Comparison of Enzyme-Linked Immunosorbent Assay Procedures for Detection of Tomato Ringspot Virus in Woody and Herbaceous Hosts

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ABSTRACT

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Two enzyme-linked immunosorbent assay (ELISA) procedures, direct and indirect, were compared in combination with two sample preparation methods, leaf disks and sap extraction to determine their relative abilities to detect tomato ringspot virus (TmRSV) in leaves of *Chenopodium quinoa*, geraniums, apples, and peaches. In sap extracts, TmRSV was detected equally well by both ELISA procedures. When leaf disks were tested, the indirect procedure was somewhat more sensitive. Single leaf disks did not provide sufficient TmRSV for efficient detection by either ELISA method; however, increasing the number of disks to five facilitated efficient detection of TmRSV in geraniums and *C. quinoa*.

During the last several years, enzyme-linked immunosorbent assay (ELISA) has gained considerable recognition and use as a diagnostic procedure for plant pathogens, most notably viruses. As the popularity of this technique has increased, many variations and modifications have been developed to increase sensitivity and sample preparation efficiency. Two of these variations have great potential value for routine virus diagnosis: first, detecting the presence of an immobilized antigen-rabbit antibody complex using enzyme-labeled goat antirabbit immunoglobulin G (IgG) (5,12), and second, using intact leaf disks as the sample source (7).

Tomato ringspot virus (TmRSV) is associated with diseases in a wide variety of plants including peach (8), grape (11), raspberry (9), geranium (1), apple (10), blueberry (4; R. F. Stouffer and C. A. Powell, *unpublished*), and cymbidium orchid (3). Because each of these crops is propagated vegetatively, effective methods for detecting TmRSV in propagating stock are a prerequisite for effective certification and control programs. In this paper, I report the comparison of ELISA variations (direct [2] and indirect [5]) in combination with two sample preparation techniques (Tissumizer [Tekmar] trituration and leaf disks) for detecting TmRSV in *Chenopodium quinoa*, geranium, apple, and peach.

MATERIALS AND METHODS

Host plants and sampling procedure. Four plant species were used as hosts for TmRSV in these studies: *C. quinoa*, Sincerity geranium (*Pelargonium* × *hortorum*), Halford peach (*Prunus persica*), and MM106 apple (*Malus sylvestris*). Unless otherwise indicated, *C. quinoa* were mechanically infected with an apple isolate of TmRSV (TmRSV-A). *C. quinoa* samples consisted of an individual leaf from each individual plant and leaf disks from another individual leaf from the same plants for the Tissumizer and leaf-disk sample preparation methods, respectively. Samples were collected from symptomatic leaves 5–7 days after inoculation. Geraniums were naturally infected acquisitions of unknown age from Pennsylvania greenhouses. Geranium samples for Tissumizer trituration consisted of a composite sample of three leaves (young, medium, and old) from each of five individual plants; the single leaf-disk sample was taken from a young leaf of each of the five plants; and the five-leaf-disk sample included disks from each of five leaves (two young, two medium, and one old) from each of the five plants. Apple and peach trees (about 1-yr-old seedlings) were infected with the TmRSV-A isolate via dagger nematode (*Xiphinema rivesi*). Samples were collected from apple and peach seedlings as described for geraniums. Apple and peach leaf samples were collected about 1 yr after viruliferous nematodes were added to potted plants and about 3 mo after virus was detected in the trees. The experiment was conducted in May. All plants were maintained in a greenhouse. Geraniums and apples showed no sysmptoms, *C. quinoa* showed ringspot symptoms, and peaches showed stunting and drastic shortening of internodes.

Sample preparation. Crude sap was prepared by triturating leaf tissue (1 g of tissue per 2 ml of buffer) with a Tissumizer in either 0.02 M potassium phosphate buffer, pH 7.4, containing 0.15 M sodium chloride, 0.05% Tween 20, and 20% polyvinylpyrrolidone, mol wt 40,000 (PBS-Tween-PVP), or 0.05 M sodium carbonate buffer, pH 9.6, for direct or indirect ELISA, respectively. The leaf-disk method consisted of floating leaf disks (14 mm in diameter) on 0.25 ml of PBS-Tween-PVP or 0.05 M sodium carbonate, pH 9.6, for direct or indirect ELISA, respectively, in wells of microtiter plates (Dynatech, Alexandria, VA) (7).

ELISA. Antiserum to TmRSV-A was prepared and fractionated as described previously (6). Indirect ELISA was performed as described by Lommel et al (5). Each sample, consisting of crude sap or leaf disks, was prepared in 0.05 M sodium carbonate buffer, pH 9.6, placed in duplicate wells of a microtiter plate, and incubated at 4 C for 16 hr. The next day, 0.25 ml of anti-TmRSV gamma-globulin (10 μg/ml) in PBS-Tween-PVP was added to each well and incubated at 37 C for 4 hr. Next, 0.25 ml of a 1/2,000 dilution of alkaline phosphatase-conjugated goat antirabbit IgG (Miles) was added to each well and incubated at 4 C for 20 hr. The next day, the enzyme substrate *p*-nitrophenyl phosphate (1 mg/ml in 10% diethanolamine, pH 9.8) was added, and the reaction was analyzed spectrophotometrically at 405 nm after 1 hr.

Direct sandwich ELISA was performed as described by Clark and Adams (2). Plates were coated at 4 C for 16 hr with anti-TmRSV gamma-globulin (10 μg/ml) in 0.05 M sodium carbonate, pH 9.6. Samples (leaf disks or crude sap) were prepared in PBS-Tween-PVP and incubated in duplicate microtiter plate wells at 4 C for 16 hr. The anti-TmRSV enzyme conjugate (1/8,000 dilution) was incubated at 37 C for 4 hr. A sample was scored positive if the A_{405} was twice that of the healthy control.

RESULTS AND DISCUSSION

Table 1 presents data from a representative experiment comparing direct or indirect ELISA in combination with Tissumizer triturates or leaf disks for detecting TmRSV. Partial or complete repeats of this experiment gave similar results. When infected tissue from any of the four hosts was homogenized with a Tissumizer and virus concentration was probably not a limiting factor, both direct and indirect ELISA detected TmRSV equally well. In several (but not all) cases, however, when leaf disks were the samples and virus concentration was probably a limiting factor, indirect

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Table 1. Absorbance values (A_{405}) obtained with different tissue preparation methods and two enzyme-linked immunosorbent assay procedures using healthy and tomato ringspot virus-infected leaves from woody and herbaceous plants

		Infected				Healthy			
Treatment	Plant	Direct (range)	Avg.	Indirect (range)	Avg.	Direct (range)	Avg.	Indirect (range)	Avg.
Tissumizer	Apple	0.17–0.22	0.20	0.19–0.21	0.20	0.00–0.03	0.01	0.00–0.03	0.02
	Peach	0.18–0.27	0.22	0.17–0.28	0.21	0.02–0.03	0.02	0.01–0.03	0.02
	Geranium	0.22–0.38	0.29	0.24–0.55	0.31	0.02–0.03	0.03	0.01–0.02	0.02
	Chenopodium	0.39–0.50	0.47	0.38–0.70	0.48	0.02–0.03	0.03	0.01–0.02	0.02
Five-leaf disks[a]	Apple	0.02–0.06	0.04	0.04–0.10	0.07	0.02–0.03	0.02	0.02–0.03	0.02
	Peach	0.05–0.08	0.06	0.04–0.18	0.11	0.00–0.03	0.02	0.01–0.03	0.02
	Geranium	0.04–0.18	0.14	0.19–0.24	0.23	0.01–0.03	0.02	0.02–0.04	0.03
	Chenopodium	0.14–0.20	0.16	0.18–0.25	0.22	0.02–0.04	0.03	0.00–0.01	0.01
One-leaf disk	Apple	0.02–0.03	0.03	0.03–0.05	0.04	0.02–0.03	0.02	0.02–0.03	0.02
	Peach	0.03–0.04	0.04	0.03–0.09	0.06	0.01–0.03	0.02	0.01–0.02	0.02
	Geranium	0.03–0.09	0.04	0.13–0.19	0.16	0.00–0.02	0.01	0.00–0.02	0.01
	Chenopodium	0.04–0.11	0.06	0.10–0.56	0.21	0.02–0.03	0.03	0.00–0.01	0.01

[a] Single leaves or leaf disks taken from five healthy and infected plants of each species.

Table 2. Detection of tomato ringspot virus (TmRSV) or tobacco ringspot virus (TbRSV) in leaf disks and sap extracts from symptomatic *Chenopdium quinoa* by direct and indirect enzyme-linked immunosorbent assay

Treatment	Direct[a]	Indirect[a]
Tissumizer	98/100	99/100
Five-leaf disks	96/100	97/100

[a] The numerator is the number of plants that were positive (A_{405} of at least twice that of the healthy control). The denominator is the number of plants tested. Healthy control readings were no higher than 0.05. Positive readings were not lower than 0.34. Direct ELISA detected only TmRSV. Indirect ELISA detected either TmRSV or TbRSV.

ELISA was slightly more sensitive than direct ELISA. Nonspecific color change in wells containing healthy tissue was the same for both ELISA methods, except indirect ELISA gave a lower background than direct ELISA when *C. quinoa* was the test plant.

The order of decreasing ability of extraction procedures to provide detectable virus was Tissumizer trituration, five leaf disks, and one leaf disk. Clear positive reactions were obtained by both ELISA procedures using sap extracts in all four hosts. A single infected leaf-disk sample yielded only clear positive results with indirect ELISA and then only with the herbaceous hosts. Increasing the number of leaf disks to five resulted in detection of TmRSV by either ELISA method in the herbaceous hosts, but virus was still not detected (A_{405} of twice that of the control) in all cases in the woody hosts. Increasing the substrate incubation time did not increase the absorbance ratio of infected to healthy samples, possibly because the coating serum and conjugated serum concentrations were optimized for a 1-hr substrate incubation.

A second experiment was performed to compare five-leaf-disk samples and Tissumizer trituration in combination with direct and indirect ELISA for routine detection of virus in orchard sites. Sap from dandelions collected from various orchards was used to mechanically inoculate *C. quinoa*. One hundred *C. quinoa* that developed necrotic local lesions were tested for virus by the procedures described in Materials and Methods, except the rabbit antibody in the indirect ELISAs contained anti-tobacco ringspot virus (TbRSV) gamma-globulin (10 μg/ml) in addition to the anti-TmRSV gamma-globulin. (Few if any of the antibodies to TbRSV react with TmRSV and vice versa.)

Tissumizer sap extracts of 98 of the 100 symptomatic *C. quinoa* were positive for TmRSV by direct ELISA (Table 2). The two symptomatic *C. quinoa* that were negative for TmRSV were confirmed to be free of TmRSV by repeated ELISA. One of these *C. quinoa* was subsequently found to contain TbRSV, and the other *C. quinoa* was found to contain an unidentified virus (neither TmRSV nor TbRSV). Thus, direct ELISA of sap extracts detected TmRSV in 100% of the symptomatic *C. quinoa* infected with TmRSV. Leaf-disk samples from 96 of the 100 symptomatic *C. quinoa* were positive for TmRSV by direct ELISA; therefore, this method failed to detect TmRSV in two TmRSV-infected plants.

Tissumizer sap extracts of 99 of the 100 symptomatic *C. quinoa* were positive by indirect ELISA. The rabbit antibody (unconjugated) in the direct ELISA was a mixture of immunoglobulins specific for TmRSV or TbRSV. Indirect ELISA detected virus in the 98 TmRSV-infected plants and the one TbRSV-infected plant. Some of the 98 TmRSV-infected *C. quinoa* may have also contained TbRSV. Indirect ELISA would not distinguish between plants with single and double infections.

Combined results confirm those of Romaine et al (7) that leaf disks are suitable samples for ELISA of herbaceous plants and that five disks are considerably better than a single disk. The leaf-disk method frequently failed to detect TmRSV in infected woody hosts, however. This is probably because the virus concentration was low or unevenly distributed. The advantages of simple and rapid sample preparation make the leaf-disk method valuable for indexing herbaceous indicators, especially when an occasional false negative can be tolerated. Results also confirm the usefulness of indirect ELISA (5), which was at least as sensitive as direct ELISA in all cases. The advantages of indirect ELISA include less strain specificity (12) and a single commercially available enzyme conjugate for detection of all viruses (5).

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