Stomatal Closure in Plants Infected with Mycoplasmalike Organisms

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We thank J. D. Castello, State University of New York College of Environmental Science and Forestry, for virus indexing, and T. A. Chen, Rutgers University, for providing stunt-affected corn plants.
Accepted for publication 7 September 1982.

ABSTRACT

Matteoni, J. A., and Sinclair, W. A. 1983. Stomatal closure in plants infected with mycoplasmalike organisms. Phytopathology 73:398-402.

Stomatal closure, as indicated by abnormally high diffusive resistance of leaf surfaces (R_l, sec·cm^{-1}) and by leaf surface replicas, was a consistent symptom of elm yellows (phloem necrosis) in *Ulmus americana* and *U. rubra*, ash yellows (ash witches'broom) in *Fraxinus americana*, infection by the elm yellows agent or the ash yellows agent in Madagascar periwinkle (*Catharanthus roseus*), X-disease in *Prunus virginiana*, and corn stunt in *Zea mays*. An abnormal increase in R_l preceded or coincided with the earliest foliar symptoms of elm yellows and ash yellows and is therefore useful for detection of these mycoplasmal infections. In each infection studied, the degree of stomatal closure increased as foliar symptoms became more intense. Xylem pressure potentials less negative than normal were consistently associated with elevated R_l in *U. americana*, but R_l was the more sensitive indicator of infection.

Additional key words: ash decline.

Relatively little is known about the physiology of the yellows diseases caused by mollicutes. Mycoplasmalike organisms (MLO) are associated with elm yellows (EY, formerly called phloem necrosis), ash yellows (AY, formerly ash witches'-broom), and X-disease of *Prunus* spp. (11,13,30). EY is lethal to American elm, *Ulmus americana* L., and four other North American elm species. Necrosis of feeder roots and of phloem in the lower trunk precede leaf epinasty and yellowing. Infected trees usually die within 1 yr after the onset of foliar symptoms, and dying trees may wilt (27). X-disease is slowly lethal to hosts such as chokecherry (*Prunus virginiana* L.) and peach (*P. persica* (L.) Batsch.) (10). In contrast, AY is nonlethal or slowly lethal (*unpublished*). Infected trees often produce brooms with small, chlorotic leaves that are sometimes simple instead of pinnately compound (13).

Foliar symptoms of EY, including yellowing, epinasty, and wilting, are similar to symptoms of water shortage in elms, particularly on poor sites (27). We found, however, that infected American elms had less negative xylem pressure potentials than healthy elms. This suggested that transpiration was blocked and led to the discovery of impaired stomatal function not only in yellows-affected American elms, but also in white ash with AY (18).

Coconut palms responded similarly to infection by MLO (19). Palms with lethal yellowing had continuously higher (less negative) xylem pressures than comparable healthy trees and did not show normal diurnal changes in xylem pressures. Stomatal closure in the green leaves of infected palms was recently reported (2).

The objectives of this research were to characterize stomatal closure associated with several MLO infections, relate closure to the abnormal xylem pressure potentials in elms, assess its usefulness for early detection of yellows-type diseases, and learn whether stomatal closure may be characteristic of yellows diseases as a group.

MATERIALS AND METHODS

Plant materials. In the field, xylem pressure potentials and leaf diffusive resistances were observed in yellows-affected American elm, red or slippery elm (*U. rubra* Muhl.), white ash, and chokecherry. Relationship of xylem pressure potential and stomatal function was determined in 37 American elms at four locations (Table 1) in central New York State. Each site had seven to 11 trees, at least three nonsymptomatic and three with yellows. In addition, seven isolated American elms and 13 red elms were observed for stomatal behavior. Diagnosis of EY in American elms was based on yellowing of leaves, phloem discoloration, and associated wintergreen odor in the lower trunk (27). Diagnosis of EY in red elms was based on yellowing, phloem discoloration, and brooms (27). Nonsymptomatic and AY-affected white ash were selected on several sites near Ithaca, NY. Affected trees had brooms with small, chlorotic leaves (13).

Ten chokecherry plants affected by X-disease were compared to 10 nonsymptomatic plants. Chokecherry produces multiple stems on common root systems, so care was taken to sample 20 different clones. Symptoms in chokecherries varied from mild chlorosis to leaf reddening, shot-hole formation, and premature release of winter buds.

Greenhouse studies of stomatal behavior in relation to symptom progression involved healthy and yellows-affected American elm, white ash, and Madagascar periwinkle (*Catharanthus roseus* (L.) G. Don 'Twinkles') and stunt-affected corn (*Zea mays* L. 'Golden Bantam'). American elms 4–6 yr old from locally collected seed were grown in a sterilized (7 hr, 121 C) peat-compost-sand mixture (1:1:1) in 3.8-L plastic cans 15.2 cm in diameter and were watered weekly with a dilute solution of balanced fertilizer. Greenhouse shading reduced the maximum light intensity to 250 μE·m^{-2}·sec^{-1}. A 16-hr photoperiod was maintained with cool-white fluorescent lights. Between growth periods, dormant trees received at least 15

TABLE 1. Xylem pressure potentials and leaf diffusive resistances of healthy and yellows-affected American elms[a]

Site	Xylem pressure potential (mPa)		Leaf diffusive resistance, R_l (sec·cm^{-1})	
	Healthy	Infected	Healthy	Infected
Roadside A	−1.42	−0.72	8.0	44.6
Roadside B	−1.48	−1.14	4.5	36.3
Vacant lot	−1.25	−0.95	4.8	21.7
Stream bank	−1.08	−0.51	1.6	21.7
Mean[b]	−1.30[c]	−0.84[c]	4.5[c]	34.1[c]

[a] Measurements for 37 trees, seven to 11 trees per site, three to seven each of healthy trees and naturally infected trees, mid-afternoons in late July.
[b] Means weighted for unequal sample sizes.
[c] Differences between corresponding means are significant at $P < 0.01$.

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wk of vernalization at 3 C with a dim-light photoperiod of 8 hr.

One-year-old white ash seedlings were collected from a population free of AY and virus symptoms, potted and grown in the same way as the elm seedlings. These seedlings were observed for virus or yellows symptoms for two seasons before use.

Periwinkles were grown from seed (W. A. Burpee, Clinton, IA 52732) in the same soil mixture in plastic pots 12.8 cm in diameter.

Three to six corn plants were grown per 9.5-L plastic pot. Nine healthy and nine infected plants were used. Plants 2–4 wk old were inoculated with the corn stunt spiroplasma by infected leafhoppers [*Graminella nigrifrons* (Forbes)].

Greenhouse inoculations. On 15 July 1980, 24 American elms were inoculated by grafting with bark patches of about 2 cm^2 from each of three different, naturally infected, symptomatic American elms. Eight control trees received patches from healthy elms. Grafts were wrapped with Parafilm® and grafting rubber. The latter was removed 4 wk later. Elms that became infected were identified by progression of typical EY symptoms (27).

Twenty-four white ash seedlings were each inoculated with one bark patch from each of two brooming white ashes on 26 July 1979. Eight control trees were either untreated or grafted with a bark patch from a healthy white ash. Trees were deemed infected if they showed symptoms of AY and if phloem sections gave a positive reaction with a Dienes' stain modified from Deely et al (9). Longitudinal and cross sections 100 μm thick were immersed for at least 10 min in stain, cleared for 10 min in 50% Karo light corn syrup (Best Foods, Div. of CPC International, Inc., Englewood Cliffs, NJ 07632) in distilled water, and mounted.

The EY agent was transmitted by splice grafting from infected stock plants of periwinkle (5) to seven other periwinkles. The AY agent, transmitted from brooming white ash to periwinkle with dodder (*Cuscuta subinclusa* Dur. and Hilg.), was further transmitted to four other periwinkles by splice grafting.

Xylem pressure potentials and diffusive resistances. Field measurements of xylem pressure potential (P, mPa) of elms were made on sunny days in late July with a pressure chamber (24) (PMS Instrument Co., Corvallis, OR 97330). Potentials of two to four shoots per tree were measured between 1100 and 1500 hr with precautions suggested by Ritchie and Hinckley (23). At the same time, a diffusive resistance porometer (Lambda LI-65) (14) was used to measure temperature and diffusive resistance to water loss (R_l, sec·cm^{-1}) of abaxial surfaces of six sunlit leaves per tree. Raw data in seconds were adjusted for temperature and converted to seconds per centimeter (21). The data were expressed as resistances because of the small amplitude of fluctuation of normal values in comparison to that of values for diseased plants. Normal values of R_l for healthy, nonwilted plants of the species studied ranged from 0.1 to 5.0 sec·cm^{-1}. For a given species, absolute values of R_l, as determined with the LI-65 instrument, were of interest only for establishing the relative magnitudes of values for healthy versus diseased plants.

Several precautions were followed in obtaining R_l data (21). For all greenhouse studies, elm and ash leaves that were fully developed before inoculation were used to reduce the possibility of R_l differences due to anatomical changes caused by infection. Measurements were made on sunlit leaves but not during frequently interrupted sunshine. Thus sudden changes in leaf surface temperature were avoided. Plants were well watered before R_l measurements, and the pressure chamber was used to confirm that high R_l values were unrelated to water shortage. Xylem pressure potentials ranged from −0.35 to −1.38 mPa for elm and −0.40 to −1.25 mPa for ash. Silicon rubber (31) or vinyl (22) leaf surface replicas were routinely made to verify stomatal closure in both field and greenhouse studies.

In mid-July and mid-September, field measurements of R_l of ash and chokecherry were recorded. Field measurements of P in ash and chokecherry were made only as necessary to confirm that stomatal closure as indicated by R_l was not associated with water deficit. Xylem pressure potentials were between −0.80 and −1.65 mPa for chokecherry and −0.50 and −1.67 mPa for white ash.

For greenhouse studies, R_l values were determined on sunlit leaves 1–3 times weekly between 1100 and 1430 hr beginning 2 wk before inoculation. Two readings on separate leaves were made per tree or six readings per plant for periwinkles. For corn plants, R_l was determined at two places on each of three leaves per plant. The leaves of healthy plants, symptomatic leaves of infected plants, and nonsymptomatic leaves of infected plants were compared. On trees, only leaves that were fully expanded before inoculation were used; trees were pruned to keep these leaves fully exposed. All plants were well watered 2–4 hr before R_l measurements. The pressure chamber was again used to verify that plants with high R_l were not under water stress.

RESULTS

Field studies. For American elms, P and R_l data were analyzed separately within the 2×4 (health×site) factorial design. The mean P of symptomatic trees, −0.84 mPa, was significantly ($P < 0.01$) less negative than the P of healthy elms, −1.30 mPa. This trend was consistent within each site. Among sites, however, mean P of symptomatic trees varied significantly, and the range of these values overlapped that of healthy trees (Table 1). No interactions of health and site were found.

The mean R_l of symptomatic elms was significantly ($P < 0.01$) greater than that of healthy elms, 34.1 and 4.5 sec·cm^{-1}, respectively (Table 1). This trend was consistent within and between sites. Again, no interactions of health and site were found. Variation increased with the magnitude of R_l. Therefore data were analyzed as independent samples with unequal variances (28).

Because stomatal resistance seemed the more sensitive indicator of disturbed water relations, further field measurements of P were made only to document plant water potential as each set of R_l data was obtained. Variation in R_l values consistently increased with magnitude of the mean.

Elevated R_l was uniform throughout the crowns of young, single-stemmed, EY-affected American elms. In multiple-stemmed trees, foliar symptoms may not be uniform. In early July, four R_l measurements each were made for healthy-appearing and symptomatic portions of the crowns of seven multiple-stemmed, infected trees. The R_l of symptomatic stems was greater than that of healthy stems but not significantly so (Table 2).

As with American elms, red elms with foliar symptoms of EY had significantly greater R_l than did healthy red elms (Table 2). Leaf surface replicas confirmed stomatal closure in infected trees of both species and revealed no anatomical differences between healthy and infected trees.

Mean R_l in leaves from white ash brooms was nearly 10 times that of healthy trees, 22.9 and 2.7 sec·cm^{-1}, respectively. Replicas confirmed stomatal closure in these leaves. Significantly elevated R_l, compared to that of healthy trees, was also detected in August in the nonbrooming crowns of yellows-affected ash trees that had basal brooms (Table 2).

Chokecherries affected by X-disease showed R_l values from 9.6 to 42.9 sec·cm^{-1}, proportional to severity of symptoms. The mean R_l of affected chokecherries was significantly greater than that of healthy plants (Table 2). Stems within a clone were selected for uniform symptom development and had uniform R_l values. Replicas showed that stomata of plants with leaf reddening were completely closed. No differences in stomatal morphology or frequency were observed.

Values of R_l for white ash seedlings with virus symptoms (mosaic, rugosity, and ringspots) were compared to values for nonsymptomatic seedlings. No significant differences were detected (Table 2).

Greenhouse studies. *Elm yellows.* Eleven of 24 inoculated American elms developed EY and died. Necrosis of fine roots began at 7 wk after inoculation, phloem discoloration at 9 wk, foliar symptoms at 10–11 wk, and death at 13 wk. Only three infected trees were alive 17 wk after inoculation. Measurements of R_l were discontinued at the end of week 15 as trees went dormant. Inoculated trees that remained healthy for two growing seasons after inoculation and had diffusive resistances similar to control trees were not considered in the analysis.

Elevated R_l was detected as early as 8 wk after inoculation. Mean

R_l of healthy and infected American elms diverged 9 wk after inoculation (Fig. 1A). Increased resistance preceded foliar symptoms by an average of 3.5 wk, with a range of 1–4 wk for individual trees. For all trees, R_l increased coincidentally with or shortly after feeder root necrosis and shortly before or at the time of phloem discoloration. Replicas revealed complete stomatal closure in trees with epinasty and yellowing. As trees died, mean values of R_l were based on successively fewer observations. Decreases in mean R_l of infected trees, as at week 13 (Fig. 1A), were the result of loss of infected trees that had high R_l before death.

Elms varied not only in the interval from inoculation to increased R_l, but also in magnitude of the increase. R_l rose as symptoms intensified. In one tree, R_l increased to more than 50 sec·cm^{-1} for 8 days (Fig. 1B). Within one additional week, leaves and buds withered, and the tree died. Three infected trees (including No. 41, Fig. 1B) showed only mild chlorosis, slight epinasty, and gradually increasing R_l (from 3.0 to 13 sec·cm^{-1}) by the end of the first growing season. These trees died shortly after leafing out the next season. In general, the ranges of R_l and the symptoms in greenhouse elms were similar to those observed in the field.

Ash yellows. Twenty of 24 AY-inoculated ash trees became infected and broomed. The remaining four trees appeared healthy, gave a negative reaction with Dienes' stain, had normal R_l (0.1–4.0 sec·cm^{-1}), and were not considered further. Symptoms in infected trees included yellowing, brooming, reduction in leaflet size and number, loss of apical dominance, and reduced shoot growth in nonbroomed parts. The infected trees were frequently induced to broom by pruning. On trees that broomed basally, fine roots also proliferated as do those of witches' broom of black locust (25).

R_l increased before or coincidentally with symptoms of yellows in ash (Fig. 2). The mean R_l of infected ashes exceeded that of healthy ones beginning in early July 1980, nearly 1 yr after inoculation. By July 10, half of the infected trees showed elevated R_l. Values for individual trees rose from normal to between 13 and 45 sec·cm^{-1} and remained high until dormancy. On the average, increased R_l preceded brooming by 6 wk, with a range of 0–21 wk. The mean date of onset of brooming was 16 August. Three trees with elevated R_l (>10 sec·cm^{-1}) during the first growth season did

TABLE 2. Comparisons of diffusive resistance of leaves of plants healthy or infected with mycoplasmalike organisms

Species of plant and source of leaf	Number of observations	Diffusive resistance, R_l (sec·cm^{-1})[a]	Level of significance
Catharanthus roseus			
Healthy plants	6	1.0 ± 0.2	0.05[b]
Plants infected by elm yellows (EY) agent	7	5.5 ± 4.2	
Shoots on plants infected by EY agent (by paired comparison)			
Healthy-appearing	7	4.2 ± 4.3[c]	0.05
Symptomatic	7	7.1 ± 4.6	
Healthy plants	5	0.1 ± 0.3	0.05
Plants infected by ash yellows (AY) agent	4	3.2 ± 1.6	
Fraxinus americana			
Healthy trees	4	2.7 ± 1.1	0.05
Brooms of AY-affected trees	4	22.9 ± 12.2	
Healthy trees (August)	15	3.1 ± 1.5	0.01
Nonbrooming crowns of AY-affected trees	14	11.0 ± 8.8	
Healthy seedlings	8	3.4 ± 1.3	NS[d]
Seedlings with virus symptoms	9	4.0 ± 1.7	
Prunus virginiana			
Healthy plants	10	3.4 ± 0.3	0.01
Plants affected by X-disease	10	18.9 ± 11.1	
Ulmus americana			
Healthy trees	20	4.5 ± 3.1[e]	0.01
Trees symptomatic with EY	17	34.1 ± 20.0	
Crowns of multiple-stemmed, EY-affected trees (by paired comparison)			
Healthy-appearing	7	3.1 ± 5.4[f]	NS
Symptomatic	7	9.2 ± 4.4	
Ulmus rubra			
Healthy trees	5	1.4 ± 1.0	0.01
Trees symptomatic with EY	8	13.7 ± 6.7	
Zea mays 'Golden Bantam'			
Healthy plants	9	6.8 ± 2.9	NS[g]
Plants affected by corn stunt			
Green areas	9	5.0 ± 1.3	0.01[g]
White-striped areas	9	40.5 ± 12.1	

[a] Mean and standard deviation.
[b] Comparisons between means based upon analyses of independent samples with unequal variance unless otherwise noted.
[c] Standard deviation of the mean difference was 1.2 sec·cm^{-1}.
[d] Not significant.
[e] Data are means from Table 1.
[f] Standard deviation of the mean difference was 4.2 sec·cm^{-1}.
[g] Mean R_l of affected plants was greater than that of healthy, and R_l of white-striped areas was greater than that of green areas, as determined by linear combination comparisons of class means (28).

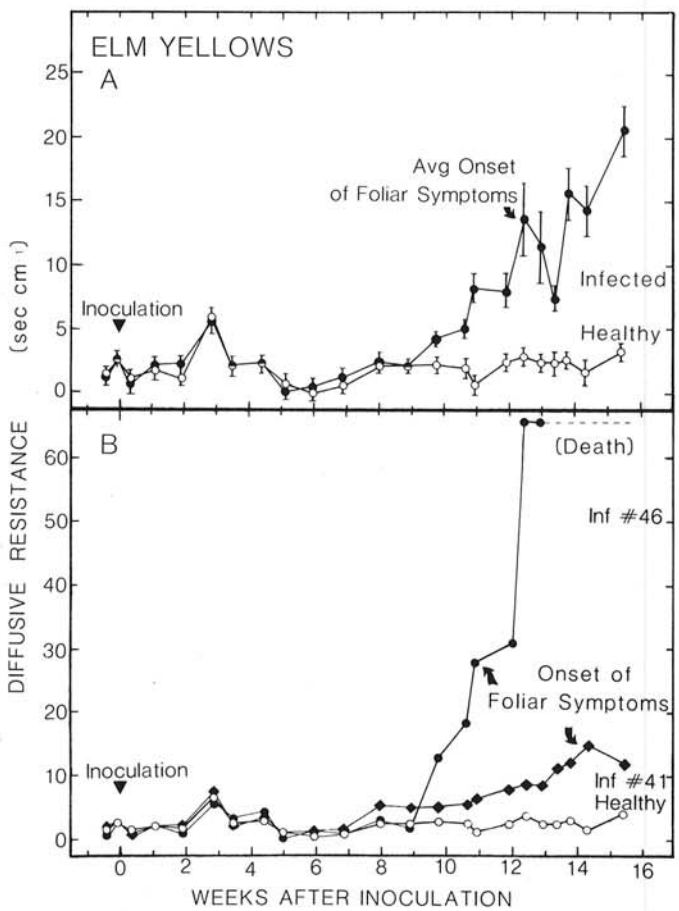

Fig. 1. A, Mean diffusive resistances (with standard errors) of abaxial leaf surfaces of eight uninoculated, healthy and 11 elm yellows (EY)-inoculated, infected American elms. **B,** Mean diffusive resistances of abaxial leaf surfaces of eight uninoculated, healthy and two individual EY-inoculated, infected American elms. Trees were inoculated 15 July 1980 by grafting with bark patches from naturally infected elms.

not broom until the second growth season, after a period of dormancy. Three trees ceased shoot growth and sprouted from the roots 2 wk before any change in R_l. When cut back, these sprouts broomed.

During the first growth season, R_l measurements were made on ash leaves formed before inoculation. In the second season, leaf surface replicas showed that 45–60% of the stomata were closed, with no changes in stomatal morphology or frequency compared to replicas of leaves formed before inoculation. Increases in R_l were then attributed to the stomatal closure.

Mosaic symptoms appeared in some inoculated ash seedlings. Tobacco mosaic virus (16) and tobacco ringspot virus (12) may infect ash. Leaf and cambium samples were serologically indexed for these viruses, but neither was detected. R_l was unrelated to virus symptoms.

Periwinkle with yellows. Symptom progression in periwinkle infected with the EY agent followed that previously described (5). Infected plants broomed, produced small chlorotic leaves, and rarely flowered. Abaxial R_l of periwinkles infected with the EY agent was greater than that of healthy plants (Table 2). Under favorable growing conditions, shoots of periwinkle with the EY agent outgrew yellows symptoms and produced healthy-appearing, floriferous shoots. Mean abaxial R_l of symptomatic shoots was greater than that of healthy-appearing shoots (Table 2).

Periwinkles graft-inoculated with the AY agent showed symptoms after 3 mo. These periwinkles broomed more severely and produced greater numbers of colorless, stunted flowers than did periwinkles infected with the EY agent. Leaf chlorosis proceeded from tip to base of individual leaves. The mean R_l of infected periwinkles was greater than that of healthy plants of comparable age and vigor (Table 2). Leaf surface replicas of periwinkle infected with either yellows agent verified stomatal closure, but no changes in stomatal morphology or frequency were evident.

Corn stunt. The stunt symptoms displayed by Golden Bantam corn were general growth suppression and white-striped areas of leaves extending distally from the stalk. Mean abaxial R_l of infected plants was significantly greater than that for healthy plants (Table 2). In infected plants, R_l was significantly greater in the white-striped areas than in green areas.

Leaf surface replicas revealed anatomical changes in addition to stomatal closure in infected corn plants. Epidermal cells in the white-striped areas had not elongated; 80% of the guard and support cell groups had not completely differentiated; and the number of stomata per unit area was 2.4 times that of healthy plants. In the green areas of infected leaves, guard and support cells were normal, but stomata were 1.7 times as numerous as in healthy plants. Mean stomatal aperture of green areas of infected plants (2.5 μm) was not significantly different from that of healthy plants (1.3 μm) grown in greenhouse light intensity of 850 $\mu E \cdot m^{-2} \cdot sec^{-1}$.

DISCUSSION

Stomatal closure independent of water deficit in leaves is a symptom of MLO pathogenesis. We observed abnormally elevated diffusive resistance in leaves of American and red elms, white ash, and chokecherry infected with their respective yellows agents and in periwinkle infected with MLO from elm or ash. In nonlethal infections (AY in white ash, and AY or EY agent infection of periwinkle), closure was only partial, but in lethal diseases (EY in American elms, X-disease in chokecherry), closure was complete. We also observed abnormally elevated R_l with corn stunt, a yellows disease of spiroplasmal etiology (6,29). Replicas of leaf surfaces showed that elevated R_l was associated with stomatal closure. MLO infection was distinguished from water stress by measuring P. In the field, infected trees had higher R_l, but less negative P than healthy trees. In the greenhouse, healthy and MLO-infected plants differed in R_l but not in P.

Basham and Eskafi (2) observed stomatal closure in MLO-infected palms. Stomata on the green leaves of palms with lethal yellowing were generally closed, even at times when stomata of healthy palms were open. Our findings for elm and ash yellows, X-disease, corn stunt, and two MLO infections of periwinkle suggest stomatal closure as a general symptom of yellows diseases caused by MLO and spiroplasmas. Closure can be easily detected by diffusive resistance measurements.

The more severe the visual symptoms in infected plants, the greater the elevation of R_l above normal. Because symptoms developed asynchronously in graft-inoculated plants, the interval from inoculation to R_l elevation varied, and so did the magnitude of R_l among plants.

In American elm, elevated R_l preceded externally visible symptoms by an average of 3.5 wk. In white ash, elevated R_l preceded brooming by an average of 6 wk, and in some trees, the interval from elevated R_l to brooming spanned a period of dormancy.

Neither the cause of stomatal closure in MLO-infected plants nor the relative importance of this closure has been determined. Toxins have been implicated in yellows disease of periwinkle caused by *Spiroplasma citri* (7). Hormone imbalance is likely. Abscisic acid accumulates in association with stomatal closure (20,26) and can cause yellowing and leaf abscission (20). Other indications of such imbalance in MLO-infected elm and ash trees are impaired translocation (4) and ontogenetic changes in phloem of elms (3), growth suppression, and broom formation. Hormonal imbalance has been detected for several mycoplasmal infections of periwinkle (8).

Several plant diseases in addition to those caused by MLO are characterized by changes in diffusive resistance to water loss associated with altered stomatal function (1). Systemic increase in diffusive resistance has been observed in plants with certain nematode (15) and virus (17) infections.

Stomatal closure in MLO diseases of plants could cause additional symptoms, the intensity of which might vary according to the degree of closure. Closure would reduce CO_2 intake, cause temperature elevation, and inhibition transport in xylem. Blocking stomatal diffusion with petroleum jelly induced yellowing in detached bean leaves (31).

Diffusive resistance measurements of stomatal closure may allow quick and early detection of diseases caused by MLO, although not to the exclusion of other possible causes. Reduced evaporative cooling of infected plants implies possible remote detection. Diffusive resistance can also provide an estimate of the relative susceptibility of plants: those most susceptible respond with complete stomatal closure. Diffusive resistance measurements

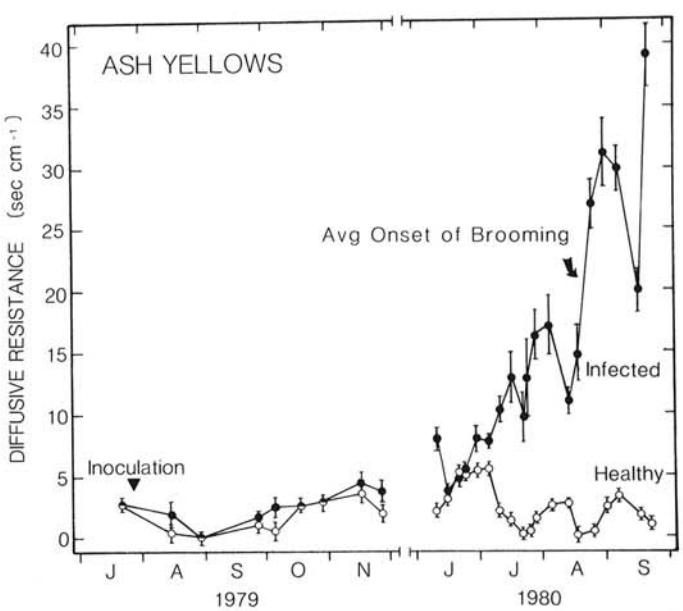

Fig. 2. Mean diffusive resistances (with standard errors) of abaxial leaf surfaces of eight uninoculated, healthy and 20 ash yellows inoculated, infected white ash seedlings. Trees were inoculated 26 July 1979 with bark patches from naturally infected ash trees.

may thus be of value in breeding programs and host range studies. Measurements of R_l may indicate presymptomatic yellows infection more conveniently than direct observation by electron microscopy or with Dienes' stain, particularly when MLO titres are low.

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