus (GBNV) and Soybean vein necrosis virus (SYNV), both belonging to the *Tospoviridae*, are seed-transmitted in soybean and peanut, respectively (Groves et al., 2016; Sastry, 2013). Despite all this evidence, some studies have reported that plants infected with TSWV cannot transmit the virus to new-generation plants (Antignus et al., 1997). The seed transmission of plant viruses depends on whether the virus can enter the plant and reach its reproductive organs, infect these organs, and whether the infected reproductive organs can proliferate and survive. Therefore, the most determining factor in seed transmission is the virus's ability to accumulate in the host reproductive organs (Cobos et al., 2019).

In this study, TSWVAntRB isolate was infected with pepper plants through mechanical inoculation. Visual and RT-PCR analyses confirmed the seed transmission of TSWV, with the virus detected in both the pepper seed and seed coat. However, TSWV was not detected in the second-generation pepper plants obtained through pollination from TSWV-infected pepper plants. Total nucleic acid isolation was performed from various parts of TSWV-infected pepper plants to determine the localization of TSWV within the pepper plants.

2. Materials and Methods

2.1. Growing conditions and plant material

The pepper cultivar Maxibell was used to investigate the seed and pollen transmissibility of the Tomato spotted wilt virus (TSWV). Maxibell seeds were generously provided by Assoc. Prof. Ümit Özyılmaz from Aydın Adnan Menderes University. (Aydın, Turkey). All plants were maintained in a growth chamber at Aydın Adnan Menderes University (Aydın, Turkey), under controlled conditions of 22±5 °C temperature, 60% relative humidity, an 8/16-hour photoperiod, and watered every 3 days with tap water. Healthy plants and virus-infected plants were segregated and placed on separate shelves within the same room.

2.2. Virus inoculation

The TSWV used in this study is a common strain (TSWVAntRB) which is shown blackspot containing light and dark-green areas in tomato. Inoculum was prepared by grinding infected tissues at the rate of 1:6 (wt/vol) tissue to buffer ratio in freshly prepared ice-cold 0.05 M potassium phosphate buffer, pH 7.0, containing 0.1% mercapto-ethanol with a chilled mortar and pestle. Debris was removed by squeezing the extract through a cotton bud. The inoculum was kept on ice until the inoculation was completed. Inoculum was applied by rubbing both surfaces of the leaf with a brush on pepper plants at four leaf stages (8 to 10 days after planting). After inoculation, the plants were sprayed with distilled water and kept in two growth chambers having the same environmental conditions (Oğuz et al., 2009).

2.3. Sample Collection

Symptomatic leaves of pepper plants were collected at 45 days post-inoculation (dpi). During the generative stage, flowers were collected from each of the TSWV-inoculated plants. Flower samples were collected during anthesis and dissected into sepals, petals, pistils, and stamens using sterilized scalpels. To prevent contamination, scalpels were sterilized with 70% ethanol and flamed to remove excess ethanol before each dissection. Total nucleic acid (TNA) was extracted from each flower part for virus detection.

Young pepper fruits were carefully separated into exocarp, mesocarp, endocarp, placenta, pedicel, and seeds using sterilized scalpels. TNA extraction was performed on each part to detect the virus. Seed coats were collected from germinating seeds, while cotyledons and leaves were collected upon the emergence of the first true leaf.

2.4. Cross-pollination

Cross-pollination between healthy and infected pepper plants was performed to investigate the vertical transmission of TSWV. Pepper plants produce perfect flowers containing both pistils and stamens within the same flower. Female flower sources were selected from healthy pepper plants that had not yet bloomed. Before pollination, all flower parts except the sepals and pistils were removed to prevent self-pollination. These female flowers were then pollinated with pollen collected from infected pepper plants.

2.5. Total nucleic acid (TNA) extraction and RT-PCR detection

Total nucleic acid samples were extracted from TSWV-infected parts of pepper plants to detect the presence of TSWV (Svanella-Dumas et al., 2000). PCR analysis was conducted using 1-Step Hot Start Master Mix® (Thermo Fisher, Massachusetts, USA). The PCR mixture included 12.5 µl of Hot Start master mix, 0.5 µl of verso enzyme, and 1.25 µl of RT-enhancer for amplification. Inoculum plant infected with TSWV was used as a positive control. The following PCR-specific primers of TSWV (Mumford et al., 1994) (F: 5'-AATTGCCTTGCAACCAATTC-3' and R: 5'-ATCAGTCGAAATGGTCGGCA-3') was used to detect a 276 base pair fragment of the TSWV L-RNA segment. The amplification conditions for the PCR reaction were as follows 50°C 15 min, 95°C 35 min for 1 cycle 94°C for 1 min, 55°C for 1min and 72°C 1 min for 35 cycles: 5 min for 72°C and each 25µl sample mixture (Fidan & Sari, 2019). Data analysis was performed using electrophoresis.

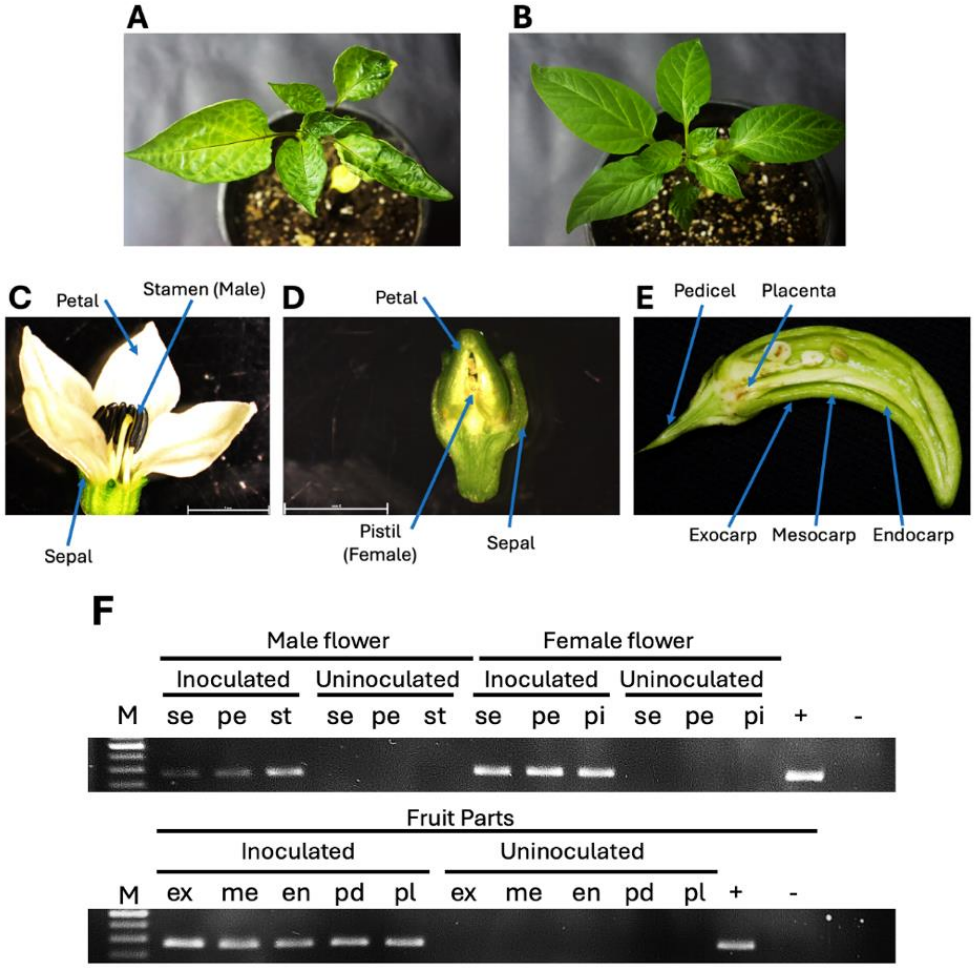

Figure 1. Symptom development, sample collection, and detection of Tomato spotted wilt virus (TSWV) in infected pepper flowers and fruits. **A,** Symptoms of a pepper leaf induced by TSWV. **B,** Healthy pepper leaf. The symptom photos were taken at 45 days post-inoculation. **C,** Pepper male flower parts. **D,** Pepper female flower parts. **E,** Pepper fruit parts. **F,** RT-PCR detection of pepper flower and fruit parts using TSWV-specific primer. M= marker; + = PCR positive control; - = PCR negative control; se = sepal; pe = petal; pi = pistil; st = stamen; ex = exocarp; me = mesocarp; en = endocarp; pd = pedicel; and pl = placenta.

Şekil 1. *Enfekteli biber çiçek ve meyvelerinde Domates lekeli solgunluk virüsü'nün (TSWV) simptom gelişimi, örnek toplanması ve tespiti.* ***A,*** *TSWV tarafından oluşturulan biber yaprağındaki simptomlar.* ***B,*** *Sağlıklı biber yaprağı. Simptom fotoğrafları inokulasyondan 45 gün sonra çekildi.* ***C,*** *Biber erkek çiçek organları.* ***D,*** *Biber dişi çiçek organları.* ***E,*** *Biber meyvelerinin parçaları.* ***F,*** *TSWV'ye özgü primer kullanılarak biber çiçeği ve meyve parçalarının RT-PCR tespiti. M= işaretleyici; + = PCR pozitif kontrol; - = PCR negatif kontrol; se = çanak yaprak; pe = taç yaprak; pi = pistil; st = stamen; ex = ekzokarp; me = mezokarp; en = endokarp; pd = pedicel; ve pl = plasenta.*

3. Results

3.1.TSWV moves to and accumulates in systemic leaves, flowers, and fruit parts

Yellowing and leaf curling symptoms were observed on pepper plants at 45 days post-inoculation (dpi) with TSWV compared with healthy peppers (Figure. 1A and B). The presence of TSWV infection was confirmed using RT-PCR with virus-specific primers. Female and male flowers, as well as immature fruit samples, were collected and carefully dissected into various parts for virus detection (Figure. 1C to E). Positive RT-PCR signals indicating TSWV presence were detected in all tested flower parts and fruit tissues (Figure. 1G). These results showed that TSWV could move to all tested flower parts and fruit tissues of pepper plants.

3.2. TSWV exists in the progenies of infected plants

Pepper plants infected with TSWV produced fewer mature seeds compared to healthy plants. Mature pepper fruits and seeds were collected at 90 days post-inoculation (dpi). Following germination, various tissues were sampled for total nucleic acid (TNA) extraction and RT-PCR analysis. TSWV was detected in seed, seed coat,cotyledon, and leaf tissues with no observable symptoms on virus-infected cotyledons(Figure.2).

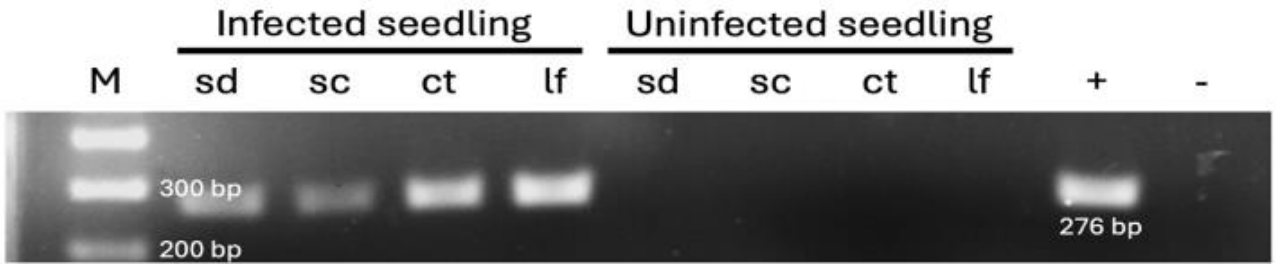

Figure 2. PCR results of whole seed (sd), seed coat (sc), cotyledon (ct), and leaf (lf) using TSWV-specific primers. Lane of DNA marker (M) is labeled. + = PCR positive control, – = PCR negative control.
Şekil 2. *TSWV'ye özgü primerler kullanılarak tüm tohum (sd), tohum kabuğu (sc), kotiledon (ct) ve yaprağın (lf) PCR sonuçları. DNA marker (M) etiketlenmiştir. + = PCR pozitif kontrol, – = PCR negatif kontrol.*

Table 1. Detection of Tomato spotted wilt virus in progenies derived from virus-infected pepper
Çizelge 1. *Virüsle enfekte olmuş biberlerden elde edilen ilk nesillerde Domates lekeli solgunluk virüsünün tespiti*

Virus	Germination rate[a]	Infection rate[b]
TSWV	117/150 (78%)	68/117 (58.12%)
Uninoculated plant	142/150 (94.67%)	0/142 (0%)

[a]Germination rate = (number of germinated seeds/numbers of tested seeds) × 100%.
[b]Infection rate = (number of seeds tested positive with PCR/number of germinated seeds) × 100%.

A total of 150 seeds derived from TSWV-infected plants were germinated and tested for virus presence, alongside 150 seeds from uninoculated plants used as a control group. Seeds from TSWV-infected plants exhibited a germination rate of 78% and an infection rate of 58.12%. In contrast, seeds from uninoculated plants showed a germination rate of 94.67%, and no virus was detected in their progeny (Table 1).

3.3. TSWV is not pollen-transmissible

To investigate the potential transmission of TSWV through pollination, pollen from virus-infected plants was collected and used for cross-pollination of female flowers on healthy plants (Figure. 3A and B). Systemic leaves and mature fruits resulting from cross-pollination were collected simultaneously. No symptoms were observed on the pepper plants six weeks after fruit collection. Analysis revealed the absence of TSWV in all tested parts of the cross-pollinated fruits and systemic leaves. Furthermore, PCR testing conducted on systemic leaves of the pollinated plants and on a subsequent self-pollinated fruit, which grew adjacent to the cross-pollinated fruit on the same stalk, yielded negative results (Figure. 3C).

4. Discussion

This study aims to determine the seed and pollen-mediated transmission ability of TSWV and to determine the localization of TSWV in pepper plants. Thus, we aimed to create a step forward by obtaining more information on TSWV transmission and localization and filling the gaps for future management of TSWV.

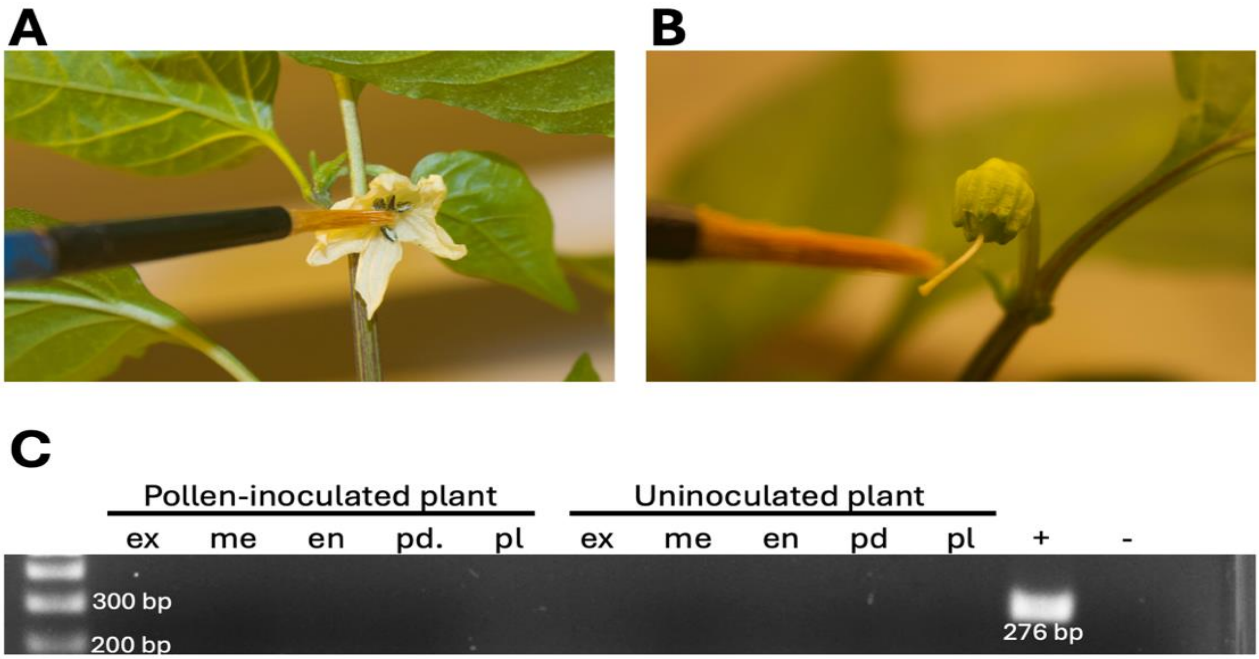

Figure. 3. Cross-pollination of TSWV-infected pepper and healthy plant and PCR detection of Tomato spotted wilt virus in pepper fruit after cross-pollination. **A,** Collection of pollen from the TSWV-infected pepper male reproductive organ (stamen). **B,** Cross-pollination of collected pollen by applying it to the female reproductive organs (pistil) of healthy pepper plants. **C,** RT-PCR detection of TSWV in inoculated pepper plants after cross-pollination using specific primers. M = marker; + = PCR positive control; – = PCR negative control; ex = exocarp; me = mesocarp; endo = endocarp; pd = pedicel; and pl = placenta.

Şekil 3. *TSWV ile enfekteli biber ve sağlıklı biberin çapraz tozlaşmasından sonra biber meyvesinde Domates lekeli solgunluk virüsünün PCR ile tespiti.* ***A,*** *TSWV ile enfekte biberin erkek üreme organından (stamen) polen toplanması.* ***B,*** *Toplanan polenin sağlıklı biber bitkilerinin dişi üreme organlarına (pistil) uygulanması yoluyla çapraz tozlaşması.* ***C,*** *Spesifik primerler kullanılarak çapraz tozlaşmadan sonra aşılanmış biber bitkilerinde TSWV'nin RT-PCR ile tespiti. M = marker; + = PCR pozitif kontrol; – = PCR negatif kontrol; ex = ekzokarp; me = mezokarp; endo = endokarp; pd = pedisel; ve pl = plasenta.*

Seed transmission occurs in 20% of plant viruses, and thus can rapidly emerge in new generations of plants (Sandra et al., 2020; Schaad, 1988). Apart from mechanic and vector transmission, seed transmission of TSWV is a gap that is not fully clarified. In this study, we provide further evidence for seed transmission of TSWV. TSWV was detected by RT-PCR in pepper seeds obtained from TSWV-infected plants (Figure 2.). TSWV was detected not only in the seed but also in the seed coat, cotyledon, and leaves. Another study showed that pepper seeds carry TSWV by RT-PCR and electron microscopy (Wang et al., 2022). In addition, studies are showing that TSWV is also carried in *Senecio cruentu* and tomato seeds (Crowley, 1957; Jones, 1944). Although seed transmission of Tomato spotted wilt virus (TSWV) has not been detected in peanuts, the virus has been detected in the testa of immature seeds and freshly harvested mature seeds. However, serological detection of the virus in testa was successful only in dried seeds. When freshly harvested mature seeds containing the infective virus in the testa were tested using growth assays, they did not transmit TSWV (Prasada Rao et al., 2009; Reddy et al., 1983). The data obtained may indicate that seed transmission may be affected by environmental conditions, genetics, and virulence, but may not be sufficient to determine the effectiveness of its spread in other pepper varieties or different plants. Although seed-mediated transmission is seen in this pepper variety, the route of seed transmission from male and female individuals needs to be further investigated.

Most studies have shown that seed-mediated transmission of plant viruses generally occurs through the embryo (Ellis et al., 2020; Johansen et al., 1994). Plant viruses enter the seed embryo either directly by entering the embryo of the seed or indirectly through pollen or egg cells (Suruthi et al., 2018). In this study, we proved that TSWV can be transmitted directly (by entering the seed embryo), but not indirectly (by pollen). Cross-pollination was carried out from infected plants to healthy plants, but no transmission was observed (Figure.3.). Replication and spread of TSWV in plants

is markedly restricted by its unique seed structure, which lacks the plasmodesmata required for effective virus movement (Carrington et al., 1996; Crowley, 1957). Therefore, the localization, accumulation, and presence of the virus in the seed and embryo may vary in different pepper varieties. TSWV was detected in cotyledons developing from infected seeds. It may occur because of factors such as low immunity in young seedlings and cotyledons, the plant's need for rapid growth, and its inability to meet the necessary nutritional needs (Zou et al., 2018). TSWV-infected pepper seeds often cannot be detected because the disease does not show any symptoms during germination (Kothandaraman et al., 2016; Suruthi et al., 2018). TSWV has been found in pepper seeds and can be transmitted symptomless to future generations without being detected by visual inspection.

In this study, the localization of TSWV in different organs of pepper was also determined. TSWV was detected in the sepal, petal, stamen, pistil (flower) and exocarp, mesocarp, endocarp, pedicel, and placenta (fruit) (Figure. 1.). The presence of TSWV in all tested parts of both flower and fruit proves that it spreads rapidly and strongly within the plant.

This study provides evidence of seed-borne transmission of Tomato spotted wilt virus (TSWV) in pepper (*Capsicum annuum* L.) plants. Additionally, TSWV was detected in the seed, seed coat, and young seedlings of the second generation of peppers, indicating a potential new mode of seed transmission in this plant. However, further investigation is needed to understand the key factors involved in this process. The timing of virus infection in crops could play a crucial role in seed transmission under field and greenhouse conditions. Our cross-pollination analyses demonstrated that TSWV is not pollen transmissible. Furthermore, this study mapped the localization of TSWV in various organs of the pepper plant, including the sepal, petal, stamen, pistil (flower), and exocarp, mesocarp, endocarp, pedicel, and placenta (fruit). Preventing seed transmission is equally critical as controlling thrips and mechanical transmission of TSWV for the seed industry, both in production and marketing.

This report represents the first documentation that TSWV is not pollen transmissible and provides insights into its localization within different parts of the pepper plant.

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