Effects of Virus and Mycoplasmalike Organism Infection on Green and White Ash

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ABSTRACT

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White ash (*Fraxinus americana*) and green ash (*F. pennsylvanica*) seedlings were inoculated with tobacco mosaic virus, tobacco ringspot virus (TRSV), tomato ringspot virus, and mycoplasmalike organisms (MLOs) alone and in all combinations. After three cycles of growth and dormancy, one or more pathogens was detected in 190 plants. TRSV was associated with chlorotic mottling in both species and with chlorotic spots, ringspots, and vein yellowing in green ash. Virus symptoms were more severe in green than white ash. MLOs were associated with interveinal chlorosis, dwarfing and glossiness of leaves, and production of axillary shoots. These and other symptoms have been observed in naturally infected ash. MLO, but not virus infection, was associated with suppressed growth, more so in white than in green ash. Neither synergistic nor additive effects of multiple pathogen infection on growth or symptom development in ash were detected.

Additional keywords: ash dieback, ash yellows, enzyme-linked immunosorbent assay.

Research since the 1970s has implicated mycoplasmalike organisms (MLOs) and viruses as potential causes of decline of white ash (*Fraxinus americana* L.) in the northeastern United States (1,3,7–10,13,15,18). Symptoms of decline include subnormal apical and radial growth and reduced leaf size, chlorosis, and viruslike foliar symptoms. The foliage appears sparse because the leaves are undersized and clumped at the ends of slow-growing shoots. The bark of declining trees on sites exposed to sun and wind becomes pinkish to orange, and annual cankers are common on the trunk and branches. Branches often exhibit a deliquescent habit. Eventually a progressive dieback of small twigs and branches begins. Epicormic sprouts may form along the main branches or trunk, and some of these sprouts on the trunk or at the root collar develop into witches'-brooms. Trees of all sizes and age classes are affected. Death usually occurs in less than 10 yr, and recovery is rare (11,15,16).

MLOs have been detected in declining white ash and in unthrifty green ash (*F. pennsylvanica* Marsh.) in New York State and in the Midwest (15,19). Mortality was significantly associated with MLO infection (15). The majority of declining ash on seven sites in New York were infected with MLOs, but other declining trees on the same site gave no diagnostic evidence of such infection (15). Additional causes of decline were suspected (15).

Viruses are widespread in the ash population affected by decline. Tobacco ringspot virus (TRSV), tobacco mosaic virus (TMV), and tomato ringspot virus (TmRSV) occur in ash in New York State (1–3). The first two viruses were associated with foliar viruslike symptoms on ash in the field, but virus infection was not correlated with dieback (1). TmRSV, first identified in stump sprouts of a white ash that had declined (9), also was associated with foliar symptoms (3,10). Trees have been reported to be simultaneously infected with viruses and MLO (9).

The objectives of this study were to identify specific symptoms or growth effects associated with coincident infection by MLOs, TMV, TRSV, and TmRSV and to determine if these viruses interact synergistically or additively with MLOs or each other in the induction of symptoms. A preliminary report has been published (4).

MATERIALS AND METHODS

Inoculation and experiment schedule. One-year-old ash seedlings (155 white ash and 155 green ash) were inoculated mechanically with purified TMV, TRSV, and/or TmRSV or were grafted with MLO-infected bark patches alone or in various combinations with the viruses. TMV was purified from tobacco (*Nicotiana tabacum* L. 'Turkish') (5); TRSV was purified from cucumber (*Cucumis sativus* L. 'National Pickling') (7); and TmRSV was purified from cowpea (*Vigna unguiculata* (L) Walp. 'Blackeye') (21). Two leaflets per ash seedling were inoculated with virus (1 mg/ml in 0.02 M phosphate buffer, pH 7.2). If a seedling received two or three viruses, then four or six leaflets were inoculated, respectively.

Seedlings were inoculated with MLOs by grafting each stem with two bark patches (about 70 sq mm each) (14) taken from branches of two white ash trees with symptoms of ash yellows (15). Each seedling was grafted twice, in June 1985 and again in April 1986.

Controls consisted of 11 seedlings of each species that were mock inoculated with phosphate buffer and grafted with pieces of their own bark. All virus inoculations and initial MLO inoculations were performed during the summer of 1985.

All plants were subjected to three growth periods of 3–5 mo duration in screened greenhouses, separated by rest periods of 3.5 mo in a cold, dark room at 1–2 C. The first growth period lasted until October 1, 1985, period two was from January to May 1986, and period three was from September 1986 to January 1987. Stem diameter of each seedling at 1.5 cm above the soil line was measured with a caliper at the beginning and end of period one and at the ends of periods two and three. Foliar symptoms and morphological abnormalities in twigs were recorded during periods two and three.

Virus and MLO detection. The pathogen status of each seedling was tested during periods two and three. Young leaves of each seedling were collected just after leaf flush and tested for viruses by direct enzyme-linked immunosorbent assay (ELISA) using horseradish peroxidase conjugated to virus-specific immunoglobulin G as the enzyme probe, as previously described (3). The sensitivity of ELISA for detection of TMV, TRSV, and TmRSV was 3, 5, and 5 ng/ml, respectively. The virus concentration in

infected seedlings was determined as described previously (18). Sample absorbance had to be above the limit of detection and at least twice the absorbance of virus-free ash leaf extract to be considered a positive test for virus.

MLOs were detected by the DAPI (4′,6-diamidino-2-phenylindole-2HCl) fluorescence test (12,17) as applied to petioles and roots. The DAPI test was performed on ash that served as sources of MLO inoculum, on seedlings that displayed stunting and/or yellowing during periods two and three, and on all MLO-inoculated seedlings at the end of the experiment.

RESULTS AND DISCUSSION

Detection of viruses and MLOs. One or more pathogens were detected in 190 of the 310 inoculated plants but not in any of the 22 control plants. In total, 41 white ash and 17 green ash seedlings became infected with TMV, 59 white ash and 71 green ash became infected with TRSV, and 16 white ash and 23 green ash became infected with TmRSV. TRSV and TmRSV were detected in 38 and 10 grafted seedlings, respectively, that had not been inoculated mechanically. This transmission occurred even though the trees used as bark donors had been tested, with negative results, by ELISA for all three viruses.

MLOs were detected in only 10 white ash and 18 green ash seedlings out of 88 grafted of each species. The low infection rates were presumed because of excessive trauma to the phloem of the small patches; in a separate study, lack of MLO transmission was associated with parenchyma formation at the union patch with stem (Sinclair, *unpublished*). In previous studies, larger, carefully handled bark patches had resulted in 50–75% rates of transmission (15).

Fifty-nine seedlings (28 green ash and 31 white ash) contained more than one pathogen. Only seven seedlings contained three pathogens, and none contained all four pathogens. The ability to establish experimental mixed infections verified field observations that a single tree can be infected with more than one pathogen (1,9).

Analyses of virus concentration in leaf tissues of infected seedlings were as follows. In white ash, TMV concentration ranged from 5 to 65 ng/ml (mean = 27 ng/ml), TRSV concentration ranged from 5 to 37 ng/ml (mean = 15 ng/ml), and TmRSV concentration ranged from 5 to 15 ng/ml (mean = 8 ng/ml). In green ash, virus concentrations were 5 to 42 ng/ml (mean = 22 ng/ml) for TMV, 5 to 14 ng/ml (mean = 7 ng/ml) for TRSV, and 5 to 11 ng/ml (mean = 9 ng/ml) for TmRSV. The concentration of TRSV in white ash was double the concentration of green ash, whereas the concentrations of TMV and TmRSV were similar in each species. TMV concentration was more than double the concentration of either ringspot virus in both species. There was no detectable effect of multiple infections (virus or MLO) on virus concentrations. Virus concentration in white ash leaf tissue was similar to concentrations found in leaves of white ash trees in the field (J. D. Castello, *unpublished*).

Symptomatology. Foliar symptoms were observed on many of the seedlings at the midpoint to end of period two and late in period three (Table 1). Symptoms were more pronounced on green ash than on white ash, more pronounced and significantly ($P < 0.05$) more frequent on inoculated than on control seedlings, more pronounced and nearly three times more frequent on graft-inoculated than on nongrafted seedlings, more pronounced during period two than period three, and more frequent on plants in which viruses and MLO were detected than on other plants. In green ash, four foliar symptoms—chlorotic mottle, chlorotic spots, ringspots, and yellowing along the major veins (Fig. 1A)—were significantly more frequent ($P < 0.05$) on plants in which pathogens were detected than in pathogen-negative seedlings. In white ash, chlorotic mottle, deformed leaflets, and glossy appearance of upper leaf surfaces were significantly more common on plants that tested positive for virus. Deformed leaflets often showed vein yellowing and chlorotic line patterns (Fig. 1B and C). Because viruslike symptoms occurred in plants that tested negative for viruses, we surmised that the ELISAs failed to detect all virus-infected plants and/or that additional viruses may have been introduced by grafting.

The relationships between pathogen detection and the occurrence of foliar symptoms were evaluated by tests for homogeneity of data in contingency tables (20), except that a *t*-test was employed for comparisons involving small portions. The contributions of individual pathogens to the incidence of specific symptoms were assessed by analyses in which chi-square was calculated from differences between observed and expected frequencies of detection of each pathogen in plants with a given symptom. The expected frequencies were those at which the pathogens were detected in plants lacking the symptom. This approach was necessary because some plants with symptoms tested negative for all viruses. Based on significant ($P < 0.05$) differences in comparative frequency of detection of plants with and without each symptom, TRSV was most closely associated with chlorotic mottling in both tree species and with chlorotic spots, ringspots, and veinal yellowing in green ash. Hibben and Hagar (8) demonstrated that TRSV was associated with chlorotic spots, blotches, mosaic, line patterns, and ringspot symptoms in mechanically inoculated white ash seedlings. Green ash was not tested in their study. Wilkinson (22) mechanically transmitted TRSV to green ash seedlings but did not record symptoms. TRSV was detected in 54% of the green ash and 40% of the white ash that showed chlorotic mottle, versus 32 and 21%, respectively, of plants that lacked this symptom. The corresponding frequencies for chlorotic spots, ringspots, and veinal yellowing in green ash compared with plants of green ash that lacked each symptom were 51, 62, and 57% versus 36, 34, and 35%, respectively. TMV, although detected 41 times in white ash and 17 times in green ash, did not occur with greater relative frequency in plants showing particular symptoms than in plants lacking those symptoms. Therefore, TMV probably did not cause any of the symptoms. These results do not support the suggested role of TMV as a cause of viruslike symptoms in white ash. TMV previously was associated with chlorotic spots, rings, line patterns, and mosaic of naturally infected white ash in the field, based on grafting from symptomatic trees to white and green ash seedlings (13). In our opinion, TRSV is the probable cause of these symptoms in ash. TmRSV was detected in green ash with several symptoms and in

TABLE 1. Occurrence of foliar symptoms in relation to detection of viruses and mycoplasmalike organisms (MLOs) in green ash and white ash

	Proportion of seedlings showing each symptom			
	Green ash, test result[a,b]		White ash, test result[a,b]	
Symptom	Positive	Negative	Positive	Negative
Anthocyanescence	0.00	0.01	0.13	0.11
Chlorosis, general	0.03	0.08	0.09	0.07
Chlorosis between large veins	0.41	0.36	0.48	0.44
Chlorosis between veinlets	0.08	0.10	0.30	0.20
Chlorotic mottle	0.45*	0.26	0.70*	0.52
Chlorotic spots	0.33*	0.16	0.18	0.13
Deformed or dwarfed leaflets	0.21	0.19	0.46*	0.25
Drooping leaves	0.04	0.06	0.02	0.08
Fluted leaflet margins	0.18	0.21	0.30	0.23
Glossy upper surface	0.18	0.08	0.20*	0.02
Large green spots	0.11	0.05	0.02	0.01
Mosaic	0.09	0.05	0.04	0.09
Ringspots	0.30*	0.10	0.03	0.02
Rugosity	0.22	0.16	0.35	0.23
Veinal yellowing	0.26*	0.10	0.18	0.13

[a] Positive by enzyme-linked immunosorbent assay for tobacco mosaic virus, tobacco ringspot virus, and/or tomato ringspot virus, or positive by DAPI (4; 6-diamidine-2-phenylindole-2HCl) test for MLOs, or negative for all three viruses and for MLOs. Proportions are based on 99 positive and 77 negative green ash, 91 positive and 87 negative white ash. All except 11 pathogen-negative seedlings of each species were previously inoculated with viruses and/or bark patches.

[b] * indicates that the value differs significantly from that in the corresponding negative column, $P < 0.05$.

white ash with glossy leaves, but the numbers of observations were so small that no conclusion about a causal role of this virus is justified.

MLOs were associated with a combination of distinctive symptoms that did not occur in plants that tested negative for these pathogens. All except one of the 28 plants in which MLOs were detected displayed interveinal chlorosis that was most severe on the youngest leaves. These leaves in 18 MLO-infected seedlings appeared abnormally glossy and were dwarfed on 15 of these seedlings (Fig. 1D, E, and F). Ten of 28 MLO-infected seedlings each produced two or more axillary shoots along the main axis of the current year's shoot during periods one or two (Fig. 1E and F), whereas only eight uninfected seedlings out of 326 did this. These axillary shoots proliferated on a few of the MLO-infected plants. Symptoms of MLO infection were more severe on white ash than on green ash and became more frequent and severe from periods two to three. Symptoms were consistent with those we have observed on white ash naturally infected with MLOs (15).

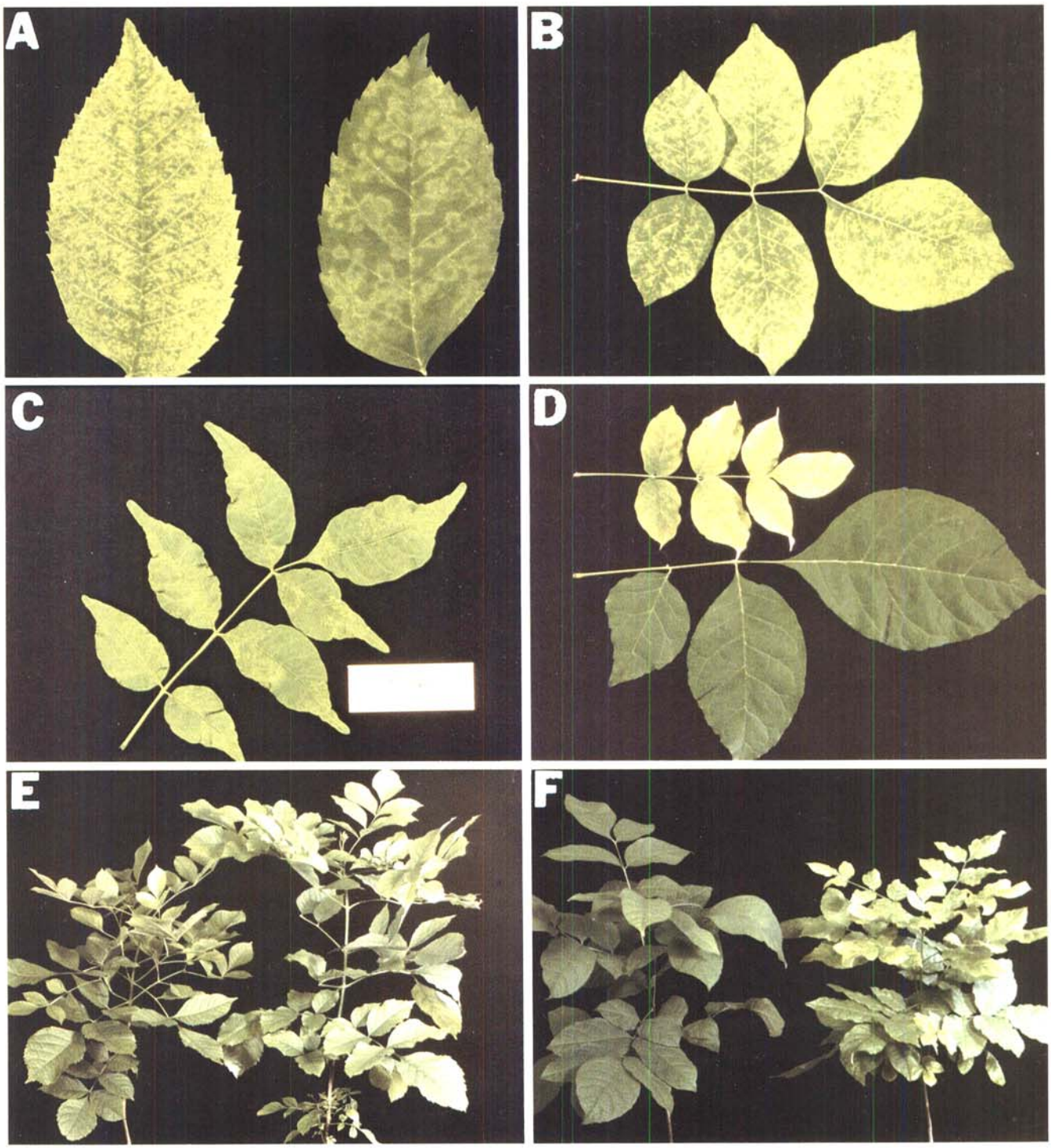

Fig. 1. Symptoms on green and white ash in which viruses and/or mycoplasmalike organisms (MLOs) were detected. **A,** Chlorotic spots (left) and ringspots on green ash leaflets. **B,** Deformed white ash leaf showing vein yellowing and chlorotic spots. **C,** Deformed white ash leaf with chlorotic line patterns. **D,** Top: dwarfed and chlorotic white ash leaf associated with MLO infection; bottom: portion of leaf from control plant. **E,** Right: MLO-infected green ash showing foliar interveinal chlorosis and production of axillary shoots; left: control plant. **F,** Right: dwarfing, interveinal chlorosis, glossy upper leaf surface, and production of axillary shoots of an MLO-infected white ash; left: control plant.

Many symptoms that developed on white ash in the greenhouse have been observed on both declining and vigorous white ash in New York and elsewhere (2,15,19). To our knowledge, the symptoms induced on green ash in the greenhouse have not been observed in the field in New York. The development of viruslike symptoms in naturally infected ash often is variable from year to year (6). Symptoms often become masked and tend to fade as the season progresses. Therefore, viruslike symptoms in naturally infected green ash may have been overlooked.

Pathogen effects on growth. For these analyses, plants were grouped according to their pathogen content based on ELISAs and DAPI tests. Pathogen-free plants were represented only by the uninoculated control groups. Inoculated plants in which no pathogens were detected were not considered. Diameter data were converted to cross-sectional (basal) area and examined by analyses of variance. These analyses were used only for seedling groups consisting of at least three plants with similar pathogen content. Differences among means were evaluated by a *t*-test taking unequal sample sizes into account (15).

The initial analyses were performed with eight groups of green ash and nine groups of white ash; each group within a tree species represented infection by a different pathogen or combination of pathogens. Basal area at the end of each period and growth rates within individual periods were examined.

In green ash, the growth rates of all groups were similar during periods one and two but diverged during period three. Cumulative basal area at the end of period three did not vary significantly among groups at the 95% confidence level, however. Therefore, the two groups containing MLOs were merged for further analysis, as were the six groups containing one or more viruses but no MLOs (Fig. 2). Analysis of variance showed significant variation among the resulting three groups ($F = 3.86$, $P = 0.024$). The difference between MLO-infected and uninoculated control plants (mean basal areas of 137 and 179 sq mm, respectively) was significant at the 95% confidence level. The mean basal area of virus-infected plants (156 sq mm) did not differ significantly from the control group or from the MLO group. No additional relationships between pathogens and growth were detected when data were analyzed after transformation to log basal area, regardless of whether the data were normalized by setting the initial basal area for each seedling equal to 1.

In white ash, significant (1% confidence level) variation in basal area among the nine groups developed during period one and increased thereafter (Fig. 3). The main sources of variation during period one were the retarded growth of the control group and the large average size of a group of three plants infected with TRSV + TmRSV + TMV. We do not know why the controls were retarded. The three large virus-infected plants had been among the largest

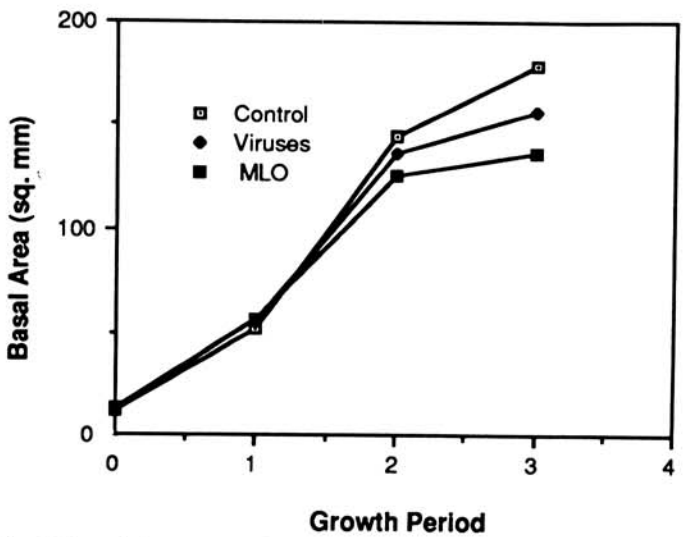

Fig. 2. Cumulative cross-sectional area (in square millimeters) of stems of control, virus-infected, and MLO-infected green ash.

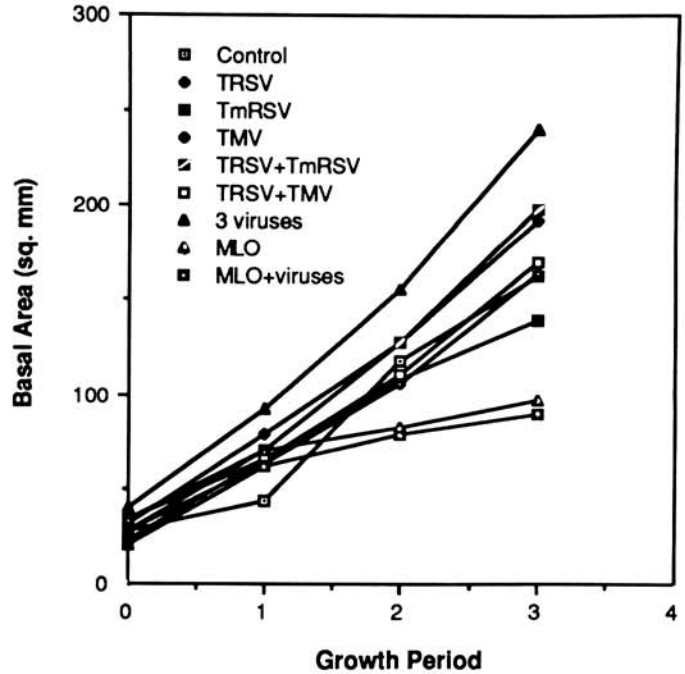

Fig. 3. Cumulative cross-sectional area (in square millimeters) of stems of control, six groups of virus-infected, and two groups of MLO-infected white ash.

when the experiment began, and their basal area merely increased as a function of their initial size. Their exponential growth rate was similar to that of plants in the control group and other virus-infected groups. During period two, the growth of MLO-infected white ash slowed in comparison with plants in other groups, and this trend continued through period three (Fig. 3). The mean basal areas of the two MLO-infected groups (90 and 97 sq mm) differed from that of the control group (163 sq mm) at the 99 and 95% confidence levels, respectively. The MLO-infected groups also grew significantly less than all virus-infected groups except one containing TmRSV. Except for the group of three large seedlings discussed above, the mean basal areas of virus-infected groups did not differ significantly from that of the control group. The slowest growing and the two fastest growing virus-infected groups differed significantly (95% confidence level) in basal area at the end of period three. Because all three groups contained TmRSV, however, we doubt that the differences were due to virus content. No other differences among virus groups were significant.

Neither additive nor synergistic effects of viruses and MLOs on ash growth or symptom development were detected. Unfortunately, however, this experiment did not adequately test for such interaction because viruses may have been present (transmitted from bark patches) in the MLO-infected, ELISA-negative seedlings that we studied.

No seedling in this study died, although several MLO-infected white ash seedlings were close to death. More time may be needed than was allotted in this study for infected ash to show the full effects of viruses and MLOs.

This study did verify that MLOs can be associated with severe debilitation of white ash, as reported previously (15). It was clear that MLO infection suppressed the growth of white ash more severely than that of green ash. This difference is consistent with informal field observations in New York State. In addition, TRSV is a cause of foliar symptoms in both white and green ash. However, any effect of virus infection on the growth of white and green ash was smaller than could be detected in our experimental design.

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