Root Necrosis and Histological Changes in Surviving Roots of White Ash Infected with Mycoplasmalike Organisms

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ABSTRACT

Root necrosis and histological changes in surviving roots of white ash (Fraxinus americana) infected with mycoplasmalike organisms that cause ash yellows were evaluated in graft-inoculated 3-yr-old plants grown in hydroponic culture and in soil, and in naturally infected trees. Dead and dying lateral roots on diseased plants in hydroponic culture were first noted at 34 days after inoculation. At 58 days, root necrosis in both hydroponic culture and soil was extensive. Leaves on some diseased plants growing in soil began wilting at 68 days. In secondary phloem of living, dead lateral roots, starch accumulation was reduced, mild hyperplasia developed, many sieve tubes and companion cells collapsed, and phloem parenchyma adjacent to collapsed cells developed thick lignified walls (pathological sclerenchyma). Taproots and stems remained alive. After vernalization, diseased plants grew feebly. The roots of naturally infected white ash in the field displayed dieback and scarce feeder roots or abnormally tapered and bushy form. These roots contained pathological sclerenchyma and scattered nonfunctional sieve tubes that autofluoresced under UV. Diseased roots of green ash (F. pennsylvanica) saplings in the field had autofluorescent sieve tubes and pathological sclerenchyma but no dieback or abnormal form. Uninfected control plants of both species had none of the above symptoms. Root damage in white ash infected with mycoplasmalike organisms precedes other symptoms and perhaps triggers branch dieback in the ash yellows syndrome.

Ash yellows, a disease induced by mycoplasmalike organisms (MLOs), causes slow growth or decline and death of white ash (Fraxinus americana L.) (16,27,41). Symptoms in aerial parts of affected trees have been described (16,20,26,27), but characteristics of diseased roots have not. Root necrosis is an early symptom in other diseases caused by MLOs, such as elm yellows and lethal yellowing of palms (12,13,30). Knowledge of the effects of MLOs on ash roots may be a key to understanding the progression of symptoms of ash yellows.

During diagnostic observations of MLO-infected white ash (40,41) we noticed morphological and anatomical abnormalities in roots. The first- or second-order lateral roots (43) of some diseased trees were short and bushy (Fig. 1), occupying a smaller volume of soil than the comparable roots of healthy trees. Distal parts of long roots were often dead. In roots 2-6 mm in diameter, sampled for diagnosis by fluorescence microscopy (40), we often noticed sieve tubes that fluoresced without the addition of any reagent (autofluorescent sieve tubes). These sieve tubes were presumed to be nonfunctional and often had collapsed. If the symptoms observed in roots were caused by MLOs, root damage might lead to the dieback of branches that often develops in ash affected by yellows. The purposes of our study were to identify and describe the morphological and histological symptoms induced by ash yellows MLOs in roots and to learn whether these symptoms precede slow stem growth and foliar symptoms. Preliminary findings were conveyed in an abstract (11).

MATERIALS AND METHODS
This study had two phases: an experiment conducted with 3-year-old white ash in hydroponic culture and in soil, and observations of roots collected from diseased and healthy white ash and green ash (F. pennsylvanica Marsh.) in the field.

For the experiment, groups of five plants were assigned to four treatments: inoculation with MLOs or mock-inoculation, followed by culture in hydroponics or in soil. The experiment was prepared as follows. Thirty dormant, healthy 3-year-old white ash, grown in a steam-sterilized soil mixture in plastic pots of 12.5 cm diameter, were removed from storage (5 mo or longer at 1-2 C) and placed in a greenhouse at 21-25 C. The photoperiod was extended to 16 hr by fluorescent lamps. After 19-22 days, the plants had begun to grow, and 20 of them were inoculated by bridge-grafting with twig segments from F. excelsior L. infected with ash yellows MLOs. The plants each received two scions approximately 2 cm long. The date of this inoculation was designated day 0. All plants were then allowed to grow for 10 days, after which the 10 remaining plants were mock-inoculated by removing and replacing strips, 20 x 2 mm, of their own bark. The 10-day interval between bridge grafting and mock-inoculation was scheduled to allow partial recovery of the bridge-grafted plants from their wounds, which were more extensive than those caused by mock-inoculation, before further manipulation.

On day 10, five bridge-grafted and five mock-inoculated plants were gently washed to remove the soil from their roots and were segregated according to treatment into two hydroponic cultures. The hydroponic system consisted of two 30-L opaque tanks, each containing 25 L of aerated circulating Hoagland's solution (22). The iron source in the nutrient solution was a Sequestrene (Ciba-Geigy Corp., Ardsley, NY), 0.02 g L-1. The solution was changed monthly. The system was housed in a controlled-environment chamber with a 16-hr photoperiod at 21 C and a night temperature of 15.6 C. The stem diameter of plants in hydroponic culture was measured with a caliper at a marked spot on the root collar on day 10 and again at the end of the experiment. Cross-sectional areas and changes in area were calculated from these measurements. The relative increase in cross-sectional area during the experiment was calculated as (final area - initial area) / initial area.

Five plants per graft treatment remained in soil in pots in the greenhouse. Ten additional bridge-grafted plants were retained in pots for sampling of roots. On day 33 all plants in both hydroponic culture and in soil were tested for mycoplasmal infection by means of the DAPI (4',6-diamidino-2-phenylindole-2HCl) fluorescence procedure, as adapted for diagnosis of ash yellows (40). Symptoms were monitored until day 116.

Because viruses could have been introduced with the MLO inoculum (16), foliar and root samples from the MLO-infected plants in hydroponic culture were assayed for the three viruses then known to occur in white ash in New York State: tobacco mosaic virus, tobacco ringspot virus, and tomato ringspot virus. A direct enzyme-linked immuno-sorbent assay for each virus was kindly performed by J. D. Castello of the State University College of Environmental Science and Forestry, Syracuse, NY. No virus was detected.

Root samples for histology were taken
from all plants in hydroponic culture on days 34, 62, and 90, and from five each of randomly selected diseased and five healthy potted seedlings on day 116. One first-order lateral root (43) of 2–4 mm diameter was excised from each plant on each day. Segments 1–2 mm long were cut from each excised root, fixed in 2.5% glutaraldehyde, pH 6.9, and stored at 8°C until they were processed for histology. Segments were then dehydrated in an ethanol series (70, 90, 95, and 100%), embedded in plastic (JB-4 Plus Embedding Kit, Polysciences, Inc., Warrington, PA), and sectioned at 5 μm. Sections were stained with one of the following: iodine-potassium iodide for starch (24); 0.03% toluidine blue in benzyl alcohol, pH 4.4, for lignin, with counterstain of 1% acid fuchsin for cytoplasmic organelles (33); or 0.5% safranin for lignin (non lignified materials were decolorized with 50% ethanol), with counterstain of iodine-potassium iodide for starch.

On day 116, two plants from each treatment were sacrificed for examination of roots and inner phloem of the stem and taproot. On day 128, the hydroponic system and the remaining 26 plants in the experiment were moved to a cold room (1–2°C) to allow the plants to undergo a period of dormancy. After 6 mo, the hydroponic system was returned to a controlled-environment chamber and the potted plants to a greenhouse, and the development of new shoots and leaves was observed.

In the field, roots of healthy and MLO-infected white ash and green ash growing near Ithaca, New York, were sampled. The disease status of each tree had been ascertained by means of the DAPI procedure (40). The white ash were in a natural stand of mixed deciduous species. The sampled trees varied from 6 to 30 cm diameter at 1.3 m above the ground, and the diseased ones exhibited branch dieback. These trees had become infected naturally at unknown times. The green ash were saplings of the cultivar Marshall Seedless, growing in a nursery. They had been inoculated by grafting with bark patches from diseased white ash 6 and 5 yr earlier and had displayed chlorosis and slow growth for 3 yr. Root segments from two healthy and two MLO-infected white ash were processed for histology as described above. Additionally, roots of five each of healthy and diseased white ash and green ash were sampled for morphological observations and fluorescence microscopy. Three first-order lateral roots, with as many higher-order laterals and fibrous roots as could be obtained, were collected from each tree, washed, and examined for dieback and abnormal form. A subsample from one root per tree was processed for fluorescence microscopy as previously described (40).

RESULTS

Root development on healthy plants in soil versus hydroponics. When the white ash were initially removed from cold storage, their fibrous roots were dead, but root regeneration from the taproots and first-order lateral roots had begun when seedlings were grafted. This regeneration in soil produced a highly branched, new fibrous root system. In hydroponic culture, lateral roots grew much longer than those in soil, often to lengths greater than 40 cm, and root branching was suppressed. Upon dissection on day 116, taproots of two control plants from hydroponic culture and two from soil had normal-appearing, opaque white cortical tissue and semitranslucent colorless tissues in the inner phloem and cambial region.

Development of ash yellows. The DAPI test revealed that roots of all plants with diseased scions were infected with MLOs 33 days after inoculation. The foliage on these plants and on the mock-inoculated controls had grown to full size and was normal in color and form. By day 34, root elongation on diseased plants in hydroponic culture had slowed, and some of the newly formed fibrous roots were dead. These changes were the first symptoms observed. Within an additional 5 days, all roots formed after the beginning of the experiment had died. First-order lateral roots that had been alive at the beginning of the experiment survived. Thereafter, until the end of the growth period (day 120), a slowly progressing dieback of first-order lateral roots occurred, until only the taproots and proximal portions of the first-order laterals remained alive (Fig. 2A). The taproots of two diseased plants that were dissected on day 116 displayed a light caramel discoloration in the inner phloem and cambial region (Fig. 2B).

The root systems of mock-inoculated plants expanded and remained healthy in appearance throughout the growth period.

Rootlet necrosis on the inoculated white ash growing in soil was extensive when these roots were first examined on day 58. Nondestructive observations

![Fig. 1. A first-order lateral root of white ash affected by ash yellows. The portion shown is approximately 60 cm long. Second- and higher-order laterals are stunted and clustered, and fibrous roots are thus concentrated in a small space.](image1)

![Fig. 2. (A) Roots of healthy (left) and MLO-infected ash from hydroponic culture 4 mo after inoculation. Nearly all fibrous roots on the diseased plant are dead. Necrotic roots visible on the healthy plant were dead before the experiment began. (B) Golden discoloration of the inner phloem of the diseased taproot shown in A; the taproot and proximal parts of first-order lateral roots are alive.](image2)
were made by temporarily removing plants from the pots and examining roots at the surface of the root ball. On day 102, an average of 98% of the visible roots of five plants that had wilted (reported below) were dead, and 68% of the roots of the 10 unwilted diseased plants were dead. The taproots remained alive. Dead roots were not seen on mock-inoculated control plants.

The foliage of plants in hydroponic culture remained normal in color and form, except for slight chlorosis in the youngest leaves of both inoculated and mock-inoculated plants, until after root necrosis was advanced. General chlorosis developed later on these plants, but its cause could not be ascertained, because mytes had begun to cause foliar damage. Of the 15 diseased plants growing in soil, 14 developed chlorotic spots, interveinal chlorosis, and/or general chlorosis by 68 days after inoculation. The leaves of five of these plants wilted and died, but the stems remained alive. Mock-inoculated plants growing in soil had chlorosis in the youngest leaves of three plants and no chlorosis in the remaining two plants 68 days after grafting.

The radial growth of diseased plants in the hydroponic system was significantly less than that of the mock-inoculated controls (mean relative increase in area at the root collar 0.47 versus 2.15, \( P = 0.05 \)). Radial growth of the seedlings potted in soil was not measured, because these seedlings had remained in the same pots for three growth cycles and were considered to be pot-bound.

When the three remaining plants in each treatment were allowed to resume growth after the 6-mo dormant period, the diseased plants produced undersized to dwarfed chlorotic leaves on stunted shoots, while the controls produced vigorous shoots and leaves of normal size and color.

**Root symptoms in the field.** All of the naturally diseased white ash had conspicuous root symptoms. Most of the long roots (43) had dead ends (Fig. 3). The inner phloem near these ends was discolored light yellow, similar to the color previously observed in taproots of graft-inoculated seedlings. Some roots had apparently failed to elongate normally, resulting in unusually tapered form, closely spaced lateral roots, and dense clusters of fibrous roots (Fig. 3). Roots with the latter three symptoms were interpreted to have been produced after the trees became diseased. Fibrous roots were scarce in samples collected from trees with severe dieback.

The roots of diseased green ash saplings differed from those of healthy green ash mainly in being somewhat shorter (the difference was not significant in the small groups compared). Tip dieback was infrequent, and fibrous roots were abundant. The inner phloem at the butt of each sapling was examined, and light yellowish discoloration was noted in only one of the diseased plants and none of the healthy controls.

**Histology.** The cell types observed in transverse sections of secondary phloem and cortex of healthy first-order lateral roots included sieve tube elements and companion cells, phloem parenchyma, thick-walled phloem fibers with small lumina, and cortical parenchyma. To assess radial growth and possible hyperplasia in phloem of surviving first-order lateral roots in hydroponic culture, we measured radial distance from the outermost lignified xylem to the first anticlinal division in each of four files of cells approximately 90° apart, located between rays, in a transverse section of one root per tree, five trees per treatment.

![Fig. 3. Portions of first-order lateral roots from white ash trees in the the field: (Left and center) Roots from trees with ash yellows, showing abnormal taper and dieback and fibrous roots either absent or abnormally clustered. The trees from which these roots came also had branch dieback. (Right) Healthy roots with well-spaced laterals and light-colored fibrous roots.](image)

![Fig. 4. Transverse sections of first-order lateral roots of white ash from hydroponic culture, 90 days after the stems were graft-inoculated with MLOs or, for healthy plants, mock-inoculated with strips of healthy bark. (A) Healthy and (B) diseased roots, stained with iodine-potassium iodide to reveal starch grains in parenchyma of the secondary phloem and cortex. (C) Secondary phloem between the vascular cambium (just beyond view at bottom) and the innermost fibers (small darts) of a diseased root, stained with toluidine blue.Collapsed sieve tubes (arrows) appear as darkly stained bands between parenchyma cells. Pathological sclerenchyma cells (large darts) are adjacent to the collapsed sieve tubes. Scale bars = 50 \( \mu m \).](image)
On day 34, the average distances to the first anticlinal divisions in roots of diseased and control plants were 41 and 48 μm, respectively, not different at \( P = 0.05 \) by \( t \) test. By day 90, however, these values had changed to 30 μm in diseased tissue and 81 μm in healthy tissue (\( P < 0.01 \)). In roots collected from soil at 116 days, the average distance from lignified xylem to the first anticlinal division was 28 μm in diseased tissue and 86 μm in healthy tissue (\( P < 0.01 \)). The diminished radial distance from lignified xylem to the first anticlinal divisions between day 34 and day 90 in diseased roots was interpreted to mean that mild hyperplasia, which was not apparent by simple inspection, had occurred. The differences between diseased and healthy roots in these radial distances also indicated a disease-associated suppression of radial growth. In roots taken from hydroponic culture on day 90, the mean radial diameters of cells along the same radii that were measured for assessment of growth and hyperplasia were 8.1 μm in diseased roots and 11.6 μm in healthy roots (\( P > 0.12 \)). Thus, hypertrophy had not occurred in the diseased tissues examined. Cell diameters were not measured in roots from soil.

Starch accumulation in diseased and healthy roots of grafted seedlings was compared by counting the number of starch granules in 15 randomly chosen cortical cells in a transverse section of one root per plant. In hydroponic culture on day 90, the mean numbers were 10.8 and 22.6 starch granules per cell in diseased and healthy roots, respectively (Fig. 4A and B) (difference significant at \( P = 0.01 \) by \( t \) test). In soil on day 116, the corresponding values, based on five observations in one root per seedling, were 2.4 and 25.7 starch granules per cortical cell, a difference significant at \( P = 0.05 \).

The most prominent histological aberrations in the grafted-inoculated white ash were seen in roots collected from soil on day 116. Collapsed sieve elements and companion cells, both scattered and in groups, were apparent, and the walls of parenchyma cells in the vicinity of the collapsed elements had become lignified (Fig. 4C). No such changes had been apparent in the last roots collected from hydroponic culture on day 90.

Roots from diseased white ash in the field were similar in histological appearance to those of grafted plants growing in soil 4 months after inoculation. Many diseased roots, but none from healthy trees, had abundant sclerenchyma in the inner phloem between the youngest band of fibers (if these were present) and the vascular cambium. This sclerenchyma often formed a continuous layer overlying a thin band of conductive phloem (Fig. 5). Some sieve tubes in diseased roots, but none in healthy controls, also autofluoresced under UV (Fig. 5B).

Neither hyperplasia nor hypertrophy was apparent. The amount of starch in small roots was highly variable within single diseased trees. Starch in diseased versus healthy trees in the field was not assessed, for reasons given in discussion. Sclerenchyma and sieve tubes that autofluoresced under UV were observed between the innermost band of phloem fibers and the vascular cambium in three of the five diseased green ash but not in the healthy controls of this species.

**DISCUSSION**

**Root necrosis in the ash yellows syndrome.** The onset of rootlet necrosis in plants in hydroponic culture only 34 days after inoculation was surprising, because we previously thought the incubation period was nearly equal in duration to one cycle of growth and dormancy (16,26). That concept was based upon the onset of foliar symptoms and slow growth in stems during the growth cycle following that in which infection began. We now understand that roots are the first organs to show symptoms of ash yellows, and that slow stem growth and the development of undersized leaves follow root damage.

From observation of the inoculated plants, it seems that most fibrous roots of highly susceptible white ash infected with ash yellows MLOs die, and the radial growth of larger roots slows markedly. Dense clusters of feeder roots, observed on severely diseased trees in the field (Figs. 1 and 3), probably formed after infection. Wilting foliage, slow growth of surviving parts, and chlorosis, observed in diseased plants growing in soil, are probably secondary symptoms induced by the loss of absorbing roots. Dieback of branches in the field may also be, in part, a consequence of root mortality. Other symptoms of ash yellows, such as loss of apical dominance, changes in leaf shape, stomatal closure, formation of witches'-brooms, and subnormal cold hardiness, probably reflect altered
hormonal balances. Symptoms in this latter group were not observed in our experimental plants.

Ash yellows may be similar to elm yellows with respect to root necrosis and wilt in highly susceptible plants. Rootlet necrosis developed in seedlings of American elm (Ulmus americana L.) 7-9 wk after inoculation with bark patches containing elm yellows MLOs (3,30). Rootlet necrosis, beginning at the tips and extending into older tissues, is also an early symptom of lethal yellowing of palms (12,13), as well as mulberry dwarf, parsley, wisteria, and broom, and witches’ broom, all caused by MLOs (44). We had occasionally seen wilted foliage on white ash saplings naturally affected by ash yellows, but only after observing wilt in experimental plants did we become confident that this symptom is part of the ash yellows syndrome. The absence of wilt in the hydroponic system apparently reflected the ability of the residual living roots to take up sufficient water in that system.

Diseased green ash, in accord with the reported tolerance of this species to MLO infection (16,39), had no dieback of roots or branches. The contrast between green ash and white ash in response to MLOs leads to the interpretation that trees with ash yellows that do not sustain significant root mortality also do not display dramatically reduced growth or dieback. This interpretation is consistent with information about pear decline caused by MLOs. The use of tolerant rootstock species protects highly susceptible pear scions from decline (17).

The finding of severe root damage caused by MLOs in white ash is consistent with the hypothesis that MLOs and drought stress interact to cause dieback of branches (18,19,27). Once the roots of a diseased tree have died back or lost the normal tendency to elongate far from the stem, the tree would be able to mobilize water only from a restricted volume of soil and would come under stress more rapidly than healthy plants during drought. The abnormal stomatal closure associated with MLO infection (26) would be expected to partially counteract the contributions of root damage and water shortage to decline. As the wilting potted trees showed, however, rootlet mortality caused by MLOs can lead to symptoms typical of severe water stress, even when the soil is consistently moist.

Histological changes in surviving roots. The premature collapse of sieve tubes and companion cells in MLO-infected ash roots was similar to that reported for other organs of various MLO-infected plants (2,4,10,21,23,28,35,36,37,42). The degree of hyperplasia in ash roots, however, was so slight that it could be described only in terms of anticlinal cell divisions abnormally close to the vascular cambium. No new tissue, like the replacement phloem characteristic of elm yellows (2,30), apple proliferation and pear decline (38), and other diseases caused by MLOs (37), was observed. The light yellow discoloration of inner phloem of taproots and surviving laterals was similar to that found in elms in early stages of elm yellows (2,30), but the discoloration in ash did not presage sheet necrosis of conductive phloem of the taproot, as occurs in elm yellows.

Starch storage was suppressed in MLO-infected ash in hydroponic culture and in soil, in comparison to healthy control plants. This suppression possibly reflected impaired translocation, as was found in elms affected by elm yellows (3). Starch in diseased versus healthy trees in the field was not studied, because differences would not have been interpretable against a background of root and branch dieback in the diseased trees. For example, low levels of starch in roots might be associated with reduced foliar area, or normal appearing amounts of starch in living roots of diseased trees (as observed in one tree) might be associated with the greatly reduced number of roots available for storage.

Development of prominently lignified walls in phloem parenchyma adjacent to collapsed sieve elements has not previously been reported for plants with mycoplasmal infections, although lignin deposition in walls of various cells in phloem of chrysanthemum (Dendranthema grandiflora Tzvelev) thought to be infected with MLOs was noticed by McGovern et al. (28). In our study, lignin in the thickened walls of cells that first differentiated as parenchyma was indicated by staining with either toluidine blue or safranin and by dull blue autofluorescence under UV radiation. We had often noticed the lignified parenchyma in naturally diseased ash, and we now consider this abnormality to be a common symptom of ash yellows. We suggest the term pathological sclerenchyma for this tissue to differentiate it from the sclerotic parenchyma (1,14) that often is seen between bands of fibers in healthy phloem. Pathological sclerenchyma develops adjacent to infected sieve tubes and is found either in the location normally occupied by phloem fibers (Fig. 5B) or between the vascular cambium and the innermost band of fibers (Fig. 4C).

Sieve tubes that autofluoresced under UV radiation were observed in naturally infected white ash and graft-inoculated green ash. Such sieve tubes, easily identified in longitudinal sections, are most often found scattered among nonfluorescing cells in conductive phloem. Sometimes they are embedded in pathological sclerenchyma in old, nonconductive secondary phloem. Sometimes they appear to have been crushed. Autofluorescent sieve tubes were noted in 45% of a group of 159 MLO-infected white ash examined previously (40). DNA can often be detected by means of the DAPI test within such sieve tubes. Therefore, we believe that the walls of sieve tubes infected with MLOs often become infused with substances that fluoresce under UV radiation.

UV-stimulated fluorescence of cell walls in phloem has been reported for a number of diseases caused by MLOs (5,6,8,9,25,28,29,31,32,34), although we found only a few published photographs that clearly show sieve tubes fluorescing without the addition of reagents. Such sieve tubes were observed by Cousin (7) in potato (Solanum tuberosum L.) plants believed to be infected by MLOs and were studied by Petzold and Marwitz (34) in various plants known to be infected. The latter authors associated the autofluorescence with fixation of diseased tissue in aldehydes. Fluorescing sieve tubes in MLO-infected ash, however, may be seen in either fixed or unfixed phloem exposed to UV radiation.

The nature of the fluorescing substances in sieve tubes is unknown. Callose does not fluoresce under UV (15). Petzold and Marwitz (34) suggested that phenolic compounds, which do fluoresce under UV, may be deposited in walls of sieve tubes affected by MLOs. McGovern et al. (28,29) observed that the walls of sieve elements and adjacent cells in chrysanthemum affected by phloem necrosis (thought to be caused by MLOs) fluoresced under UV and reacted with reagents that indicated the presence of lignin. Fluorescing sieve tubes in ash, although often found adjacent to pathological sclerenchyma, contain different substances in their walls, because they fluoresce differently. Walls of sclerenchyma fluoresce dull blue, similar to xylem; the sieve tubes fluoresce white initially and light tan later, sometimes with a pinkish tinge in old tissue. Sclerenchyma stains blue with toluidine blue or pink with safranin, indicating lignin, whereas nearby phloem sieve tubes are not differentially stained in either nonlignified elements by these stains.

Autofluorescent sieve tubes are occasionally observed in white ash in which MLOs are undetectable (40). Therefore, MLO infection is not the only stimulus capable of inducing the chemical changes that permit fluorescence of sieve tubes under UV radiation. The autofluorescent sieve tubes not associated with MLO infection, however, are usually widely scattered and not accompanied by sclerenchyma.

We conclude that diagnostic histological and morphological changes in roots of ash affected by ash yellows precede the development of symptoms in aboveground parts and may be responsible for some of the latter
symptoms. The changes in roots, more dramatic in white ash than in the more tolerant green ash, are generally similar to those recorded for other plants infected by MLOs.

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LITERATURE CITED