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Short communication

A rapid and efficient inoculation method for *Tomato spotted wilt tospovirus*

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Abstract

A rapid and efficient method of inoculation for *Tomato spotted wilt tospovirus* (TSWV) was achieved by applying the inoculum with a device consisting of a spray gun, an atomizer and a CO_2-powered sprayer. The inoculum contained infected leaf sap prepared in 0.1 M phosphate buffer, pH 7.0, 0.2% sodium sulfite and 0.01 M 2-mercaptoethanol (1 g: 10 ml) and 1% each of Celite 545 and Carborundum 320 grit. The spray application of chilled inoculum at the rate of 1.1 ml/plant and at an air pressure of 4.1 bar resulted in systemic infection nearly to a 100% of the tobacco (*Nicotiana tabacum*) plants inoculated. The inoculation procedure was successfully applied to two other important host species of TSWV, peanut (*Arachis hypogaea*) and tomato (*Lycopersicon esculentum*), where 75.0–100% and 72.2–91.6% plants developed systemic infection, respectively. The approach facilitated a much faster inoculation of test plants with TSWV as it was estimated to be about 50 times quicker (depending on the plant species) than the hand inoculation. The procedure is suitable for rapid and simultaneous inoculation of a large number of test plants with TSWV and should facilitate screening of germplasm and breeding lines for virus resistance.

Keywords: *Tomato spotted wilt virus*; Tospovirus; *Bunyaviridae*; Virus inoculation

Tomato spotted wilt virus (TSWV) is an ambisense RNA virus containing a tripartite genome packaged in a quasi-spherical, enveloped particle of 80–110 nm size (Moyer, 1999; Pappu, in press). TSWV belongs to the genus *Tospovirus* and family Bunyaviridae and is economically one of the most important plant viruses worldwide (Mumford et al., 1996; Sherwood et al., 2000). TSWV has a very broad host range infecting as many as 900 plant species (Peters, 1998). Several species of thrips are reported to transmit TSWV (Ullman et al., 2002; Whitfield et al., 2005). In the southern United States, TSWV is one of the most economically important pathogens in several crops such as peanut (*Arachis hypogaea* L.), pepper (*Capsicum annuum* L.), tobacco (*Nicotiana tabacum* L.) and tomato (*Lycopersicon esculentum* Mill.). Control of TSWV is difficult through application of insecticides alone (Culbreath et al., 1991; McPherson et al., 1992, 1999). However, a combination of tactics that include cultural practices, vector control and use of virus-resistant cultivars resulted in reduced impact of TSWV (Culbreath et al., 1999; Riley and Pappu, 2000). Resistant cultivar is considered as the most important tool for management of TSWV. Continuous deployment of new resistant cultivars is necessary as the virus is known to evolve and break down resistance rapidly (Choe et al., 1996; Latham and Jones, 1998; Hobbs et al., 1994; Moury et al., 1997; Hoffmann et al., 2001a). Identification of sources of virus resistance is achieved by screening germplasm either by sap inoculation with the virus under greenhouse conditions or in the field under natural, thrips-mediated infection.

Mechanical or manual transmission of TSWV is not very efficient in certain host species, resulting in many 'escapes'. For example, several workers reported escapes to mechanical transmission of TSWV to peanut (Halliwell and Philley, 1974; Nome et al., 1985; Dubern et al., 1987; Clemente et al., 1990; Pereira, 1993; Hoffmann et al., 1998; Mandal et al., 2001). In a previous study, several factors such as the plant growth environment, plant growth stage, source of inoculum, antioxidants and abrasives were found to influence the rate of mechanical transmission of TSWV to peanut (Mandal et al., 2001). Evaluation of these factors resulted in development of an efficient sap inoculation protocol, which was also found suitable for inoculation with the different isolates of TSWV to peanut, pepper,

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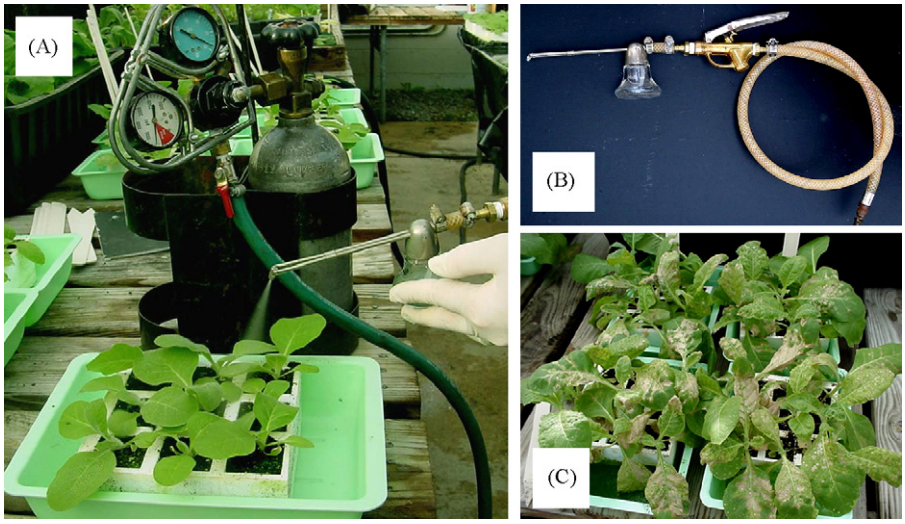

Fig. 1. Pressurized, spray inoculation of *Tomato spotted wilt virus* (TSWV). (A) Inoculation of tobacco test plants with the inoculation device consisting of an atomizer and a spraying gun (B) connected to a CO_2 powered sprayer. All the inoculated tobacco plants showed symptoms of TSWV at 14 days post inoculation (C).

tobacco, and tomato cultivars (Mandal et al., 2001; Mandal et al., 2006). However, in this inoculation procedure, the inoculum was manually applied to each leaf of a test plant, which is time consuming. Hand inoculation of a single peanut seedling requires rubbing of 8–12 leaflets on both the surfaces, which is painstaking when a large number of plants are to be inoculated. Hand inoculation depends on the skill of the individual as uneven hand pressure while rubbing lamina often causes damage to the leaves; especially those that are small and delicate. The objective of the present study was to develop a rapid method for mechanical inoculation with TSWV that overcomes the limitation of hand inoculation and greatly improves the efficiency of mechanical inoculation of test plants.

The GAL isolate of TSWV (Mandal et al., 2006) causing severe necrosis and death of tobacco seedlings was maintained in the greenhouse by periodic hand-inoculation of tobacco seedlings, cv. K326. Freshly infected leaves showing systemic necrosis were used as the source of inoculum. The inoculation device was constructed by connecting a spray gun and an atomizer with a CO_2 powered sprayer (Fig. 1). The inoculum was prepared by grinding infected leaves at the rate of 1.0 g tissue and 10 ml of 0.1 M phosphate buffer, pH 7.0 containing 0.2% sodium sulfite and 0.01 M 2-mercaptoethanol using a chilled pestle and mortar. The extract was filtered through cheesecloth and 1% each of Celite 545 and Carborundum 320 grit (Fisher Scientific, Fair Lawn, NJ) were added to the inoculum. The inoculum was maintained on ice until it was transferred to the atomizer.

The test plants of tobacco cv. K326 were used to determine the appropriate pressure and volume of inoculum required for efficient transmission. The test plants were grown in the greenhouse in Styrofoam float-tray (Speedling Inc., Sun City, FL, USA) with 6.45 cm^2 cell size containing media mix consisting of Canadian sphagnum peat moss (75–85%), perlite (15–20%) and vermiculite (5–10%) (Berger Peat Moss-Lee Berger Itee, Saint-Modeste, Quebec, Canada). Forty-day-old seedlings were inoculated at three different air pressures, 3.4, 4.1 and 5.5 bar. Inoculation was accomplished by spraying 15 ml of inoculum from a height of 10 to 12 cm above the plant canopy. The effective volume of inoculum was determined by spraying inoculum at the rate of 139, 278, 556 and 1111 μl/plant (2.5, 5.0, 10.0 and 20 ml of inoculum to 18 test plants each) at the air pressure of 4.1 bar. Eighteen test plants were inoculated in three independent trials for each treatment. After inoculation, plants were allowed for symptom expression in a temperature-controlled greenhouse (25–30 °C). Systemic infection was determined at 15 days post inoculation (dpi) by enzyme linked immunosorbent assay (ELISA) using a TSWV-specific kit from Agdia (Elkhart, IN) (Mandal et al., 2006). Data were analyzed by the GLM procedure of SAS (SAS Institute Inc., Version 7, Cary, NC).

The inoculated tobacco seedlings produced a systemic foliar necrosis at 10–14 dpi. At 3.4 bar of air pressure, 100, 94.4 and 55.5% plants produced systemic infection in the three different trials. At 4.1 bar all the inoculated plants produced systemic infection in the two trials and 94.4% plants produced systemic infection in the third trial. At 5.5 bar, only 55.5, 77.7 and 61.1% plants produced systemic infection in each of the three trials. Since spray inoculation at 4.1 bar consistently resulted in near 100% transmission (Fig. 2), subsequent inoculation experiments were conducted at 4.1 bar.

The inoculum volume, when used at the rate of 139 μ/plant, 33.3–50% plants developed systemic infection. The increase of twofold of inoculum volume (278 μl/plant) resulted in a significant increase of transmission (66.6%). Another twofold increase of the inoculum volume (556 μl/plant) resulted in 83.3–100% of plants systemically infected. Finally, the inoculum volume of 1111 μl/plant resulted in systemic infection in all the plants in the three trials. The data showed that the level of transmission increased with the increase of inoculum volume (Fig. 2).

The above experiments helped select an appropriate air pressure and inoculum volume for achieving the highest rate of transmission of TSWV. These criteria were subsequently used to validate the inoculation procedure by inoculating more batches of tobacco plants and two other important hosts of TSWV, peanut and tomato. Peanut seeds (cv. Georgia Green) were sown in

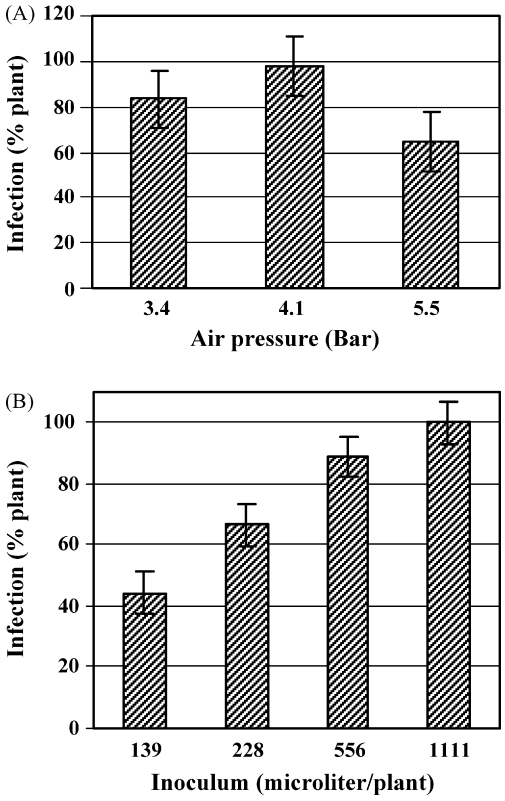

Fig. 2. *Tomato spotted wilt virus* (TSWV) infection in tobacco cv. K326 at (A) different air pressure (bar) and (B) different volumes of inoculum (μl/plant) at 4.1 bar. Values are mean of three independent trials, where 18 plants were inoculated in each of three trials. LSD = 31.9 for bars and LSD = 15.7 for volumes at $P = 0.05$.

pots (25 cm diameter) containing media mix. The seedlings (2–3 leaf stage) were taken out of the potting mixture and laid down closely in a plastic tray. The inoculum (1 ml/plant) was sprayed at 4.1 bar from 3 to 5 cm above the plant surface. The inoculated surface of the plants was turned up side down and another round of spray was applied. Inoculated plants were transplanted (8 plants/pots) and placed in the greenhouse for symptom expression. The test plants of tobacco cv. K326 and tomato cv. Sunny were grown in Styrofoam float-tray and inoculum (1 ml/plant) was sprayed at 4.1 bar over the seedlings (30–40 days old) from 10 to 12 cm above the plant canopy. The inoculated plants were allowed to grow in the same flats in the greenhouse and systemic infection was determined testing the newer, uninoculated leaves by ELISA. The times required for the spray as well as hand application of inoculum on peanut, tobacco, tomato were recorded.

Inoculation of potted seedlings of peanut resulted in low to moderate levels of transmission: only 25–60% of plants developed systemic infection in five trials (data not shown). The spray inoculation was not effective in standing peanut seedlings presumably due to the size and hardiness of the peanut leaves. To ensure a better penetration of the inoculum, spraying was conducted at a close proximity on the plants which were laid horizontally on a plastic tray. This simple modification enhanced the transmission to 75–100% of the inoculated plants (Table 1). The inoculation to tobacco plants resulted in transmission of

Table 1
Transmission of *Tomato spotted wilt virus* to different host plants by spray inoculation

Host[a]	No. infected[b]/no. inoculated[c] trials			Mean (%)	Incubation (days)
	I	II	III		
Peanut[d]	18/24	20/24	24/24	86.1	9–15
Tobacco	36/36	36/36	34/36	98.1	7–15
Tomato	26/36	33/36	30/36	82.4	12–20

[a] Test plants were 3–4 days old for peanut cv. Georgia Green, 40 days old for tobacco cv. K326 and 30 days old for tomato cv. Sunny.
[b] Determined by enzyme linked immunosorbent assay.
[c] Inoculum was applied at the rate of 1 ml/plant at an air pressure of 4.1 bar.
[d] Inoculum was sprayed at a distance from 3 to 5 cm of the uprooted peanut plants that were laid down on a plastic tray. Tobacco and tomato plants were sprayed at a distance of 10–12 cm of the standing plants.

94.4–100% of the inoculated plants. The size, shape and softness of tobacco leaf facilitated a good coverage and penetration of the inoculum which might have resulted in the high rate of transmission in tobacco. In tomato, 72.2–91.6% plants developed systemic infection. The manual (by hand) inoculation of the same cultivars of peanut, tobacco and tomato with the same isolate of TSWV resulted in systemic infection in 80–100, 100 and 70–100% of plants, respectively (Mandal et al., 2006). Therefore, the transmission rate of TSWV in spray inoculation was similar to that with the hand inoculation. However, automated inoculation facilitated rapid and simultaneous inoculation of a large number of test plants. The time taken for spraying of 20 ml of inoculum at 4.1 bar was about 30 s, which means the flow rate of inoculum was about 0.666 ml/s. Therefore, inoculation of a single plant with 1 ml of inoculum would require about 1.5 s, whereas hand inoculation of a single seedling of peanut (three quadrifoliates), tobacco (three leaves) or tomato (three compound leaves) required 70, 14 and 27 s, respectively. The pressurized spray inoculation technique is, therefore, 9.33, 18.0 and 46.66 times quicker than the hand-inoculation in tobacco, tomato and peanut, respectively.

Transmission of *Tobacco mosaic virus* to cucumber plants by spraying with an airbrush was reported by Lindner and Kirkpatrick (1959). Successful biolistic inoculation was demonstrated for *Potato leafroll virus* to tobacco (Hoffmann et al., 2001b), *Barley yellow dwarf virus* to wheat (Helleco-Kervarrec et al., 2002) and *Cucumber mosaic virus* to gladiolus (Aebig et al., 2005). In the present study, we successfully demonstrated an efficient and rapid inoculation of TSWV by pressure spraying of inoculum using a simple device and showed its applicability to inoculating important host plants of TSWV. While the conventional hand inoculation method involves inoculating plants one at a time, the method presented here allows simultaneous inoculation of a group of test plants with TSWV. The procedure should be useful for transmission studies and for the rapid screening for resistance against TSWV in various hosts.

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