Seasonal Variations in Susceptibility and in Internal Inoculum Densities in Maple Species Inoculated with *Verticillium dahliae*

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ABSTRACT

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Differences in susceptibility to *Verticillium dahliae* between potted Norway (*Acer platanoides*) and silver (*A. saccharinum*) maple seedlings were determined by the percentages of infected trees, foliar symptoms, infected stem sections, and height of fungus movement in the stems. Data were pooled for four inoculation times in each growing season in 1983, 1984, and 1987. Norway and silver maples did not differ in percentage of infected trees, but *Verticillium* was recovered from a higher percentage of stem sections and the fungus grew to a higher percentage of the stem length in Norway maples. Similar comparisons were made in sugar (*A. saccharum*), Norway, and silver maples in 1987. Sugar maple was the most susceptible species by all criteria. A seasonal decline in susceptibility was observed for sugar and Norway maples but not for silver maples. *Verticillium* produced foliar symptoms in sugar but not in silver or Norway maple seedlings. Inoculum densities in the stems of silver and sugar maples were compared and expressed as total numbers of colony-forming units. Numbers of both short-lived conidia and mycelial fragments and microsclerotia were determined, and the number of colony-forming units isolated was higher from sugar than from silver maples.

Increased use of maple species and cultivars in landscape plantings has been associated with increased incidence of Verticillium wilt caused by *Verticillium dahliae* Kleb. Reports of the relative susceptibility of maple species to *V. dahliae* are conflicting. Dochinger (4) found in field studies that Verticillium wilt was most prevalent in silver maples (*Acer saccharinum* L.), followed by Norway (*A. platanoides* L.), red (*A. rubrum* L.), and sugar (*A. saccharum* Marsh.). Hoitink et al (8) reported that Norway and sugar maple cultivars and seedlings were very susceptible and that infected silver and red maples were symptomless. Smith and Neeley (20) also observed that infected silver maples remained symptomless.

Internal inoculum densities of *Verticillium* spp. have been studied in herbaceous hosts but not in woody hosts. Schnathorst and Mathre (17) reported that inoculum densities in susceptible cotton cultivars were 20 times those in resistant ones. Pegg and Street (13), however, found higher counts in resistant than in susceptible hops. Davis et al (3) reported that internal inoculum concentrations in potato were correlated with symptoms. Brandt and Reese (2) noted lower inoculum levels in resistant than in susceptible mint but no difference in fungus movement. Newcombe et al (11) reported a quantitative measure of invasiveness of *Verticillium* in alfalfa related to resistance.

Whereas information is lacking for maples, seasonal variations in the susceptibility of elm species to Dutch elm disease have been reported (18,19,22). Smalley and Kais (19) suggested that knowledge of seasonal cycles in disease susceptibility could facilitate evaluation of resistance.

This study reports the relationship between seasonal stages of tree development and internal inoculum densities and disease development in three maple species inoculated with *V. dahliae*.

MATERIALS AND METHODS

Seasonal variations in susceptibility. Studies were conducted in 1983, 1984, and 1987 to determine seasonal variations in susceptibility of maple seedlings to Verticillium wilt. Silver and Norway maples were compared in 1983, 1984, and 1987 and silver, Norway, and sugar maples in 1987.

Dormant maple seedlings 45–60 cm tall were planted in 2-L pots in a 2:2:1 (v/v) peat, perlite, soil mix and placed in a lathhouse. In the 1983 and 1984 studies, inoculum consisted of a mixture of *Verticillium* isolates from *Viburnum* spp., potato, and Norway, sugar, and Japanese (*A. palmatum* Thunb.) maples; in the 1987 study, isolates from Norway, sugar, and silver maples were used. *V. dahliae* was grown from mycelium in 50 ml of Czapek Dox broth in 125-ml Erlenmeyer flasks, incubated on rotary shakers for 4 days at 22 ± 1 C. Equal volumes of each spore suspension were mixed and diluted to a final inoculum concentration of 1.25–2.00 × 10^6 spores per milliliter. Then, 2 ml of inoculum was placed in a serum cap affixed to the lower stem (5), and a scalpel wound was made on either side of the stem, below the inoculum level, at the base of the cap. Water controls were provided for each species.

Inoculations were made between the half- and full-leaf stage and 28, 56, and 84 days later. The number of replicates per inoculation time was 10, 12, and 20 in 1983, 1984, and 1987, respectively. Plants were arranged in a completely randomized block design in a lathhouse. Foliar symptoms were rated on a scale of 0–100% in 5% increments, with 0 = no foliar involvement and 100 = total foliar involvement.

Trees were harvested 8 wk after inoculation by severing the stem at the inoculation point. Stems were cut into five equal parts, the bark was removed, and a 2.5-cm section was cut from the top of each part. Sections were arranged in serial order and frozen at −20 C. The frozen serial sections were dipped in boiling water (10), split longitudinally, and plated onto Czapek Dox agar amended with 100 μg/ml of novobicin and streptomycin. Plates were incubated at 18 C and observed periodically for the presence of *Verticillium*. Susceptibility was rated on the percentage of infected plants, stem sections, and foliar symptoms and on the height of fungus movement up the stem.

Disease and internal inoculum development. Two studies were initiated to monitor disease development and internal inoculum densities in sugar and silver maple seedlings. In the first study, data on the percentage of infected trees, foliar symptoms, infected stem sections, height of fungus movement in stems, and inoculum density were compared at the end of the growing season the year of and the year after inoculation. Seedlings 40–70 cm tall were planted in 2-L pots and inoculated as previously described. Inoculum was a mixed conidial suspen-

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sion of five isolates from sugar and silver maples adjusted to a concentration of 1 $\times 10^6$ conidia per milliliter. Eighty trees of each species were inoculated on 2 June 1986 at full-leaf stage. Twenty water controls were provided for each species. Plants were arranged in a completely randomized design in a lathhouse.

One-half of the treated and control trees of each species were harvested after leaf drop in 1986 and the other half after leaf drop in 1987. Stems were debarked and cut into 2.5-cm sections, which were arranged in serial order and stored at −20 C. Distribution of *Verticillium* within the stem was determined by isolations from every sixth stem section as described above.

Internal inoculum density in each stem was determined by grinding the remaining frozen stem sections in a Wiley mill (model 3). Sawdust was collected through a 2-mm screen. Then, 1 g of sawdust from each stem was dispensed into 10 petri dishes (15 × 100 mm). Modified sodium polypectate agar (SPA [13]), at 45 C, was poured into each dish. Modified SPA was made with 20 g/L of agar and adjustment of the pH to 5.0. Tergitol, 1 ml, was sterilized in 100 ml of water. This solution and 200 μg of streptomycin sulfate with 50 mg of choramphenicol were added to the other ingredients and sterilized in 900 ml of water. Petri dishes were incubated for 2 wk at 18 C. Germinating colony-forming units were counted with a ×7 stereomicroscope.

In the second study, percentages of infected trees and inoculum densities were determined five times in 1987 between inoculation at full-leaf stage and leaf drop. Dormant sugar and silver maples, 60–90 cm tall, were planted, and 125 trees of each species were inoculated as previously described. Foliar symptoms were rated 9 and 15 wk after inoculation. At 7, 26, 70, 118, and 173 days after inoculation, 25 trees of each species were excised at the inoculation point. Stems were debarked, cut into 5 cm sections, placed in plastic bags, and stored at −20 C. The frozen tissue was ground in a Wiley mill as previously described. Two 1-g samples from each tree were collected and plated in modified SPA as previously described. The first sample, used to determine the total number of colony-forming units per gram of sawdust, was plated immediately after grinding. The second, used to estimate the number of microsclerotia, was dried in a petri dish at 25 C for 14 days before plating. Preliminary experiments indicated that conidia and mycelial fragments lost viability after drying.

RESULTS

Seasonal variations in susceptibility. Seasonal fluctuations in susceptibility in Norway, silver, and sugar maples are shown in Figure 1. Differences within and between species were noted during the growing season, depending on the criterion for susceptibility. For 1983, 1984, and 1987, there was a significant reduction in the percentage of infected Norway maples, but not of silver maples, between the first and last inoculation times. The percentage of infected Norway maples was significantly less than that of silver maples only after the last inoculation time. No foliar symptoms were noted in either species during any test year. The percentage of infected stem sections in both Norway and silver maples declined significantly between the first and last inoculations, whereas the height of *Verticillium* growth in the stems declined significantly in Norway maples only. For these two criteria, silver maples were less susceptible than Norway maples only after the initial inoculation.

In the 1987 test, the percentages of infected sugar and Norway maples, but not of silver maples, declined between the first and last inoculations. For the

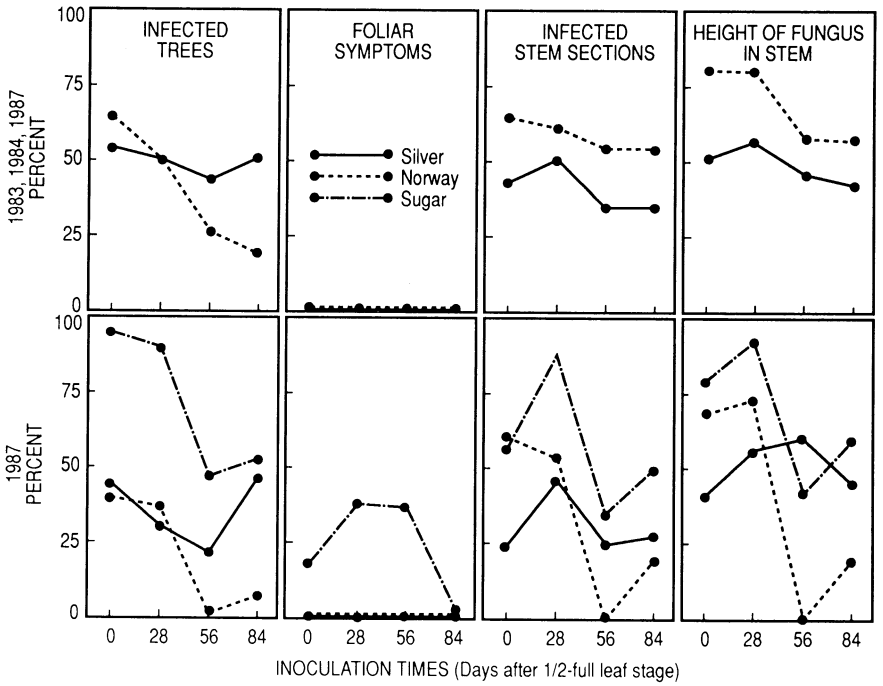

Fig. 1. Patterns of seasonal susceptibility to *Verticillium dahliae* in Norway, silver, and sugar maple seedlings. Means of (top row) 1983, 1984, and 1987 inoculations with silver and Norway maples and (bottom row) 1987 inoculations with silver, Norway, and sugar maples. Data points represent values for criteria of susceptibility. Infected trees = trees from which *Verticillium* was reisolated; foliar symptoms = from no symptoms (0%) to total foliar involvement (100%); infected stem sections = isolations from sections sampled equidistant along the stem; height of fungus in stem = maximum height as a percentage of stem length.

Table 1. Mean susceptibility of Norway, silver, and sugar maples inoculated periodically through the growing season with *Verticillium dahliae*[x]

Inoculation years / Species	Criteria of susceptibility[y] Infected trees (%)	Foliar symptoms (%)	Infected stem sections (%)	Height of fungus movement in stem (%)
1983, 1984, and 1987				
Norway	41 a[z]	0 a	60 a	71 a
Silver	51 a	0 a	42 b	51 b
1987				
Norway	20 a	0 a	34 a	41 a
Silver	35 a	0 a	31 a	51 a
Sugar	70 b	22 b	59 b	69 b

[x]Trees were inoculated at half- to full-leaf stage and 28, 56, or 84 days later and harvested 8 wk after inoculation.

[y]Infected trees = trees from which *Verticillium* was reisolated; foliar symptoms = from no symptoms (0%) to total foliar involvement (100%); infected stem sections = isolations from sections sampled equidistant along the stem; height of fungus movement in stem = maximum height as a percentage of stem length.

[z]Means of all inoculation dates in 1983, 1984, and 1987 for Norway and silver maples and in 1987 for Norway, silver, and sugar maples. Numbers in a column followed by the same letter are not significantly different ($P = 0.05$) according to least squares means.

first two inoculations, the percentages of infected Norway and silver maples were similar and significantly less than the percentage of infected sugar maples. After the last inoculation, the percentages of infected sugar and silver maples were greater than that of Norway maple but did not differ from one another. Sugar maple was the only species to show foliar symptoms, and the percentage declined from a high of 37% after the day-28 inoculation to 0% after the day-84 inoculation. Both sugar and Norway maples showed greater seasonal declines than silver maples in the percentage of infected stem sections. At the beginning of the season, *Verticillium* was isolated from similar percentages of sugar and Norway maple stem sections but from significantly fewer silver maple stem sections. After the day-84 inoculation, *Verticillium* was recovered from significantly more sugar maple stem sections than from either silver or Norway maple stem sections; the latter two species did not differ from one another. The height of fungus growth declined in stems of Norway and sugar maples but not in stems of silver maples. After day-56 and day-84 inoculations, heights of fungus growth in sugar and silver maples were similar. Fungus growth in Norway maple stems was significantly lower than that in the other two species.

Mean data for inoculation dates in 1983, 1984, and 1987 for Norway and silver maples and for sugar, Norway, and silver maples inoculated in 1987 are compared in Table 1. There were no significant differences between Norway and silver maples in numbers of infected trees nor were foliar symptoms noted in either species in any year. However, *Verticillium* was recovered from significantly more stem sections and grew to a higher percentage of the stem length in Norway maples than in silver maples. In 1987, sugar maple was the most susceptible species by all criteria, and no significant differences were noted between silver and Norway maples.

Disease and internal inoculum development. The susceptibilities of sugar and silver maples inoculated in 1986 and harvested at the end of the 1986 and 1987 growing seasons are compared in Table 2. There was no significant difference in the percentages of infected sugar and silver maples in either 1986 or 1987. However, the percentage of infected trees for both species was significantly lower in 1987 than in 1986. The mean percentages of foliar symptoms in sugar maples in 1986 and 1987 were 20 and 18%, respectively; silver maples were symptomless in both years. *Verticillium* was recovered from a significantly higher percentage of sugar maple stem sections than from silver maple stem sections in both years. Height of fungus growth was greater in sugar than in silver maples at the end of both growing seasons but was less in both species in 1987 than in 1986. Inoculum density was much greater in sugar maple than in silver maple at the end of both years and declined in both species between the end of 1986 and 1987.

Changes in percentage of infected trees and inoculum density in silver and sugar maples during a growing season are shown in Table 3. In sugar maples, both total colony-forming units and microsclerotia per gram of sawdust increased up to 70 days after inoculation to 223.9 and 98.2, respectively, and then declined. In silver maples, both colony-forming units and microsclerotia declined throughout the growing season. The percentages of infected sugar maples remained between 80 and 92% from the first through the last harvest, whereas the percentages of infected silver maples declined from 84 to 16%. No foliar symptoms were noted in any silver maples, whereas sugar maples averaged 17 and 15% foliar symptoms 9 and 15 wk, respectively, after inoculation.

Table 2. Disease development and inoculum concentrations in sugar and silver maples inoculated with *Verticillium dahliae*[x]

Year harvested Species	Criteria of susceptibility[y]				
	Infected trees (%)	Foliar symptoms (%)	Infected stem sections (%)	Height of fungus movement in stem (%)	Colony-forming units per gram of sawdust
1986					
Sugar	95 a[z]	21 a	73 a	74 a	622 a
Silver	95 a	0 b	36 b	40 b	28 b
1987					
Sugar	70 b	18 a	42 b	47 b	85 c
Silver	50 b	0 b	16 c	22 c	15 d

[x]One-half of the seedlings inoculated in June 1986 were harvested at leaf fall in 1986 and the remainder in 1987.
[y]Infected trees = trees from which *Verticillium* was reisolated; foliar symptoms = mean of three readings in 1986 or 1987, from no symptoms (0%) to total foliar involvement (100%); infected stem sections = isolations from sections sampled equidistant along the stem; height of fungus movement in stem = maximum height as a percentage of stem length.
[z]Numbers in a column followed by the same letter are not significantly different ($P = 0.05$) by chi-square or least squares means.

Table 3. Percentage of infected trees and *Verticillium dahliae* inoculum concentrations in sugar and silver maples harvested during the growing season

Number of days after inoculation when harvested[x]	Infected trees (%)		Average no. cfu/g of sawdust[y]			
			Before drying		After drying	
	Sugar maple	Silver maple	Sugar maple	Silver maple	Sugar maple	Silver maple
7	80 a[z]	84 a	30.8 a	8.4 a	2.6 a	0.90 a
26	80 a	48 a	76.2 b	3.1 a	49.0 b	0.90 a
70	84 a	80 a	223.9 c	3.6 a	98.2 c	0.20 a
118	92 a	68 a	90.8 b	2.8 a	30.3 b	0.30 a
173	80 a	16 b	87.1 b	0.4 a	57.8 b	0.04 a

[x]Twenty-five trees of each species were harvested at each date.
[y]One of two samples of tree stems ground into sawdust was plated immediately to determine total colony-forming units and the other was dried before plating to determine numbers of microsclerotia.
[z]Numbers in a column followed by the same letter are not significantly different ($P = 0.05$) by chi-square or least squares means.

DISCUSSION

We found that the susceptibility of species varied with the disease criteria and the year and time of inoculation. Thus, the means of all inoculation dates in 1983, 1984, and 1987 for Norway and silver maples did not differ in percentages of infected trees and of foliar symptoms. However, height of fungus growth was greater in Norway maples than in silver maples. In 1987, mean data for all criteria suggested that Norway and silver maples were equally susceptible. However, mean values masked significant seasonal differences between and within species. Seasonal declines in susceptibility of Norway maples resulted in fewer infected Norway maples than silver maples after late-year inoculations. Height of fungal growth was greater in Norway maples than in silver maples in early-season but not in late-season inoculations.

Seasonal differences in susceptibility may be influenced by anatomical and physiological changes in the trees and provide useful information relating to mechanisms for resistance. Regulski and Peterson (15) reported constant growth of *V. dahliae* on silver maple sap at bud swell through full-leaf stage; growth on sugar and Norway maple sap declined.

They concluded that sap nutrients may be a factor in disease susceptibility. We found declines in pathogenicity in Norway and sugar maples, but not in silver maples, that may corroborate their in vitro results.

Smith and Neely (20) concluded that declines in growth of *Verticillium* in several tree species late in the season were correlated with increases in temperatures. This may explain declines in susceptibility in Norway and sugar maples, but late-season declines were not noted in silver maples.

Foliar development and vascular development are correlated in several tree genera. Foliage develops as a flush in sugar and Norway maples and slows later in the season, whereas development is slower and more continuous in silver maples (P. Larsen, *personal communication*). Declines in susceptibility of sugar and Norway maples may be correlated with differences in rates of vascular development, as occurs with susceptibility of elms to Dutch elm disease (1,9).

Lack of foliar symptoms in silver and Norway maples suggests low inoculum concentrations and/or retarded vertical colonization. Silver, but not Norway, maple has been reported as a symptomless host (20). Newcombe and Robb (12) found resistance in alfalfa to be associated with restricted vertical fungus movement. Hall and Busch (6) reported that leaf colonization was necessary for foliar symptom expression in some hosts.

Height of fungus movement and foliar symptoms were not correlated (Fig. 1). Inoculum concentrations were more closely correlated with foliar symptoms. Inoculum concentrations in symptomatic sugar maples at the end of the first and second years after inoculation were 22 and six times, respectively, greater than those in symptomless silver maples (Table 2). We do not have similar information for Norway maples. By other criteria, however, Norway maples ranked more susceptible than silver maples but less susceptible than sugar maples. Possibly, a threshold inoculum concentration, necessary for foliar symptoms expression, was not attained in Norway maples. Those reporting foliar symptoms in Norway maples used larger plants (8,20,21) that may support faster inoculum buildup.

Inoculum concentrations were higher in sugar maples than in silver maples after only 7 days. Inoculum increased in sugar maples but declined in silver maples, becoming nearly undetectable by 173 days. Because initial inoculum was from both species, isolate specificity cannot explain differences in subsequent inoculum densities. Lack of fungus proliferation and loss of viability in silver maples may have resulted from an inadequate nutrient base or from antifungal substances produced by the plant or by xylem-colonizing microflora (7).

Microsclerotia formation varies with the host. Rankin (14) reported microsclerotia in tracheae and tracheids of diseased maples. Schnathorst (16) reported that microsclerotia generally form saprophytically after moistening of dead host tissue. We found that patterns of microsclerotia development after inoculation of sugar and silver maples were similar to those of total colony-forming units but that concentrations were lower.

Because disease development appears to be associated with inoculum density, understanding fluctuations in inoculum densities and periods of susceptibility may help elucidate the basis for tree resistance.

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