Detection of Tobacco Mosaic and Tobacco Ringspot Viruses in White Ash Trees by Enzyme-Linked Immunosorbent Assay

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ABSTRACT

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Tobacco mosaic virus (TMV) or tobacco ringspot virus (TbRSV), or both, were detected by enzyme-linked immunosorbent assay (ELISA) at various times during the 1981 growing season in tissue samples taken from 21 of 23 white ash (*Fraxinus americana*) trees. Virus infection was not correlated with twig and branch dieback; however, all but two trees with viruslike foliar symptoms were infected with either or both viruses. Neither virus was associated consistently with any specific foliar symptom. TbRSV was detected most consistently in root tissue samples. TMV was detected with equal frequency in both root and aboveground tissues. Peak periods of virus detection occurred at 3- to 5-wk intervals throughout the growing season.

Additional key words: ash dieback, contributing factors, predisposing

Both tobacco mosaic virus (TMV) and tobacco ringspot virus (TbRSV) have been transmitted to herbaceous hosts from leaves of white ash (*Fraxinus americana* L.) that show foliar symptoms of virus infection, including line patterns, mosaic, chlorotic spots, mottling, ring spots, chlorosis, red and green rings, and premature autumn coloration (7,10). Foliar symptoms often were associated with twig and branch mortality, reduced apical growth, and branch and trunk cankers (12,14). This syndrome is called ash dieback. Both TMV and TbRSV were detected singly and together in white ash trees with and without dieback symptoms in central New York (1,4). The objectives of this study were to determine 1) if either TMV or TbRSV infection was correlated with a particular symptom of ash dieback and 2) the optimum season and the best tissue from which to detect these viruses in white ash.

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MATERIALS AND METHODS

Sampling procedures. Twenty-three white ash trees were selected, 18 in two previously established dieback plots (17) and five in one new plot, all near the towns of LaFayette and Tully, NY. Trees were selected to represent all stages of the ash dieback syndrome. Each tree was rated for dieback severity according to the system of Silverborg and Ross (17), which is based on percentage of branches and twigs dead or dying back. In addition, foliar symptoms were observed and recorded weekly for all trees from 18 May through 5 October 1981. Twenty grams of leaf and bark (cambium and phloem) tissue from small twigs was collected weekly from each of one to three branches from all trees. Fully expanded leaves were collected whenever possible. About 10 g of roots (small roots <5 mm in diameter) also were collected weekly from 18 trees at a depth of 20–30 cm. All samples were placed in individual plastic bags, labeled, sealed, brought to the laboratory, rinsed in tap water, air-dried, and frozen at −20 C. Tissue samples were collected from trees in the three plots on different days during each week.

Sample processing. For each tree and collection date, 4 g of leaf tissue, 2 g of bark tissue, and 2 g of root tissue were placed in separate plastic vials. Extraction buffer (phosphate-buffered saline, pH 7.4, + 0.05% Tween-20 + 1% β-mercaptoethanol) was added to each sample to give a 1:5 ratio of tissue:buffer. Each sample was triturated with a Janke and Kunkel Tissumizer (Tekmar Co., Cincinnati, OH), then refrozen at −20 C until indexed for TMV and TbRSV by enzyme-linked immunosorbent assay (ELISA). All samples collected from one tree (weeks 1–21) were processed at one time, then refrozen until processing of samples from all trees was completed. In addition, indexing of processed tissue samples from one tree was completed before indexing of samples collected from

another tree began. All samples were indexed over a period of 7 wk.

Virus purification and antiserum production. A local tobacco isolate of TMV was purified from *Nicotiana tabacum* L. 'Turkish' by the procedure of Gooding and Hebert (6), and followed by log-linear sucrose density-gradient centrifugation (3) and equilibrium density-gradient centrifugation in CsCl (16).

An ash isolate of TbRSV (obtained from C. R. Hibben, Brooklyn Botanic Garden Research Center, Ossining, NY) was purified from infected cucumber (*Cucumis sativus* L. 'National Pickling') by the procedure of Stace-Smith et al (18) and further purified as described for TMV.

Antiserum to each virus was produced in rabbits by administering four weekly subcutaneous and intramuscular injections followed by an intravenous injection of virus in the marginal ear vein 7 wk after the initial injection. Subcutaneous and intramuscular injections consisted of 0.2–0.5 mg of purified virus in 0.5 ml of 0.05 M Tris-HCl buffer (pH 7.4) emulsified in 0.5 ml of Freund's complete adjuvant. Intravenous injections consisted of 0.1 mg of purified virus in 0.5 ml of buffer and were administered without adjuvant. The rabbits were bled 3–5 days after the final injection, and the serum was collected after low-speed centrifugation. The antisera were cross-absorbed with purified healthy tobacco or cucumber protein prepared according to the method of Shepard (15). Specific antibody titer was determined by standard microprecipitin procedure (2).

ELISA. The ELISA technique of Clark and Adams (5) was modified as follows: Horseradish peroxidase (HRPO) was conjugated to the gamma-globulin fraction (γG) (13) after ammonium sulfate precipitation and dialysis. Gamma-globulin obtained from one bleeding was conjugated and used for all tests. Nunc Immunoplate I microtiter plates (Vangard International, Neptune, NJ) were used throughout this study. Plate wells were each coated with 100 μl of purified gamma-globulin (anti-TMV γG, 20 μg/ml, or anti-TbRSV γG, 5 μg/ml) in coating buffer (0.05 M carbonate buffer, pH 9.6), covered with Parafilm, and incubated at 37 C for 4 hr. Plates then were washed with phosphate-buffered saline (pH 7.4) containing Tween 20 (0.05%) and bovine serum albumin (10 mg/ml) (PBS-T-BSA) three times for 4 min each wash. Samples were prepared in extraction buffer as described and added at 50 μl/well, two wells per sample. The plates were covered with Parafilm and incubated overnight at 4 C. The plates then were washed as before in PBS-Tween 20, and 50 μl of conjugate diluted in PBS-T-BSA (anti-TMV γG:HRPO-1/100, anti-TbRSV γG: HRPO-1/200) was added per well. The plates were incubated for 3 hr at 22 C. Plates were washed again and 100 μl of substrate (0.4 ml of *o*-dianisidine [Sigma, St. Louis, MO] and 50 μl 3% H_2O_2 in 20 ml 0.1 M citrate buffer, pH 5.4 [13]) was added to each well. The plates were covered and incubated in the dark at 22 C for 1 hr. The reaction was stopped by adding 50 μl of 0.1 M NaOH to each well. Results were recorded visually and the absorbance at 460 nm (A_{460}) was measured and recorded with a Titertek Multiskan plate reader (Flow Labs, McLean, VA).

Positive, negative, and buffer controls were included on each plate. Buffer controls consisted of two wells that contained extraction buffer. Because we did not have a consistent source of known virus-free white ash tissue when these tests were made, we used healthy tobacco sap for negative controls. Negative controls consisted of two wells that contained healthy tobacco sap diluted 1/10 in extraction buffer. Positive controls consisted of purified TMV or TbRSV diluted to 2,000 ng/ml in extraction buffer (two wells per plate). A sample was considered positive for virus if the A_{460} of the sample well was greater than twice the average A_{460} of the buffer and healthy tobacco control wells included on the plate in which the sample was tested. The limit of sensitivity of the ELISA for detection of TMV and TbRSV was determined by dilution and assay of purified virus preparations.

Table 1. Mean absorbance values obtained in enzyme-linked immunosorbent assay with healthy tobacco sap, extraction buffer, and purified preparations of tobacco mosaic virus (TMV) and tobacco ringspot virus (TbRSV)[a]

Virus conc. (ng/ml)	A_{460} values	
	TMV	TbRSV
2,000.0	0.313 ± 0.011	0.599 ± 0.040
200.0	0.192 ± 0.016	0.579 ± 0.017
20.0	0.092 ± 0.004	0.363 ± 0.039
10.0	0.070 ± 0.006	0.219 ± 0.015
5.0	0.080 ± 0.002	0.141 ± 0.020
2.5	0.065 ± 0.009	0.100 ± 0.018
Buffer	0.064 ± 0.028	0.058 ± 0.026
Healthy tobacco	0.066 ± 0.026	0.062 ± 0.025

[a] Mean absorbance values and standard deviations calculated from six wells for each virus at each concentration, except A_{460} values of the 2,000 ng/ml preparations, which were calculated from 179 wells on 89 plates for each virus. The mean absorbance and standard deviations of healthy tobacco and buffer controls were each calculated from 168 wells on 84 plates.

RESULTS

Antisera produced against purified TMV and TbRSV had specific microprecipitin titers of 1:2,000 and 1:1,000, respectively. The limit of sensitivity for virus detection in ELISA was about 20–200 ng/ml for TMV and 2.5–5.0 ng/ml for TbRSV (Table 1).

Seventeen of 23 trees indexed by ELISA were evaluated as infected with both viruses, two were infected with TMV only, and two were infected with TbRSV only. Neither virus was detected in two trees (Table 2).

Infection with either one or both viruses was not correlated with twig and branch dieback because 12 of 21 infected trees were in class 1 (no twig or branch dieback, Table 2). Neither virus was detected in two of seven trees with severe dieback (class 4).

All 23 trees sampled in 1981 displayed foliar symptoms, which began to appear in mid-June (week 5). The first symptoms observed were leaf chlorosis and mottling, which intensified over time. Symptoms that developed throughout the season were the same as reported by others (7,10). All foliar symptoms were more pronounced from mid-July to leaf fall in October. Virus infection as determined by ELISA was correlated with the presence of foliar symptoms in all but two trees in which neither virus was detected. However, neither virus was consistently associated with any one foliar symptom over time, and there was no correlation between particular foliar symptoms and dual infection. Although foliar symptoms increased in severity over time, an increase in A_{460} values of either virus in aboveground tissues collected over time was not detected (Table 3).

In total, 2,054 leaf and bark samples and 361 root samples were indexed for TMV and TbRSV by ELISA. Both viruses were detected in bark and leaf tissues. However, fewer than 4% of all bark samples collected indexed positive for either virus. Therefore, the results of bark and leaf tissue indexing were combined to generate a comparison between root and aboveground tissues as

Table 2. Correlation between dieback class and presence of tobacco mosaic virus (TMV) or tobacco ringspot virus (TbRSV)

Disease class[a]	No. trees	No. trees infected			
		TMV	TbRSV	Both viruses	Neither virus
1	12	1	2	9	0
2	2	0	0	2	0
3	2	0	0	2	0
4	7	1	0	4	2

[a] Class 1 = no twig and branch dieback, class 2 = some dead twigs and branches, class 3 = less than 50% of the twigs and branches are dead, and class 4 = more than 50% of the twigs and branches are dead.

virus sources. Figure 1 summarizes the weekly indexing results from aboveground tissues of 19 trees infected with TMV (Fig. 1A) and 19 trees infected with TbRSV (Fig. 1B). The mean percentage of aboveground tissue samples that indexed positive for TMV was 16.7 ± 0.7%; for TbRSV, the mean was 13.4 ± 6.2%. In general, TMV was detected more frequently in aboveground tissues of infected trees later in the growing season (slope of the linear regression line in Figure 1A is significant at $P = 0.0005$, $r = 0.707$, one-tail Student's t test). The frequency of detection of TbRSV in aboveground tissues was not as well correlated with time (slope of the linear regression line, solid line, in Figure 1B is less significant, $P > 0.05$, $r = 0.315$). The ability to detect both viruses within aboveground tissues of infected trees appeared to occur in pulses. The pulses occurred at 3- to 5-wk intervals, with peak virus detection at weeks 7, 10, 14, and 18 for TMV and weeks 6, 10, 15, and 19 for TbRSV (Fig. 1A,B). The pulses of peak TbRSV detection in aboveground tissues were pronounced (Fig. 1B, broken line), whereas the pulses of TMV detection were less distinct (Fig. 1A).

Roots of 18 trees were sampled and indexed by ELISA for both viruses. TMV was detected in roots from 13 of 18 trees. The frequency of detection of TMV in root tissue was not correlated with time (slope of the linear regression line in Figure 2A, solid line, is not significant, $P > 0.1$, $r = 0.239$, one-tail Student's t test). The mean percentage of root samples that indexed positive for TMV was 18.1 ± 8.4%. TbRSV was detected in roots from 16 of 18 trees sampled. TbRSV was detected more frequently than TMV from roots collected later in the growing season (slope of the linear regression line in Figure 2B is significant at $0.1 > P > 0.05$, $r = 0.359$). The mean percentage of root samples that indexed positive for TbRSV was 50.3 ± 10.6%. Detection of both viruses within the roots of white ash also occurred in pulses at 3- to 5-wk intervals, with peak periods of virus detection at week 6 for TMV and weeks 9, 14, and 19 for both viruses (Fig. 2). Peak periods of TMV detection in root tissue were pronounced (Fig. 2A, broken line), whereas peak periods of TbRSV detection were less distinct (Fig. 2B).

Distribution of both viruses among tissues of infected trees varied among trees. In 10 of 21 virus-infected trees, both viruses were present in leaf and bark tissues from all branches sampled as well as in root tissue. Absorbance values of tissue samples that indexed positive for virus, from a representative tree in which both viruses were detected, are presented in Table 3. In two trees, TbRSV was the only virus detected (Table 2, class 1). In these two trees, the virus was consistently

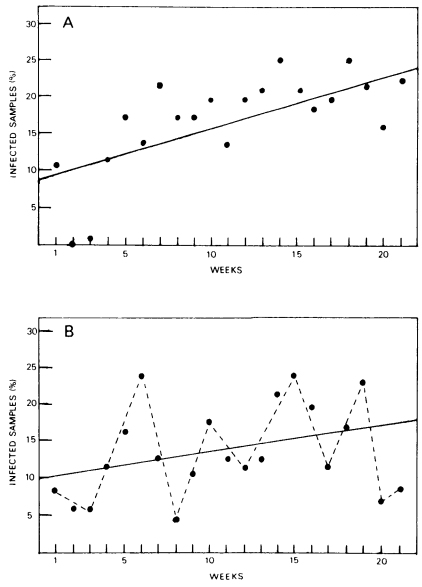

Fig. 1. Percentage of aboveground tissue samples collected weekly (18 May through 5 October 1981) from 19 white ash trees that indexed positive by ELISA for **(A)** tobacco mosaic virus ($P = 0.0005$, $r = 0.707$) and **(B)** tobacco ringspot virus (TbRSV) ($P > 0.05$, $r = 0.315$). The broken line (---) indicates the cyclical nature of TbRSV detection within these tissues.

Table 3. Absorbance values of aboveground tissue samples collected weekly from three branches of one tree and root tissue samples also collected weekly from the same tree that indexed positive for tobacco mosaic virus (TMV) or tobacco ringspot virus (TbRSV) and displayed foliar viruslike symptoms

	TbRSV				TMV			
	No. samples rated positive		Avg. A_{460}[a] of positive samples		No. samples rated positive		Avg. A_{460} of positive samples	
Week	Above-ground[b]	Root[c]	Above-ground	Root	Above-ground	Root	Above-ground	Root
1[d]	0	1	···	0.116	0	0	···	···
2	0	0	···	···	0	0	···	···
3	0	0	···	···	0	0	···	···
4	0	0	···	···	0	0	···	···
5	0	1	···	0.126	0	0	···	···
6	4	1	0.089	0.125	3	0	0.067	···
7	1	1	0.087	0.109	3	0	0.067	···
8	1	1	0.085	0.093	2	1	0.069	0.080
9	1	1	0.084	0.103	2	1	0.071	0.076
10	0	1	···	0.084	0	0	···	···
11	0	1	···	0.086	0	1	···	0.076
12	0	1	···	0.095	1	1	0.086	0.079
13	0	1	···	0.131	1	1	0.074	0.095
14	2	1	0.096	0.114	0	1	···	0.089
15	3	1	0.073	0.113	1	1	0.081	0.083
16	3	1	0.084	0.078	0	0	···	···
17	2	1	0.080	0.121	0	1	···	0.088
18	0	1	···	0.109	3	1	0.069	0.079
19	2	1	0.090	0.090	3	1	0.066	0.081
20	0	1	···	0.102	1	1	0.074	0.074
21	0	1	···	0.078	2	1	0.071	0.076

[a] A_{460} values represent an average of two wells per sample. A_{460} values greater than twice (>3 standard deviations) the average A_{460} value of buffer and healthy tobacco control wells on each plate were considered positive for virus. Each tissue sample was evaluated against control wells on the plate in which the sample was tested. The mean A_{460} value and standard deviation of all buffer and healthy tobacco control wells (44 wells on 11 plates) was 0.033 ± 0.010.

[b] Six aboveground tissue samples were collected weekly from this tree (three bark and three leaf tissue samples) and indexed for both viruses.

[c] One root tissue sample was collected weekly from this tree and indexed for both viruses.

[d] Weekly collections were conducted from 18 May through 5 October 1981.

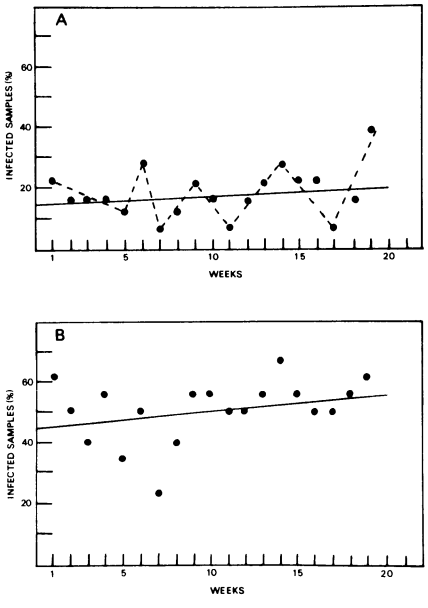

Fig. 2. Percentage of root tissue samples collected weekly (18 May through 23 September 1981) from **(A)** 13 white ash trees that indexed positive by ELISA for tobacco mosaic virus (TMV) in root tissue ($P > 0.1$, $r = 0.239$) and from **(B)** 16 white ash trees that indexed positive for tobacco ringspot virus in root tissue ($0.1 > P > 0.05$, $r = 0.359$). The broken line (---) indicates the cyclical nature of TMV detection within root tissue.

detected in root tissue but was not detected in aboveground tissues. In the nine remaining virus-infected trees, either or both viruses were detected sporadically in root or aboveground tissues from one or two of the branches sampled.

DISCUSSION

Both viruses appeared uniformly distributed within many virus-infected white ash trees sampled. Both viruses were detected within leaf and bark tissues of all branches sampled as well as in root tissue in 10 of 23 trees indexed. TMV, but not TbRSV, was detected more frequently from aboveground tissues collected in late summer (Fig. 1A). Distribution of TMV in aboveground tissues either became more uniform or the concentration of TMV in these tissues increased as the season progressed. It was not possible to estimate the concentration of either virus in tissue samples because of interplate variability in A_{460} values and because dilutions of purified virus were not included on each plate.

Foliar symptoms, possibly attributed to virus infection, first appeared in mid-June (week 5) and became more severe late in the growing season. The first appearance of foliar symptoms preceded a peak in detection of both viruses by 1–2 wk (Fig. 1). However, the more frequent detection of TMV in symptomatic foliage collected in late summer indicates that TMV may be responsible for the foliar symptoms observed. Although foliar symptoms were observed on all 23 trees indexed, neither virus was detected in two trees, and only TbRSV was detected in two trees (Table 2). TMV may have been present in these trees but at concentrations below the limits of detection by our ELISA system. Foliar symptoms may also have been caused by other viruses or pathogens. Recently, Hibben (8) reported the transmission of tomato ringspot virus from white ash trees with dieback symptoms. Mycoplasmalike organisms also are associated with yellows symptoms in white ash in New York State (8,12).

Both viruses were also detected in root tissue. TbRSV was detected more often in root tissue than TMV (50.3 ± 10.6% vs. 18.1 ± 8.4%, respectively). In addition, TbRSV, but not TMV, was detected more frequently from root tissue collected late in summer (Fig. 2B) and more frequently from root tissue than aboveground tissues (50.3 ± 10.6% vs. 13.4 ± 6.2%). TMV was detected with about equal frequency in both root and aboveground tissues (16.7 ± 0.7% vs. 18.1 ± 8.4%, respectively). In two trees, only TbRSV was detected in root tissue, with no evidence of either virus in aboveground tissues. These data suggest that initial infection of white ash trees with TbRSV may occur through the root system. TbRSV is vectored by the plant-parasitic dagger nematode *Xiphinema americanum* Cobb. This nematode is present in New York State in soil beneath white ash trees with dieback symptoms (9,14).

Pulses of virus detection that occurred at 3- to 5-wk intervals in both root and aboveground tissues of white ash trees are difficult to explain. Because of the manner in which tissue samples were collected, processed, and indexed, it seems unlikely that the pulsing phenomenon represents a sampling or indexing artifact. It is possible that pulses of increased virus detection may be caused by increases in viral replication or movement of virus within or among tissues in response to tree metabolism as influenced by environmental conditions.

Although virus infection was associated with foliar symptoms, it was not associated with twig and branch dieback. Trees in all dieback classes were virus-infected, including 12 trees without symptoms of branch dieback (Table 2). These 12 trees may subsequently develop the typical dieback syndrome and will be monitored for development of typical ash dieback symptoms. At this time, the role of TMV and TbRSV in the etiology of ash dieback still remains equivocal and awaits the results of inoculation studies with these viruses in white ash, already in progress. Manion (11) suggested that viruses are likely to be long-term contributing factors in decline-dieback diseases. The combined effects of predisposing and inciting factors such as drought, insect defoliation, or other pathogens may induce the typical dieback syndrome and enhance the impact of contributing factors like viruses (11).

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