Tissue Blot Immunoassay for Detection of *Tomato spotted wilt virus* in *Ranunculus asiaticus* and Other Ornamentals

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ABSTRACT

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A tissue blot immunoassay (TBIA) was developed to detect *Tomato spotted wilt virus* (TSWV) in *Ranunculus asiaticus* tubers and other ornamentals. TBIA was comparable to double-antibody sandwich enzyme-linked immunosorbent assay for accuracy and reliability. A nondestructive sampling method was used with *R. asiaticus* tubers to determine: (i) the relationship between tuber infection and size; (ii) the distribution of TSWV in tubers; and (iii) the relationship between tuber infection and tuber germination. Small tubers had a higher percentage (44%) of infection than large tubers (19%). When destructive sampling was acceptable, the central stem tissue was the most reliable to test. TSWV infection was associated with a significant reduction of tuber germination. Among the tubers that tested positive for TSWV infection, 48% of those that germinated produced foliage in which TSWV was detected. The remaining 52% of the infected tubers planted that germinated developed into plants that were asymptomatic and in which TSWV was not detectable after germination. Only 4% of tubers that tested negative produced infected plants after germination. Our results indicate that TBIA can be used in TSWV management programs to identify infected plants and to index tuber crops.

Additional keywords: *Frankliniella occidentalis*, tospovirus

Tomato spotted wilt virus (TSWV), the type member of the genus *Tospovirus* (family *Bunyaviridae*) (6), is an increasing problem for production of agricultural and ornamental crops worldwide (5,7). The virus has an extremely wide host range of over 650 species, including dicots and monocots (7). TSWV causes diverse symptoms that include ring spots, stem necrosis, mosaic, petiole necrosis, and stunting, which may vary with the host. For example, a survey of 511 commercial greenhouses in Pennsylvania revealed that only 38% of the plants infected with tospoviruses exhibited the characteristic ringspot symptom associated with TSWV infection (9). Virus infection also decreases flower quality and the number of flowers per stem (15). In addition, symptom expression in some crops can be delayed for up to 2 months postinfection (e.g., chrysanthemum

and dahlia), resulting in infected plants serving as symptomless reservoirs of TSWV (1,15,25). Similarities between some tospovirus symptoms and those associated with fungal, bacterial, or other viral pathogens further confound diagnosis (9,27).

Propagation and distribution of infected cuttings can result in TSWV spread (9), because plants are often vegetatively propagated and cuttings frequently do not express symptoms until they are rooted and moved into pots. The virus can spread rapidly to other plants before the grower is aware that a problem exists. Other propagation materials such as tubers, corms, rhizomes, and bulbs rarely show symptoms, but can be important virus sources (19). Because of the worldwide movement of propagative materials and the potential for TSWV spread, a simple and rapid diagnostic tool is needed.

TSWV is known to be transmitted by eight species of thrips (Thysanoptera: Thripidae) (17,26). The most prominent vector of TSWV in agricultural and ornamental production in the United States is *Frankliniella occidentalis* (Pergande), the western flower thrips (WFT) (19). Other thrips species are important in specific

locales and on particular crops, virus species, and/or isolates (20,26). Adults transmit TSWV only if they acquire the virus as immatures (first and second larval instars) (21). After larval acquisition, the virus replicates in thrips cells, and adult thrips can transmit the virus for the duration of their lives (22).

Ranunculus asiaticus L. is an ornamental crop grown for tuber production, potted plants, and cut flowers. Production is located principally in Western Europe, South Africa, California, Israel, and Japan (16). TSWV has been a serious problem in *R. asiaticus* production, but the impact of infection on the crop has not been well studied. *R. asiaticus* seeds are planted for tuber production, and tubers with desirable characteristics such as flower color and number of stems are selected and dried. These tubers are then planted to produce seed for the next year. TSWV could persist in these tubers, thus serving as a source for virus in production areas. Consequently, a rapid and nondestructive technique to determine whether TSWV persists in tubers after drying and whether infected tubers can serve as a virus source is needed.

Several techniques are available for the detection of tospoviruses, including reverse-transcriptase polymerase chain reaction (RT-PCR) (18), enzyme-linked immunosorbent assay (ELISA) (3,8), and tissue blot immunoassay (TBIA) (11,14). An ELISA kit developed for growers is also commercially available (28). While these methods are all reliable, most require tissue preparations that can be difficult with woody or dormant tissues, and some, like RT-PCR, require specialized equipment and facilities. Our overall goal was to develop a TBIA that would work with woody and dormant tissues (e.g., tubers and bulbs) and be feasible for pest management practitioners to use in early detection and indexing.

The advantages of TBIA are many. TBIA requires minimal sample preparation; the volume of antibody needed for detection is less than that needed for double antibody sandwich (DAS)-ELISA; and the format has potential for use in the field. The nonstructural (NSs) protein encoded

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by the S RNA of TSWV is abundant in infected plant and thrips cells, and it forms abundant paracrystalline inclusions in infected cells (12,22,23). Thus, the objectives of this research were to (i) develop a TBIA to detect TSWV NSs in herbaceous plants, tubers, and bulbs; (ii) compare the reliability of the TBIA with that of DAS-ELISA; and (iii) determine how long the NSs protein could be detected with TBIA after blotting. We also report application of TBIA for detection of TSWV in ornamentals and *R. asiaticus* tubers, distribution of the virus in infected tuber tissue, and the effect of TSWV infection on tuber germination.

MATERIALS AND METHODS

Plant material. Herbaceous crop plants and weeds with virus symptoms were sampled from nursery production areas (both greenhouse and field) in North San Diego County, CA, and tested for TSWV by TBIA and ELISA. Plants species tested included: *Begonia* × *hiemalis*, *Chrysanthemum* sp., *Eustoma* sp., *Impatiens* sp., *Kalanchoe* sp., *Limonium latifolium* (Sm.) Ktze., *Malva parviflora* L., *Senecio cruentus* DC., *Datura stramonium* L., and *Tropaeolum* sp. Many of these plants are nursery crops and did not require identification because they were labeled by the grower; those that were weeds were identified by the farm advisor for horticultural crops in San Diego County (K. Robb). We used *Emilia fosbergii* Nicolson and TSWV isolate (TSWV-MT2) maintained as described (21), as a positive control in TBIA and ELISA.

R. asiaticus cv. Telecote was used in all the experiments described. *R. asiaticus* tubers from the 1996 and 1997 crop years were dried and stored consistent with commercial practices by placing tubers in paper bags at room temperature away from direct light. Tubers are viable for 2 years or more after harvesting if properly stored (16). Tubers from the 1996 crop were stored for 2 years before testing, and tubers from 1997 were stored for 1 year before testing. Ranunculus tubers have multiple tuberous rootlets, ranging anywhere from 5 to 40 rootlets that are attached to central stem tissue (Fig. 1A). Two rootlets from opposite sides of the tuber were removed for TBIA without destroying the tuber integrity. The remaining tuber was planted to obtain biological confirmation of infection and germination efficiency.

TBIA. Leaf samples from herbaceous plants were rolled into a tight bundle, sliced with a razor blade, and the cut area lightly blotted onto 0.45-μm Nitro ME nitrocellulose (NC) membranes (Micron Separations, Inc., Westboro, MA) for 3 to 5 s. Rootlets sampled from dried *R. asiaticus* tubers were incubated for 16 to 20 h in deionized (DI) water at room temperature (RT) in 24-well microtiter plates (Costar Corporation, Cambridge, MA). A cross

section of each rootlet was then cut with a razor blade, and the cut surface was lightly pressed onto the NC membrane.

The blotted membranes were air-dried at RT, placed in glass hybridization tubes, and allowed to react with a series of reagents described below. All incubation and wash steps were carried out at RT in a hybridization oven (Lab-Line, Barnstead, Dubuque, IA) at 16 rpm. Membranes were exposed to reagents sequentially, and the reagents were poured in and out of the tube without removing the membrane. First, membranes were blocked with 10 ml of 1% (wt/vol) bovine serum albumin (BSA) in phosphate buffered saline (PBS) (0.14 M sodium chloride, 1.0 mM potassium phosphate, 8.0 mM sodium phosphate, and 2.5 mM potassium chloride at pH 7.5) for 1 h. Second, the membranes were treated with the appropriate dilution of anti-NSs monoclonal antibody from a stock solution (MAb at 1 mg/ml, provided by John Sherwood) based on preliminary experiments (2). Although concentrations of stock antibody were standardized, working dilutions varied with antibody batch and ranged from 1:100 to 1:2,000. Antibodies were diluted in 4 ml of antibody dilution buffer (PBS, 0.1% Empigen BB [Calbiochem, La Jolla, CA] and 0.1% BSA). The membranes were then incubated with the antibody for 2 h. After reacting membranes with NSs antisera, membranes were washed three times in

PBS with 0.5% Tween (PBST), 5 min/wash as described above followed by incubation in 4 ml of a 1:2,000 dilution (in antibody dilution buffer) of rabbit anti-mouse alkaline phosphatase conjugate (Biorad, Richmond, CA) for 1 h. The membranes were washed again in PBST (3 times, 5 min/wash). Alkaline phosphatase substrate was added (Sigma Fast BCIP/NBT tablets, b5655), and the membranes were incubated for 5 to 15 min. The membranes were then rinsed in water and examined after air drying at RT. TSWV-infected and -noninfected *E. fosbergii* plants and/or *R. asiaticus* tubers were used to confirm the validity of the assay. The presence of a purple precipitate indicated that the NSs protein was present and that the sample was infected with TSWV. When the portion of the plant infected was small, the resulting positive reaction was small, and blots were examined using a dissecting microscope to confirm the presence of a positive reaction. In most cases, positive reactions were visible by eye and did not require magnification. To test for nonspecific reactions between plant samples and the substrate, blotted plant samples were reacted with only the substrate. A purple precipitate indicated a nonspecific reaction.

In the field, the entire procedure was performed in disposable 50-ml polypropylene tubes (Sarstedt, Newton, NC). The tubes were incubated in a pocket or backpack and

Fig. 1. A, *Ranunculus asiaticus* tuber with two rootlets removed for virus testing. B, Results of tissue blot immunoassay (TBIA) of leaf tissue. *Tomato spotted wilt virus* (TSWV) infected samples developed a purple color, whereas healthy *Emilia* did not. *Kalanchoe* (*) had a nonspecific reaction with the substrate causing a purple color to develop when non-TSWV infected plants were sampled. C, Results of TBIA after tuber samples were blotted on membrane, stored from 3 to 84 days, and processed. +, Rootlets infected with TSWV. -, Rootlets not infected with TSWV.

agitated periodically. Actively growing plants did not require the presoaking step required for testing of dried tubers. None of the other incubation steps were altered.

Comparison of TBIA and ELISA. TBIA results using NSs MAb were compared with DAS-ELISA using a polyclonal antibody to TSWV virions provided by John Cho, University of Hawaii, Maui Branch Station, Kula, HI (4,8). This DAS-ELISA was selected for comparison because it is commonly used for detection of TSWV (4). Controls used were TSWV-infected and -noninfected *R. asiaticus*, and *E. fosbergii* leaf tissue maintained as described earlier. A total of 540 tubers were tested by both methods. Tuber rootlets were soaked, blotted for TBIA, and the same rootlets were triturated in 1 ml of ELISA extraction buffer. DAS-ELISA was performed as previously described (4,8). Samples (150 μ l per well) were incubated in 96-well microtiter plates previously coated with 150 μ l of anti-TSWV antibody (1 mg/ml) diluted 1:2,000. Plates were washed three times with PBST, and then anti-TSWV antibody conjugated to alkaline phosphatase was added at 1:8,000 dilution. The plates were washed three times with PBST, and then substrate (*p*-nitrophenyl phosphate, 1 mg/ml) was added. The DAS-ELISA reactions were allowed to progress for 1 h at RT, and then absorbance was measured at 405 nm using a Biorad 550 microplate reader. Samples tested by DAS-ELISA were considered positive for TSWV infection when the absorbance readings exceeded two times the mean of the negative controls plus two standard deviations.

Longevity of blotted membranes. TSWV-infected and -noninfected tubers were blotted on membranes for TBIA as described. The membranes were stored in sealed plastic bags at 4°C or RT for 3, 5, 7, 28, 63, and 84 days. Membranes were then tested using the TBIA procedure described. This experiment was replicated five times, with three TSWV-infected and three TSWV-noninfected tubers included per replication.

Distribution of TSWV in *R. asiaticus* tubers. Rootlets and central stems were

sampled from randomly selected tubers to determine the distribution of TSWV in the tissue. Tubers were soaked in DI water for 24 h, and then each rootlet and the central stem portion of each tuber were blotted and tested by TBIA (N = 159 tubers). Each rootlet was tested individually. To standardize for variation in the number of rootlets per tuber (the number of rootlets per tuber varied from 5 to 26), the number of infected rootlets per tuber was expressed as percent rootlets infected.

***R. asiaticus* tuber size and TSWV infection.** To determine if tuber size was influenced by virus infection, tubers were randomly sampled from three commercial size categories: jumbo (7 cm diameter and larger), #1's (6 to 7 cm), and #2's (5 to 6 cm) in 1996 and 1997. TBIA was performed on two rootlets from opposite sides of the tuber (Fig. 1A).

Impact of TSWV infection on tuber germination. Tubers were tested for TSWV infection with TBIA by sampling two rootlets per tuber. The tubers were then planted in Sunshine Mix no. 1 (Fison Western Corp., Vancouver, B.C.), watered thoroughly, and placed in a growth chamber at 10°C with 24 h of light for germination, which are ideal conditions for tuber germination (16). After 3 weeks, germination was assessed and leaf tissue from each tuber was tested for TSWV by TBIA. Symptomatic tissue was selected for testing, and if no symptoms were apparent, tissue was arbitrarily selected for testing.

Data analysis. All analyses were performed using Statistical Analysis Software (SAS Institute, Cary, NC) Procedures version 6.09. Results of ELISA and TBIA were analyzed using a chi-square (χ^2) test to evaluate the null hypothesis that TBIA and ELISA do not produce similar results. Logistic regression methods using the Genmod procedure were used to analyze

germination, year, size, and infection because these data can be expressed categorically and have a binomial distribution.

RESULTS

Tissue blot immunoassay. Use of the NSs antibody resulted in strong positive reactions (purple staining) with infected plants and no reaction with noninfected plants (Fig. 1B) when *Ranunculus*, *Begonia* $\times$ *hiemalis*, *Chrysanthemum* sp., *Eustoma* sp., *Impatiens* sp., *M. parviflora*, *S. cruentus*, and *Tropaeolum* sp., *D. stramonium*, and *E. fosbergii* were tested. The plants tested exhibited a variety of TSWV symptoms; some were characteristic of TSWV infection and the plants were not confirmed as infected by TSWV until tested. False positive results occurred only with blotted samples of *L. latifolium* and *Kalanchoe* sp., which were indistinguishable from a true positive reaction. This reaction shown in Figure 1B was due to a nonspecific reaction between plant sap and the substrate. All blots processed in polypropylene tubes in the field produced similar results to those processed in the glass tubes in the hybridization oven.

Comparison of TBIA and ELISA. Our results indicate that TBIA is a reliable alternative to ELISA for detection of TSWV. When the same *Ranunculus* tuber samples were tested by TBIA and ELISA, there was a 96.5% agreement between the two assays (N = 540, $\chi^2 = 450$, $P = 0.0001$). Among the samples, 26.5% (143 of 540) were positive and 70% (378 of 540) were negative by both assays. Among the remaining 3.5% of the samples for which the two assays provided different results, the differences between the two methods were split: 1.5% (8 of 540) tested positive with TBIA and negative with ELISA, and 2% (11 of 540) were positive by ELISA and negative by TBIA.

Longevity of blotted membranes. The NSs protein was reliably detected on membranes after samples were blotted and stored for as long as 84 days at RT or 4°C (Fig. 1C). As in previous experiments, negative controls (*E. fosbergii* and *R. asiaticus*) showed no reaction.

Presence and distribution of TSWV in *R. asiaticus* tubers. TSWV was detectable in tubers after 2 years of storage. The virus was not uniformly distributed in *Ranunculus* tubers, and the percentage of infected rootlets per tuber varied. TSWV was detected in more than half the rootlets per tuber for the majority of infected tubers assayed (Table 1). When one or more rootlets had detectable amounts of TSWV, the central stem portion always tested positive.

***R. asiaticus* tuber size and TSWV infection.** There was a significant correlation between tuber size and incidence of TSWV infection in 1997 ($\chi^2 = 140$, $P = 0.0001$). The smallest tubers (size grade #2), had a threefold greater incidence of TSWV than the larger tubers in 1997 (Fig. 2). The

Fig. 2. Percent *Tomato spotted wilt virus* (TSWV) infection in *Ranunculus asiaticus* tubers of different size grades [jumbo [7 cm diameter and larger], #1 [6 to 7 cm], and #2 [5 to 6 cm]] harvested from the 1996 and 1997 crops. Tubers were tested by tissue blot immunoassay of two rootlets from opposite sides of the tuber. Each bar represents a population of tubers, and bars with different letters are statistically different ($P \leq 0.05$).

Table 1. The percentage of *Tomato spotted wilt virus* (TSWV)-infected *Ranunculus asiaticus* rootlets per tuber

Rootlets infected (%) ^{y,z}	Central stems infected (%) ^z	No. of tubers
100	100	31
99-75	100	26
74-50	100	14
49-25	100	13
24-1	100	13
0	0	62

^y Rootlet number per tuber varies (from 5 rootlets per tuber up to 26 rootlets per tuber) so the percentage of rootlets infected is presented.

^z All tuber material was tested for TSWV infection by tissue blot immunoassay.

small tubers had the highest incidence of TSWV in 1996 and 1997. No significant difference occurred between jumbo and #1 tubers for virus incidence in 1996 and 1997. Year of harvest was also significant with regard to infection of the tubers, with the 1996 crop having higher incidence of TSWV than the 1997 crop ($\chi^2 = 132$, $P = 0.0001$).

Impact of TSWV infection on germination. Tuber germination for TSWV-infected *R. asiaticus* was significantly reduced ($N = 411$, $\chi^2 = 20.6539$, $P = 0.0001$). Thirty-nine percent (41 of 105) of tubers testing positive before planting germinated, compared with 85% (261 of 306) that tested negative. Size alone was not found to be a significant factor related to tuber germination ($\chi^2 = 2.3471$, $P = 0.3093$). Size and infection negatively impacted germination of #2 (small) tubers harvested in 1997, with smaller infected tubers being less likely to germinate ($\chi^2 = 10.6$, $P = 0.0048$) (Fig. 3B). In 1996, all tubers had low percentage of germination regardless of tuber size ($\chi^2 = 3.7$, $P = 0.1584$) (Fig. 3A), and TSWV-infected tubers had reduced germination when compared to noninfected tubers.

Ninety-six percent of the tubers that were negative for TSWV (by testing two rootlets from opposite sides with TBIA) produced symptomless foliage after germination and TSWV was not detected in the leaf tissue. Four percent of the tubers testing negative prior to planting had TSWV-infected foliage after germination. Among the 41 tubers that were positive by TBIA and still germinated, 48% had foliage symptomatic of TSWV infection after germination and tested positive for TSWV. The remaining 52% had foliage that did not have symptoms of TSWV, and the foliage tested negative for TSWV.

DISCUSSION

Serological tests are the most widely used method to diagnose TSWV in plants and insects (2,3,8,28). Our results show that TBIA detection of the NSs protein is a reliable method to detect TSWV in foliage and tuberous tissues from many plant species, although care must be taken with some plant species due to nonspecific reactions. Because false positives may occur with some plants (e.g., *Kalanchoe* sp. and *L. latifolium*), plant species need to be first tested by reacting a blotted membrane with substrate alone to determine if plant proteins are reacting with the substrate. The TBIA and DAS-ELISA were equivalent in accuracy and reliability. One advantage of TBIA is that less sample preparation is required than with assays that require grinding of samples in buffer. The ease of sampling and processing makes TBIA a useful tool for growers and pest control advisors, e.g., assays can be performed in disposable bottles without any special laboratory facilities. Thus, TBIA could

increase the efficiency of indexing and become an integral part of TSWV management programs.

The NSs protein was stable on membranes and could be detected after 84 days of storage. This is consistent with findings for other proteins that were detected up to 6 months after blotting (10). While an important use of TBIA is for rapid diagnosis, some other applications of TBIA for TSWV diagnosis will benefit from the stability of the NSs protein on the membrane. For example, samples can be blotted in one location and sent elsewhere for testing, or batch processing over time could be applied to indexing large numbers of plants.

TSWV is unevenly distributed in *Ranunculus* tubers, which is consistent with other research showing that TSWV is unevenly distributed in many plant species (13,25,27). Postgermination testing of tubers revealed that, in most cases, our nondestructive two rootlet sample detected infected tubers. Germination assays showed that only 4% of the tubers that tested negative by TBIA produced symptomatic infected foliage upon germination. It is likely that these tubers had a low number of infected rootlets and thus were not detected using the two rootlet sampling method. This hypothesis is consistent with our finding that 13% of the total tubers in our in-

vestigation had very few infected rootlets (less than 24% of the total rootlets per tuber tested positive). To accurately detect infected tubers with a low number of infected rootlets, more rootlets must be tested. To improve accuracy, at least six rootlets would need to be sampled, which in some cases (e.g., small tubers), might be too destructive. Large-scale commercial testing of *R. asiaticus* using this TBIA and the two rootlet sampling method is ongoing. Germination trials in the field using tubers from this large-scale testing will ultimately help determine whether sampling two rootlets per tuber is commercially acceptable. In situations where destructive sampling could be used, e.g., for random sampling of tuber batches, testing the center portion of the tuber, in which virus was detected in 100% of the known TSWV-infected samples, would be the most reliable sampling method.

A greater number of smaller tubers (grade #2) were TSWV-infected than larger tubers (grades #1 and jumbo). This indicates that TSWV infection affects the size of the tuber. However, tuber size alone cannot be used to determine if tubers are infected because cultural practices, environmental conditions, and genetics may also affect tuber size. This information could be used to direct and concentrate indexing efforts. For example, larger tubers

Fig. 3. Percent germination of tubers that were grown under controlled-environment conditions. Tubers of different grades (jumbo, #1, and #2) and from the 1996 and 1997 harvest were analyzed. Tubers were tested by tissue blot immunoassay before planting. Each bar represents a population of tubers, and bars with different letters are significantly different ($P \leq 0.05$). A, Tubers harvested in 1996. B, Tubers harvested in 1997.

selected for seed production for the next year may be a source of TSWV in production areas. We recommend that these tubers be tested before planting in production areas and infected tubers culled.

The germination experiments conducted in a growth chamber indicated that TSWV persists in *Ranunculus* tubers after 2 years of storage and that tuber germination was adversely affected by TSWV infection. Germination was least in the smallest infected tubers (grade #2) from the 1996 harvest (a year in which there was a high incidence of TSWV). Germination of larger tubers (grades #1 and jumbo) was not as severely affected by infection, perhaps because these tubers have greater reserves. It seems likely that this attribute would result in a higher probability of some non-infected rootlets being present that could successfully germinate. Because TSWV negatively impacts tuber germination, we suspect that infected rootlets on these tubers simply did not germinate. Similar observations have been made with other TSWV-infected bulb and tuber crops (24,25). *Dahlia*, *Hippeastrum*, *Nerine*, and *Amaryllis* bulbs and tubers infected with TSWV commonly produce plants and daughter tubers that are not infected with TSWV (25).

The TBIA can be used for TSWV monitoring and indexing programs to identify potential sources of TSWV. This may allow rapid removal of inoculum sources and large-scale indexing of propagative materials. Because there is minimal sample preparation and laboratory facilities are not required, TBIA could be an important component of TSWV management programs.

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