# Etiology of Bronze Leaf Disease of *Populus*

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## ABSTRACT

Smith, J. A., Blanchette, R. A., Ostry, M. E., and Anderson, N. A. 2002. Etiology of bronze leaf disease of *Populus*. Plant Dis. 86:462-469.

Bronze leaf disease is a potentially destructive disorder of the *Populus* section of the genus *Populus*. The causal agent has been reported to be *Apioplagiostoma populi* (anamorph: *Discula* sp.). Based on etiological and symptomological studies, field observations of symptom development suggest that the pathogen moves systemically in the host. This was verified by graft experiments where symptoms progressed from the scion into the elongating stem. A bronze-pigmented vascular discoloration was observed in symptomatic leaves and branches. Dieback of affected stems also was common. Spore-trap studies elucidated the timing and necessary weather conditions of *A. populi* ascospore dispersal in relation to infection and symptom development. Exposure–tree experiments revealed that ascospores of *A. populi* are the primary inoculum and resulting infection causes distinctive disease symptoms on affected trees. Perithecia of *A. populi* were observed on overwintered symptomatic leaves, but were not observed on asymptomatic leaves. Acervular conidiomata were observed on symptomatic leaves during August and September. Although *A. populi* ascospores germinated in vitro, *A. populi* was not recovered from symptomatic tissue. Isolations from diseased leaves consistently yielded *Epicoccum nigrum*, but the role of this species is unclear. Inoculations of susceptible plants with *E. nigrum* conidia failed to reproduce symptoms, but inoculations with ascospores of *A. populi* produced symptoms typical of bronze leaf disease and Koch's postulates were performed.

Additional keywords: Ascomycetes, aspen, Diaporthales, disease resistance, *Leuce* section, systemic fungi

Bronze leaf disease of *Populus* L. is a potentially damaging disease with poorly understood etiology. The name of the disease is descriptive of the diseased leaves that exhibit a bronze color. The disease affects several species and most hybrids (Table 1) in the section *Populus* (formerly section *Leuce*). Both indigenous species of aspen (*P. grandidentata* Michx. and *P. tremuloides* Michx.) are susceptible, but the disease is particularly severe on hybrids between these species. The disease was first observed in Massachusetts (4) and Ontario (6) in 1957 and has since been reported in Iowa, Michigan, Wisconsin (11), Minnesota, Ohio, Pennsylvania (21), Vermont, New York, and Quebec (20). There are no reports of the disease in either the western United States or western Canada. The current known distribution of the disease corresponds closely with the range of bigtooth aspen (*P. grandidentata*).

Symptoms of bronze leaf disease begin in midsummer when leaves turn orange-brown or reddish-brown from the margins

inward (6,20) and many leaves on affected trees are underdeveloped (6). By late summer (September in Minnesota), affected leaves become dark reddish-brown, and dry from the margins toward the midrib and curl inward (4,6,20). The midrib and petiole often remain green (18,20). Symptomatic leaves may be scattered throughout the crown or confined to one branch (6,20). Affected leaves are often concentrated in the lower crown initially, but eventually develop throughout the tree. Many symptomatic leaves remain attached during the winter (20) and may be present at bud break the following spring. A uniform symptom pattern on scattered branches suggests that the pathogen may infect stems or buds (20). Dieback of branches is associated with the disease (20) and susceptible clones often decline in less than a decade. Vascular discoloration has been observed in some diseased plants (18), but it is not known if this symptom is useful in diagnosis.

The etiology and epidemiology of the disease have not been studied. There are several reports of leaf bronzing as a general symptom of diseased *Populus* spp. in the literature (2,3,9,13,14,16); however, it is not clear whether all accounts refer to the same disorder. Many of these reports implicate different pathogens with the disease. In addition, Koch's postulates have not been demonstrated with any of the pathogens. Two reports confirm the presence of similar fungi. Cash and Waterman (4) observed and described a new species of ascomycete (Diaporthales, Gnomoniaceae), which they named *Plagiostoma populi* (syn. *Apioplagiostoma populi* (Cash and Waterman) Barr) on overwintered leaves of *Populus tremuloides* × *grandidentata* exhibiting leaf bronzing symptoms in Massachusetts. Acervular conidiomata of an unidentified imperfect fungus also were observed on symptomatic leaves (4). Attempts to isolate these fungi from tissue and spores met with failure. Dance (6) reported that a *Gnomonia* sp. and *Gloeosporium* sp. were present on bronzed leaves of hybrid *Populus* spp. in Canada. It is likely that the *Gnomonia* sp. identified by Dance is actually *A. populi*, because there are few differences in the morphological descriptions by the authors. The *Gloeosporium* sp. also is very similar to the unidentified imperfect fungus observed by Cash and Waterman (4).

Attempts by Dance (6) to isolate these fungi from diseased leaves also were unsuccessful. Marks et al. (13) cites the Dance report as associated with a shoot blight of uncertain etiology. The shoot blight described (13) probably was correctly attributed to *Colletotrichum gloeosporoides* (Penz.) Penz. and Sacc. in Penz. (13). *Gnomonia* spp. or *A. populi* were not found associated with symptomatic plants in the shoot blight study—supporting the differentiation of these two diseases. Sinclair et al. (20) pointed out that *C. gloeosporoides* might be present in shoots exhibiting bronze leaf disease.

Other foliar diseases on *Populus* spp. have been described that exhibit symptoms similar to those described by Cash and Waterman (4). Boyer (2,3) described a necrotic leaf spot disease on *P. tremuloides*, *P. tremula* L. × *tremuloides,* and *P. alba* L. × *grandidentata* that produced symptoms that superficially resembled bronze leaf disease. However, discrete necrotic lesions, atypical of bronze leaf disease, also were observed and *A. populi* was not observed on overwintered symptomatic leaves. Ink spot disease caused by *Ciborinia* spp. can superficially resemble bronze leaf disease during severe epidemics (M. E. Ostry, *unpublished data*), but development of sclerotia distinguishes this disease. Sinclair et al. (20) refer to *A. populi* as the putative causal agent of bronze leaf disease and refer to the unidentified conidial state as *Discula* (syn.

*Gloeosporium*). Sinclair et al. (20) also state that the conidia may serve as spermatia, although this has not been substantiated. The inability to germinate ascospores of *A. populi* has hindered attempts to determine the imperfect state of the fungus and determine what role it plays in pathogenesis.

At least two viruses have been associated with leaf bronzing symptoms. An unidentified potyvirus has been isolated from *P. × euramericana* (Dode) Guinier, *P. grandidentata,* and *P. tremuloides* in Wisconsin (1,14), and tobacco necrosis virus (TNV) particles have been isolated from declining *P. tremuloides* in the Rocky Mountain states (9). There are several other described viruses in aspen that are associated with branch dieback (1,16), but the symptoms are atypical of bronze leaf disease. Additionally, inoculation of susceptible hosts with each of these viruses has not resulted in leaf bronzing symptoms. Attempts to identify virus particles consistently associated with symptomatic samples in the current study were unsuccessful.

The objectives of this investigation were to (i) determine the role of *A. populi* in disease development and elucidate its life cycle, (ii) describe symptomology and epidemiology of the disease in the field, and (iii) determine the modes of transmission of the pathogen.

## MATERIALS AND METHODS

**Description of *Populus* spp. examined.** Study materials were seven clones that have exhibited symptoms of bronze leaf disease for at least the last 12 years at the University of Minnesota Rosemount Experiment Station in Dakota County, MN. Three plantations were included in this study: a 15-year-old planting that was coppiced in the spring of 1990, containing *P. alba* L. × *glandulosa* Uyeki (AK 30), *P. alba* × *glandulosa* (AK 41), and *P. alba* × *sieboldii* Miquel (AS 34); a 24-year-old planting including the symptomatic clones *P. alba* (4877) and *P. alba* × *grandidentata* 'Crandon' (Crandon); and a 14-year-old plantation of seedlings of *P. grandidentata* × *tremuloides* (BT × T) and the reciprocal cross (X 25).

**Symptom development.** *Foliar symptoms and sample collections.* Foliar symptoms of bronze leaf disease and tree phenology were monitored from bud break until leaf fall during 1999 and 2000. Seven ramets representing each of the symptomatic clones and hybrids were marked using metal tags. From mid-April to mid-October, the trees were evaluated once a week and leaf disease symptoms were recorded using the following 0-to-10 scale: 1 = no symptoms; 2 = leaf dull green, surface dried and roughened; 3 = interveinal chlorosis or reddening of <25% of leaf blade area; 4 = 25 to 50 % of leaf surface reddish or chlorotic; 5 = leaf >50% reddish-chlorotic, closer to veins and midrib; 6 = 100% reddish-chlorotic later becoming bronze colored; 7 = leaf is <25% dark reddish-brown, mostly along margins; 8 = 25 to 50% of leaf dark reddish-brown, particularly along the margins; 9 = leaf 50 to 80% dark reddish-brown; and 10 = leaf dark reddish-brown except <20% near petiole and midrib. Rating assignment was based on the most advanced symptoms present. Disease symptom patterns were recorded in September 1999 on each of the ramets. Six symptomatic branches 40 to 60 cm in length per tree were selected; two branches from the lower third, two from the middle, and two from upper third of the crown. The leaves were rated using the 0-to-10 scale. It was determined whether each leaf was preformed (originating from previous year's wood) or neoformed (originating from current year's wood) by evaluating leaf morphology and proximity to bud scars.

Each week, two branches approximately 60 to 80 cm in length, exhibiting typical symptoms, were collected, placed in plastic bags, and transported to the laboratory. One branch with symptomatic leaves was preserved as a herbarium specimen and the other stored at 4°C for histological and microscopic observations. Other collections of branches and leaves were made periodically during 1999 and 2000 for isolations and examinations for fruit body development.

*Vascular discoloration and branch dieback.* In September 2000, branches 40 to 60 cm long were collected from symptomatic trees and evaluated for the presence of vascular discoloration. Two branches from the lower third, two from the middle, and two from the upper third of the crown were collected and brought back to the laboratory. The branches were divided into four equal sections, 1-cm segments were cut from the terminal margin of each section, and the bark was removed. The segments were evaluated visually to determine whether the xylem or pith was discolored. The cross-section was evaluated and the percentage of the cross-section area discolored was estimated. The age of the wood was determined for each segment. The adjacent foliar symptom pattern was described by determining whether all of the leaves within 10 cm of the segment were asymptomatic, partially symptomatic, or all symptomatic.

A study was initiated in September 1999 to determine the incidence of dieback associated with bronze leaf disease. With the exception of X 25, clones and hybrids used in the studies described above were used in this experiment, and an asymptomatic *P. tremuloides* in an adjacent planting was selected as a control. Twenty-five lateral branches, 40 to 60 cm in length with all of the leaves symptomatic but no visible injuries, were selected and marked with yarn. Branches of the *P. tremuloides* (control) were completely asymptomatic. In June 2000, the branches were examined for the incidence and severity of dieback. The following rating system was used: 0 = no dieback, 1 = partial dieback, and 2 = completely dead. Branches also were evaluated for the presence of other pathogens or wounds.

**Isolations from plant tissue and spores.** *Isolations from symptomatic tissue.* During 1999 and 2000, isolations were attempted from symptomatic bud, leaf, and stem tissue. During the summer of 1999, attempts to isolate fungi from symptomatic BT × T leaf tissue were made weekly from the onset of symptom development to senescence (224 attempted isolations total). Isolations also were attempted from an equal number of asymptomatic leaves. Other attempts to isolate from symptomatic leaves from various species and locations were made. The leaves were

**Table 1.** Susceptible *Populus* spp. and hybrids reported or observed by authors exhibiting symptoms of bronze leaf disease and their location[a]

| Species[b] | Location[c] |
| --- | --- |
| *P. alba*[d] | MI, MN, WI |
| *P. canescens* | MI, MN, OH, WI |
| *P. grandidentata* | IA, MN, WI |
| *P. tremula* | MN, OH |
| *P. tremuloides* | MN, VT |
| *P. alba* × *grandidentata* | MI, MN, OH, PA, WI, ON, PQ |
| *P. alba* × *tremuloides* | MI, WI |
| *P. alba* × *sieboldii* | MN, WI |
| *P. alba* × *davidiana* | MN, WI |
| *P. canescens* × *grandidentata* | MI, MN, WI, ON |
| *P. grandidentata* × *tremuloides* | MA, MN, NY |
| *P. grandidentata* × *davidiana* | MI, WI |
| *P. tremula* × *tremuloides* | MN |
| *P. tremuloides* × *canescens* | MI, WI |
| *P. tremuloides* × *davidiana* | MI, WI |

[a] References are noted in text; all other observations made by authors.

[b] All taxa listed belong to section *Populus*.

[c] IA = Iowa, MI = Michigan, MN = Minnesota, NY = New York, OH = Ohio, ON = Ontario, PA = Pennsylvania, PQ = Quebec, VT = Vermont, WI = Wisconsin.

[d] 4877 is a putative *P. alba* clone, but with leaves typical of *P. alba* × *grandidentata* hybrids.

rinsed with water and pieces of approximately 10 mm$^2$ were cut from four areas on the leaf from the petiole to the tip. The leaf pieces were sterilized in 1% sodium hypochlorite for 30 s and rinsed with sterile distilled water. The leaf pieces were placed on malt agar containing 0.005% streptomycin-sulfate and 0.005% penicillin-G antibiotics. The plates were incubated for 14 days at 23°C. Several attempts to isolate from symptomatic dormant buds and stem tissue also were made during 1999 and 2000. Buds were collected from the base of symptomatic leaves and outer bud scales were removed. The bark was removed from the stem and stem segments were cut lengthwise into four sections. Isolations from buds and stem tissue were done using the technique described above.

*Spore germination.* Attempts were made in spring 2000 to germinate *A. populi* ascospores. The following media were used: malt extract agar (MEA), potato dextrose agar (PDA), Czapek's agar (CA), nutrient agar (NA), water agar (WA), and leaf disk agar (LDA). LDA was made by collecting young, healthy Crandon leaves from greenhouse-grown plants and placing them under running water for 20 min, then in a 1% sodium hypochlorite solution for 15 s, followed by a second rinse with distilled, sterile H$_2$O. A sterile 15-mm cork borer was used to cut disks out of the leaves. The disks then were placed on small drops of petroleum jelly placed on the bottom of a petri plate to hold the leaf disks in place. A layer of 2% Difco Noble agar with 0.05% streptomycin-sulfate and penicillin-G was poured over the leaf disks.

Overwintered leaves with mature asci and ascospores were brought into the lab, placed in a jar with a screen lid, and placed under running water for 30 min. After rinsing with sterile distilled water, a 15-mm cork borer was used to cut the leaves into disks. The disks were placed on moistened Whatman #1 filter paper placed on the lid of an inverted plastic petri plate containing the media. When LDA was used, the disks were aligned so that the tissue containing the ascospores was directly under the healthy leaf disks in the media. The plates were placed in incubators set at 15, 18, 20, and 23°C with continuous light. After 24 h, the diseased leaf disks and filter paper were removed. The plates were observed for the presence of ascospores at ×200 magnification using a light microscope. Germinated ascospores were transferred to MEA with a sterilized scalpel. The plates were incubated at 23°C under continuous light, and fungal colonies were examined weekly for the development of fruit bodies.

An attempt also was made to germinate spores from acervular conidiomata on symptomatic leaves in August 2000. Leaves containing the acervular-conidiomata were rinsed with sterile distilled water for 1 h. The leaves were then soaked in sterile distilled water for 2 h and then

placed in petri plates with moist filter paper. The petri plates were placed in an incubator set at 18°C overnight. The following day, a sterile needle was used to transfer spores and entire fruiting bodies to plates of LDA, WA, MEA, PDA, and CA. The plates were incubated at 18, 20, and 23°C with continuous light for 14 days.

**Spore dispersal and disease development.** *A. populi ascospore dispersal.* Ascospore dispersal of *A. populi* was monitored using petroleum jelly-coated microscope slides (17) during 1999 and 2000. Four traps were placed in or near each of the symptomatic clones. No spore traps were placed near clone AK 41 because few symptomatic leaves were present on this clone. Under each tree, two spore traps were placed at approximately 0.5 m above the ground, oriented horizontally by metal clips attached to metal stakes. The other two traps were placed approximately 2 m above the ground in the tree and positioned horizontally with metal clips attached to small branches. Two traps, one on the ground and one in the tree crown, were oriented with the petroleum jelly facing up, and two other slides similarly located were oriented with the jelly facing down. The traps were changed weekly beginning in late April and ending in early October.

The traps were stained with 1% phloxine and examined under a light microscope at ×400 magnification. Each slide was examined along three transects at the 20-, 30-, and 40-stage marker. The number of *A. populi* ascospores were counted on each transect, and the presence of any other spores consistently observed were noted. The numbers of spores counted per slide were tallied, and the sum of all weekly slides was calculated. Rainfall and weekly mean daily temperatures were collected from a weather station approximately 1 km from the study site.

*Exposure trees.* Greenhouse-grown *P. alba × grandidentata* clones Crandon, Shimek, Sherrill, and *P. alba* 4877 (obtained from Iowa State University Department of Forestry) were exposed to natural *A. populi* ascospore dispersal during spring of 2000 to investigate timing in the infection process and symptom development. Softwood cuttings were taken from terminal shoots of asymptomatic, greenhouse-grown stock plants. The basal ends of the cuttings were dipped in 1% sodium hypochlorite solution and treated with Hormex #1 (1,000 ppm indole butyric acid [IBA]), inserted in 1:1 (peat:vermiculite) mix, and placed under mist. After 3 weeks, the rooted cuttings were transferred to Sunshine SB 40 potting mix and grown in 10-cm plastic containers for approximately 2 months under lights in the greenhouse. The plants were fertilized with a soluble 10-10-20 fertilizer approximately every 2 weeks. After 2 months, the plants were repotted into 7.5-liter plastic containers and grown for 3.5 months in the greenhouse.

In mid-April of 2000, the plants were cut back to promote a new flush of growth and to simulate bud break in the field. The four clones were exposed to natural inoculum for six 2-week intervals, beginning 27 April. The experimental design included four replicates in a randomized four-block design. The potted plants were placed inside a fenced enclosure under infected trees of clones AK 30, AK 41, and AS 34. Within the enclosure, four wooden frames that held four potted plants each were placed in plastic saucers with 2-liter plastic bottles attached to each plant to provide irrigation from beneath the pots. In the middle of each frame were two square wooden frames covered by large mesh wire. One frame was placed on the ground and the other was placed approximately 1 m above it. Inside each frame were 100 leaves containing perithecia of *A. populi*. Two petroleum jelly-coated slides (one facing up and one down) were placed 5 cm above the frames to trap *A. populi* ascospores and to determine timing of inoculum release during the exposure intervals. Control plants of each clone were placed outside of the greenhouse at St. Paul at each of the exposure dates. After each 2-week interval, the plants exposed were brought back to St. Paul and also maintained outside. All plants were rated for leaf symptoms four times starting in late July and ending in late September using the 0-to-10 rating scale. After the growing season ended, the plants were planted in the field nearby and monitored the following summer for symptoms.

*Fruitbody development.* In November 1999, 250 symptomatic leaves were collected from AK 30, AS 34, 4877, Crandon, and BT × T (750 total). Foliage from an adjacent asymptomatic *P. tremuloides* was used as a control. The leaves were placed in mesh bags and overwintered outside. In May 2000, the leaves were examined for the presence of *A. populi* perithecia at ×10 magnification. The number of leaves of each clone containing perithecia was determined. A subset of leaves also was brought inside periodically during the late winter and early spring of 2000 to document the development of *A. populi*. Symptomatic leaves also were examined during the summer of 1999 and 2000 for the presence of conidiomata.

**Artificial inoculation studies.** *A. populi inoculations.* In May and June 2000, healthy 'Crandon' and '4877' plants were inoculated with *A. populi* ascospores. Two treatments were tested with eight replications of four plants and a control per treatment per clone. Eight-week-old greenhouse grown plants were placed in a growth chamber set at 18°C (treatment 1) or a mist bench in the greenhouse (treatment 2). The plants were placed in a tray of water and petroleum jelly-coated slides were positioned horizontally approximately 10 cm above the soil and approxi-

mately 10 cm below the top of the plants. Leaves containing perithecia and ascospores of *A. populi* were suspended directly above the plants. Control plants were maintained on a greenhouse bench. After 7 days, the plants were removed from the inoculation chambers and maintained in the greenhouse until August, when they were placed outside. In September, the plants were rated for symptoms using the 0-to-10 rating scale. After the growing season ended, the plants were planted in the field nearby and monitored for symptoms in 2001.

*Systemic transmission.* In February 1999, dormant cuttings were rooted in the greenhouse from branches collected randomly from symptomatic trees in the field. The following July, symptoms of bronze leaf disease developed on these trees in the greenhouse. These results and field observations in 1999 indicated that the pathogen might move systemically in affected trees. A study of the systemic transmission of the disease agent was initiated in September 1999. Branches with symptomatic leaves, 20 to 40 cm in length and 0.6 to 0.8 cm in diameter, were marked on AK 30, AS 34, 4877, Crandon, and BT × T using yarn. Stems from an asymptomatic clone of *P. tremula* × *tremuloides* also were marked for a control. In February 2000, marked stems were collected and grafted onto asymptomatic *P. tremuloides* seedlings and cutting-grown *P. alba* 'Raket' rootstock growing in 7.5-liter plastic pots. Thirty replications were made per clone per rootstock. Half of the grafts were whip and half were cleft grafts. The grafts were tied with rubber strips and the unions were sealed with LacBalsam sealant and maintained in the greenhouse under artificial lights. The surviving plants were maintained in the greenhouse until 1 July. With the exception of BT × T, for each of the clones, one group of eight plants was placed outside of the greenhouse on 1 July, 15 July, and 1 August. One group of eight plants of BT × T was placed outside on 1 July. The grafts were examined for symptoms throughout the rest of the growing season and the length of shoot growth with symptomatic leaves was recorded. At the end of the growing season, the plants were planted in the field nearby and monitored for symptoms in 2001.

## RESULTS

**Symptom development.** *Foliar symptoms and branch dieback.* In 1999, bud break on AK 41 and AS 34 was occurring on 28 April; on 4877, AK 30, and Crandon on 30 April; and BT × T and X 25 on 5 May. The onset of bronze leaf disease symptoms in 1999 occurred on AK 30 and AS 34 on 26 May, and Crandon and 4877 on 9 June as chlorotic, stunted leaves from preformed buds. These symptoms slowly progressed to an orange-reddish color, and were restricted to interveinal regions (rat-

ing 4) by mid-July. Symptoms first developed on BT × T and AK 41 on 23 June when leaves turned dull drab-green (rating 1), and symptoms developed on X 25 (rating 2) on 30 June. The symptomatic leaves of clones BT × T, AK 41, and X 25 also turned orange to reddish (rating 4) by 11 August. The symptoms progressed until leaves turned dark reddish-brown (rating 10) from the margins and curled inward on all clones and seedling trees by 15 September. At this date, only the regions near the mid-rib and petiole remained green.

Bud break was occurring on all clones and seedling trees on 4 May 2000. Symptoms first developed on Crandon (rating 1), AS 34 (rating 2), and 4877 (rating 1) on 22 June. Symptoms developed on AK 30 (rating 2) on 29 June, on BT × T (rating 2) on 13 July, and on AK 41 (rating 1) and X 25 (rating 2) on 19 July. Leaves became dark reddish-brown (rating 10) on all clones and seedling trees by 1 September. Of the 720 symptomatic leaves evaluated in September 1999, 23% (n = 163) were neoformed and 77% (n = 557) were preformed. The mean symptom rating for the neoformed leaves was 4 and for preformed leaves it was 10.

Mean incidence of partial and complete branch dieback (rating of 1 or 2) on all clones was 72% compared with 4% on healthy controls. Dieback occurred on 1- to 3-year-old wood with 46% completely dead and 26% partially dead. Crandon had the highest number of completely dead branches (60%), whereas AS 34 had the lowest (38%).

*Vascular discoloration.* A bronze vascular discoloration was observed in all symptomatic leaves examined. The xylem of affected branches of BT × T was also consistently discolored. BT × T had 91% of stems with symptomatic leaves exhibiting discoloration; however, Crandon had only 10% of stems with symptomatic leaves exhibiting discoloration. Branches from healthy controls had only 2% discoloration of the xylem. Most discoloration in the branches was confined to the xylem; however, some branches also had abnormal discoloration in the pith. Staining was found only on stem segments that had symptomatic leaves within 10 cm of the segment examined. The oldest branches had the highest percentage of samples with discoloration and the youngest branches had the lowest. No wounds were found associated with branches exhibiting discoloration.

**Isolations from plant tissue and spores.** *Isolations from symptomatic tissue.* *Epicoccum nigrum* was the most consistently isolated fungus from symptomatic tissue. The isolates obtained produced a bright orange-red pigment in culture that diffused into the agar. Weekly isolations from symptomatic BT × T leaves (n = 224) yielded 155 isolates, of which 63 (66%) were *E. nigrum*. *Alternaria* spp. also were

isolated, although less frequently (11%), and the remaining isolates included *Penicillium* spp. (5%), *Aspergillus* spp. (3%), *Fusarium* spp. (3%), *Phoma exigua* (1%), and unidentified taxa (11%). Isolations from healthy leaves of BT × T (n = 224) resulted in 110 isolates; 8 (7%) were identified as *E. nigrum*, and the remaining isolates included *Alternaria* spp. (55%), *Penicillium* spp. (18%), *Fusarium* spp. (11%), and unidentified taxa (9%). Isolations from healthy (n = 480) and diseased leaf tissue (n = 480) from several different hybrids and species exhibiting bronze leaf disease symptoms from various locations resulted in 417 isolates from diseased leaves and 267 isolates from healthy leaves. Of the isolates obtained from diseased leaves, 308 (77%) were *E. nigrum*; the remaining isolates were identified as *Alternaria* spp. (8%), *Penicillium* spp. (2%), *Fusarium* spp. (1%), and unidentified taxa (12%). In comparison, only 33 (12%) isolates of *E. nigrum* were recovered from healthy leaves, and the remaining isolates were *Alternaria* spp. (46%), *Penicillium* spp. (21%), *Fusarium* spp. (7%), and unidentified taxa (14%). *E. nigrum* was also isolated from buds and branches of affected trees, but recovery was less frequent than from leaves.

Of the 432 isolations attempted from branches with symptomatic leaves, 210 isolates were obtained and 21 (10%) were identified as *E. nigrum*; the remaining isolates were identified as *Alternaria* spp. (44%), *Fusarium* spp. (17%), *Penicillium* spp. (9%), *Aspergillus* spp. (6%), and unidentified (24%). No *E. nigrum* isolates were obtained from asymptomatic controls, although 171 isolates were obtained and identified as *Alternaria* spp. (56%), *Fusarium* spp. (18%), *Penicillium* spp. (11%), and unidentified taxa (15%). Isolations attempted from buds (n = 108) located at the base of symptomatic and asymptomatic leaves yielded 92 isolates from diseased tissue and 47 from healthy tissue. Of the 92 isolates obtained from buds at the bases of diseased leaves, 7 (8%) were identified as *E. nigrum*. The remaining isolates were identified as *Alternaria* spp. (80%), *Fusarium* spp. (8%), *Penicillium* spp. (2%), and unidentified taxa (2%). Of the 47 isolates obtained from healthy tissue, none were identified as *E. nigrum*. The remaining isolates were identified as *Alternaria* (71%), *Penicillium* spp. (15%), *Aspergillus* spp. (5%), *Fusarium* spp. (4%), and unidentified taxa (5%).

*Spore germination.* *A. populi* ascospores were observed on all media at temperatures of 15, 18, and 20°C, but none were discharged at 23°C. However, germination occurred only on LDA plates incubated at 15, 18, or 20°C. Once transferred to MEA or LDA, the germinating ascospores either became contaminated or failed to establish cultures. Attempts to establish cultures from the acervular conidiomata resulted in

either no cultures or contamination by *Alternaria* spp.

**Spore dispersal and disease development.** *A. populi ascospore dispersal.* Ascospores of *A. populi* were dispersed during April and May in 1999 and April through June in 2000 (Fig. 1). In 1999, most ascospores (66%) were trapped between 27 April and 3 May. In 2000 the peak period of spore dispersal (30%) was 4 through 10 May. Rainfall occurred each week during spore dispersal in both 1999 and 2000. As mean weekly temperatures approached 20°C, the number of ascospores trapped decreased. The dispersal of ascospores occurred during bud break of susceptible *Populus* spp.

*Exposure trees.* All trees from exposure intervals 1 to 4 (20 April through 14 June) developed symptoms, and 75% of the trees from interval 5 (15 to 28 June) developed symptoms. However, no symptoms were observed on trees from exposure interval 6 (28 June to 12 July) and the controls. Ascospores of *A. populi* were found on traps from intervals 1, 2, 3, 4, and 5, but not 6. At the time of the first rating (31 July), symptoms had developed only on trees from exposure interval 1 (20 April to 3 May). By the second rating (25 August), symptoms had developed on trees from

intervals 2, 3, and 4 (4 May to 14 June). On 15 September (third rating), symptoms were developing on trees from interval 5. Infected exposure trees had individual symptomatic leaves randomly distributed throughout the crown. No symptoms developed on leaves that flushed after the trees were brought back to St. Paul, and no systemic symptoms were present during 2000. The plants were planted in the field at the end of the 2000 field season and systemic symptoms were observed in 2001 on the infected plants, but no symptoms were observed on the asymptomatic plants.

*Fruitbody development.* *A. populi* perithecia (Fig. 2B) consistently were observed on symptomatic leaves collected periodically during spring 1999 and 2000. All 750 symptomatic leaves examined in May 2000 contained perithecia of *A. populi*, but the 250 control leaves did not. *Discula*-like acervular conidiomata (Fig. 2A) were found on symptomatic leaves in September and October in 1999 and 2000; however, their distribution on leaves was much less uniform than the perithecia of *A. populi*.

**Artificial inoculation studies.** *A. populi inoculations.* By September 2000, typical bronze leaf disease symptoms developed on 13% of the trees inoculated in May and June 2000 with *A. populi* by suspending

leaves with perithecia over susceptible trees in the growth chamber (treatment 1). The symptoms (scattered symptomatic leaves, but no systemic symptoms) observed resembled those on the trees exposed to natural inoculum. Perithecia were observed on the diseased leaves of growth chamber plants the following May (2001). No symptoms were observed on controls or trees inoculated in the mist bench (treatment 2). At the end of the field season in 2000, the plants from treatment 1, treatment 2, and controls were planted in the field. Systemic symptoms were observed in 2001 on the same plants from treatment 1 that developed symptoms in 2000, but none of the plants from treatment 2 were symptomatic.

*Systemic transmission.* Bronze leaf disease symptoms developed on all clones grafted with scions collected from symptomatic trees, whereas no symptoms developed on the rootstock plants grafted with scions from the asymptomatic clone. Crandon had the highest percentage of grafts that developed symptoms (95%), followed by BT × T (86%) and 4877 (71%). Symptoms on all grafts developed first on the leaves closest to the graft union and then progressed toward the terminal leaves over time (Fig. 2C). The mean percentage of shoot length (from buds on scion) with symptomatic leaves was highest on BT × T (89%) and lowest on 4877 (25%). Some grafts developed multiple shoots, and symptoms generally progressed on all shoots of an affected tree. Symptoms developed first on the trees brought out of the greenhouse first (1 July), followed by trees brought out later (15 July and 1 August). The grafts were planted in the field at the end of summer 2000. Symptoms were observed in 2001 on the same plants that developed symptoms in 2000, and the disease symptoms had progressed into the current year's (2001) growth. Perithecia of *A. populi* were observed in May of 2001 on the diseased leaves that developed on the grafts in 2000.

## DISCUSSION

*A. populi* consistently was associated with symptomatic tissue, confirming earlier reports that linked this pathogen with the disease. The exposure experiments, artificial inoculation, and systemic transmission studies implicate *A. populi* ascospores as the inoculum and demonstrate systemic movement of the pathogen within the host. Perithecia of the fungus were seen in diseased leaves from field-exposed trees, growth-chamber inoculated trees (treatment 1) in May 2001, and on graft-inoculated trees, but no perithecia were observed in leaves from controls. Thus, Koch's postulates have been performed with *A. populi*, confirming its role in bronze leaf disease.

The disease cycle has been clarified in this study (Fig. 3). Initial leaf infection by
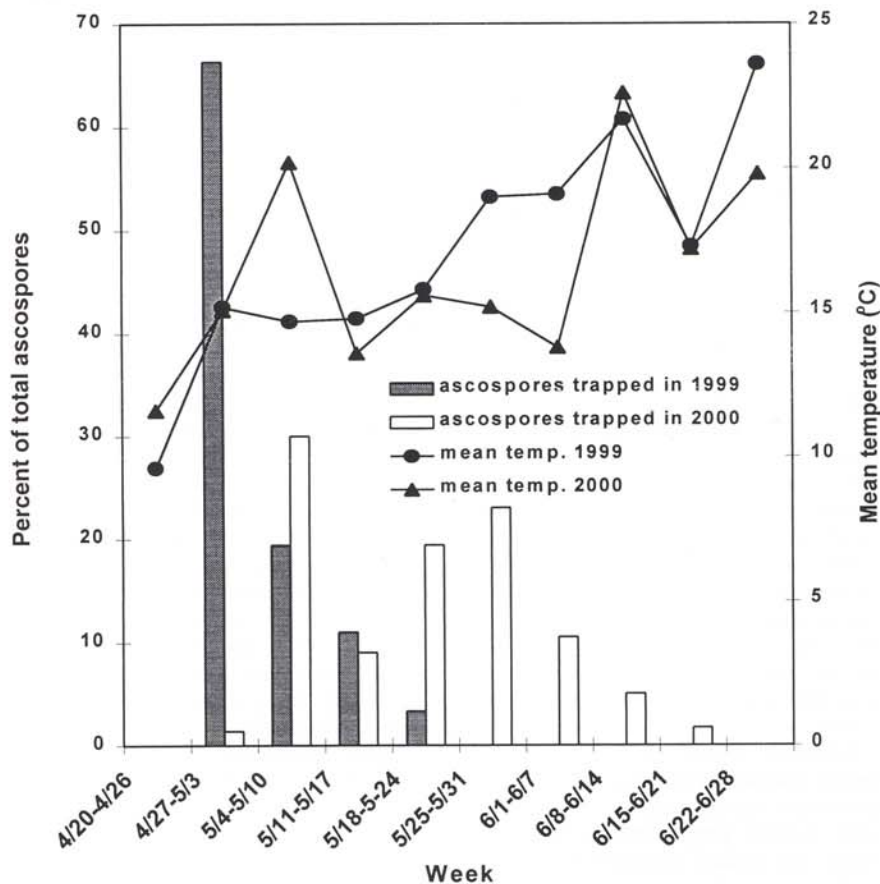


**Fig. 1.** Percentage of total *Apioplagiostoma populi* ascospores trapped versus mean weekly temperature in 1999 and 2000. Spore traps were changed weekly during 1999 and 2000. The total number of spores was tallied, and the number counted each week is represented as a percentage of the total for the year. Temperature data are taken from a weather station located approximately 0.8 km from the study site.

ascospores is followed by systemic movement of the pathogen as illustrated by progression of symptoms to developing leaves. The individual infected (preformed) leaves scattered throughout the crown result from infection by windborne ascospores. This is supported by the exposure tree experiment, in which trees exhibited symptoms similar to naturally infected trees. Ascospore dispersal of *A. populi* coincided with bud break, and infection of individual leaves is responsible for the scattered symptomatic, preformed leaves commonly observed in the field. Neoformed leaves present, when exposed to inoculum, also were infected on the exposure trees, and this suggests that infection is independent of leaf age.

Environmental factors also may play a role in disease development. *A. populi* ascospore dispersal is influenced by weather, with at least some rainfall necessary for ascospore liberation and dispersal. There may be an optimum temperature of 18°C for ascospore dispersal. Most ascospore liberation and dispersal in the laboratory and field were at temperatures near 18°C. In addition, in the laboratory, most germination occurred at 18°C, and germination rates dropped significantly as temperature increased. Symptom development on the first set of exposure trees (those exposed to inoculum first), followed later by symptom development on the other sets of exposure trees (exposed to inoculum later), suggests that a latent period separates infection and symptom development.

The development of symptoms on preformed leaves, followed by development on adjacent, neoformed leaves, suggests that the pathogen systemically invades developing tissue during the growing season. The systemic symptom progression observed in the field was confirmed by the grafting experiment, proving that the pathogen moves systemically within an infected shoot. In this experiment, the symptoms always developed in the leaves on the scion, closest to the graft union, and progressed into leaves along new growth from the scion later in the season. The field studies indicate that death of symptomatic stems does occur. Vascular discoloration in woody tissue adjacent to symptomatic leaves provides further evidence that the pathogen was present in stem tissue. The studies on the systemic movement of the pathogen indicate that the pathogen may overwinter in stems or buds.

The suggestion that *Discula* sp. is the anamorph of *A. populi* is based on the observation of acervular conidiomata on the surface of symptomatic leaves. Several other teleomorph genera related to *Apioplagiostoma* have *Discula* anamorphs, such as *Apiognomonia, Gnomonia,* and *Gnomoniella* spp. For instance, *Gnomoniella fraxini* Redlin and Stack and *Discula fraxinea* (Peck) Redlin and Stack, causal agents of ash anthracnose (19), have *Discula* anamorphs. However, on known hosts, *Discula* spp. cause markedly different symptoms than bronze leaf disease. It also has been suggested that the conidia in acervular conidiomata described by Cash and Waterman (4) and Dance (6) may serve as spermatia (20). This is a possibility because the conidia did not germinate and *Discula* spp. could not be recovered from symptomatic tissue in this study, and germination has not been reported previously in the literature (4,6). The inability to isolate the *Discula*-like fungus presents a challenge to understanding the life cycle of
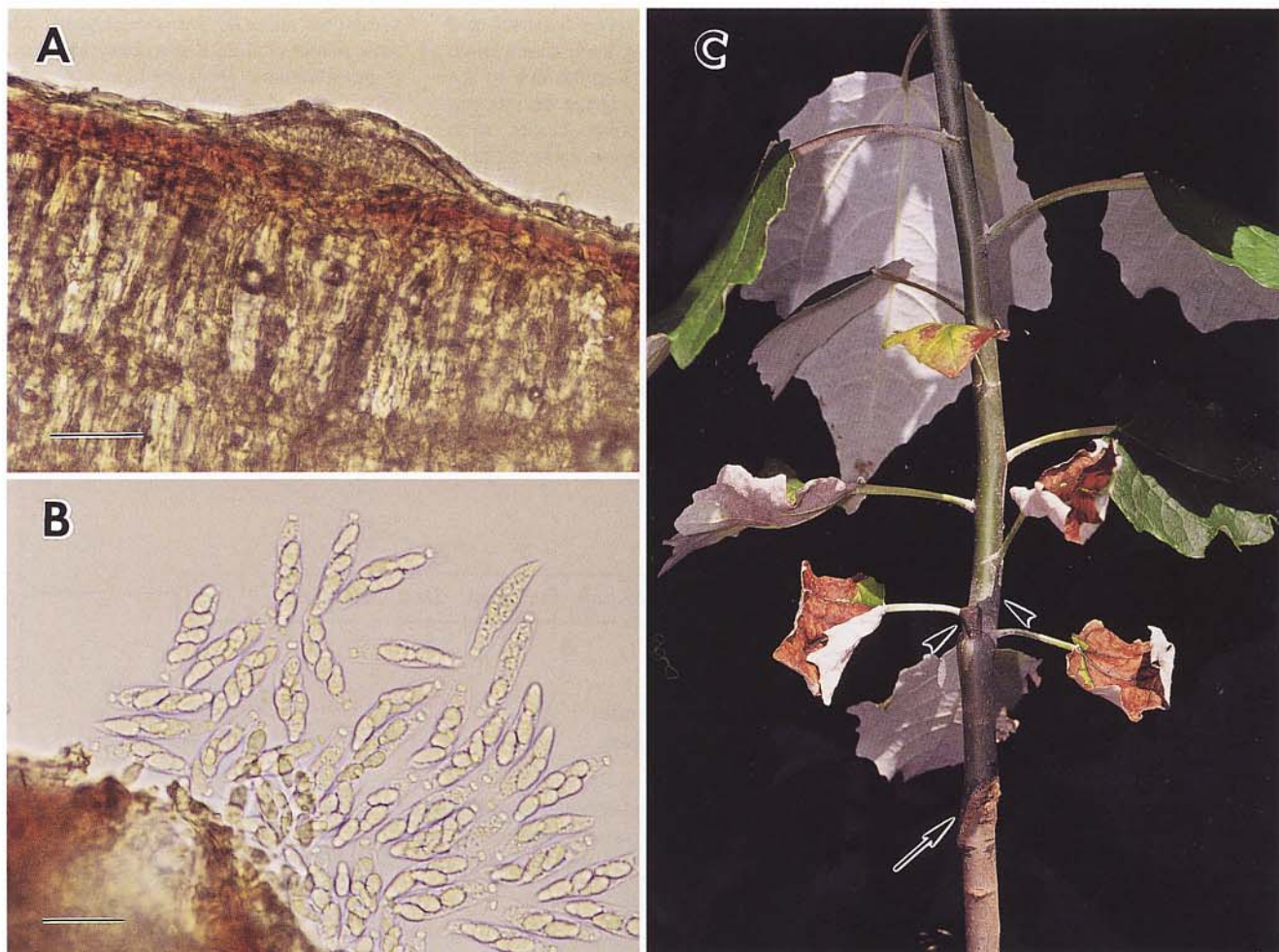


**Fig. 2. A,** Acervular conidioma as seen on the upper surface of a diseased leaf in late August. **B,** Perithecium, asci, and ascospores of *Apioplagiostoma populi* in overwintered leaf in May. **C,** Progression of systemic symptoms from infected scion into elongating shoot (arrow at graft union). During the growing season, symptoms moved into leaves along the elongating shoot originating from the bud at the end of the dormant scion (arrow heads). Scale bar in lower left of A and B equals 25 µm.

*A. populi.* The possibility that *A. populi* is a fastidious species is suggested by our failure to isolate the fungus. However, the germination of spores on LDA suggests that the fungus may have strict requirements for growth on artificial media, and host material is necessary to stimulate germination.

Several other diseases are caused by *Apioplagiostoma* or *Plagiostoma* spp. worldwide. *Apioplagiostoma aceriferum* (Cooke) Barr was described on *Acer campestre* L. leaves in Europe (15). *Plagiostoma perseae* Hyde causes leaf spots on avocado in New Guinea (10), *P. castanicola* Fakirova is found on overwintered leaves of *Castanea sativa* Mill. in Bulgaria (7), *P. solidaginis* Cooke and Barr is found on overwintered stems of *Solidago sempervirens* L. in Connecticut (5), and *P. euphorbiae* (Fuckel) Fuckel is found on *Euphorbia verrucosa* L. (8). A leaf-spotting disease on palm in north Queensland, Australia, also is reportedly caused by *Maculatipalma fronsicola* Frolich and Hyde (8), a genus closely related to *Apioplagiostoma*. Of the above, only *P. euphorbiae* has a putative anamorph state, *Discosia euphorbiae* Fuckel (8), and none has been isolated from plant tissue or spores. No other species of *Apioplagiostoma* or *Plagiostoma* are known to occur on *Populus*, and there are no other reports of these species causing systemic diseases of woody plants. These genera require further investigation to define relationships between teleomorphic and associated anamorphic fungi in relation to disease development.

The frequent isolation of *E. nigrum* from symptomatic leaves adds complexity to understanding this disease. It is well known that *E. nigrum* is a common endophyte that subsequently develops on senescent leaves (22,23). It is also known that *E. nigrum* produces the antibiotic flavipin (12), and this may be responsible for the frequent isolation of *E. nigrum*. The frequent isolation of this species from numerous geographic locations in the current study suggests that the fungus may be associated with bronze leaf disease. The distinctive orange to red pigment produced in culture is very similar to the discoloration observed in vascular tissues of diseased plants. However, the discoloration observed in vascular tissues may be a nonspecific host response to invasion by fungi, including *A. populi*. In addition, it does not appear that *E. nigrum* is responsible for the initial infection for several reasons. First, during the exposure experiments, trees only were infected when ascospores of *A. populi* were observed on spore traps. However, conidia of *E. nigrum* consistently were observed on spore traps prior to and after the period of ascospore dispersal, and the trees exposed during these times failed to develop symptoms. Second, due to the frequent isolation of *E. nigrum* from symptomatic tissue, susceptible clones were inoculated with *E. nigrum* spore suspensions and mycelial plugs, but no infection was observed on these trees (21). Third, although *E. nigrum* frequently was isolated from leaf tissue, it was not commonly isolated from stem or bud tissue. Given that the cause of bronze leaf disease spreads systemically, if *E. nigrum* were the causal agent, it would be readily isolated from stem and bud tissue.

Susceptibility among clones varies. Clone AK 41 exhibited the least incidence of disease and lowest symptom severity. This clone has leaves that are very similar to *P. alba*, with thick pubescence on the undersides of the leaves. It also was observed that cultivated *P. alba* clones 'Nivea' and 'Pyramidalis' do not become symptomatic. Clone 4877 is susceptible, but it is much closer morphologically to hybrids between *P. alba* and *P. grandidentata*, or *P. × canescens* Smith, than it is to *P. alba*. Numerous, large *P. × canescens* have been observed in Minnesota with symptoms of bronze leaf disease. The cross *P. grandidentata × tremuloides* (BT × T) was much more susceptible to the disease than a *P. tremuloides × grandidentata* cross (X 25). The female parent of BT × T was a *P. grandidentata* on the St. Paul campus of the University of Minnesota that exhibited symptoms annually for several years. The male parent of X 25 was a nonsymptomatic *P. grandidentata* from northern Minnesota in an area where bronze leaf disease occurs. Thus, selective breeding may produce resistant clones.
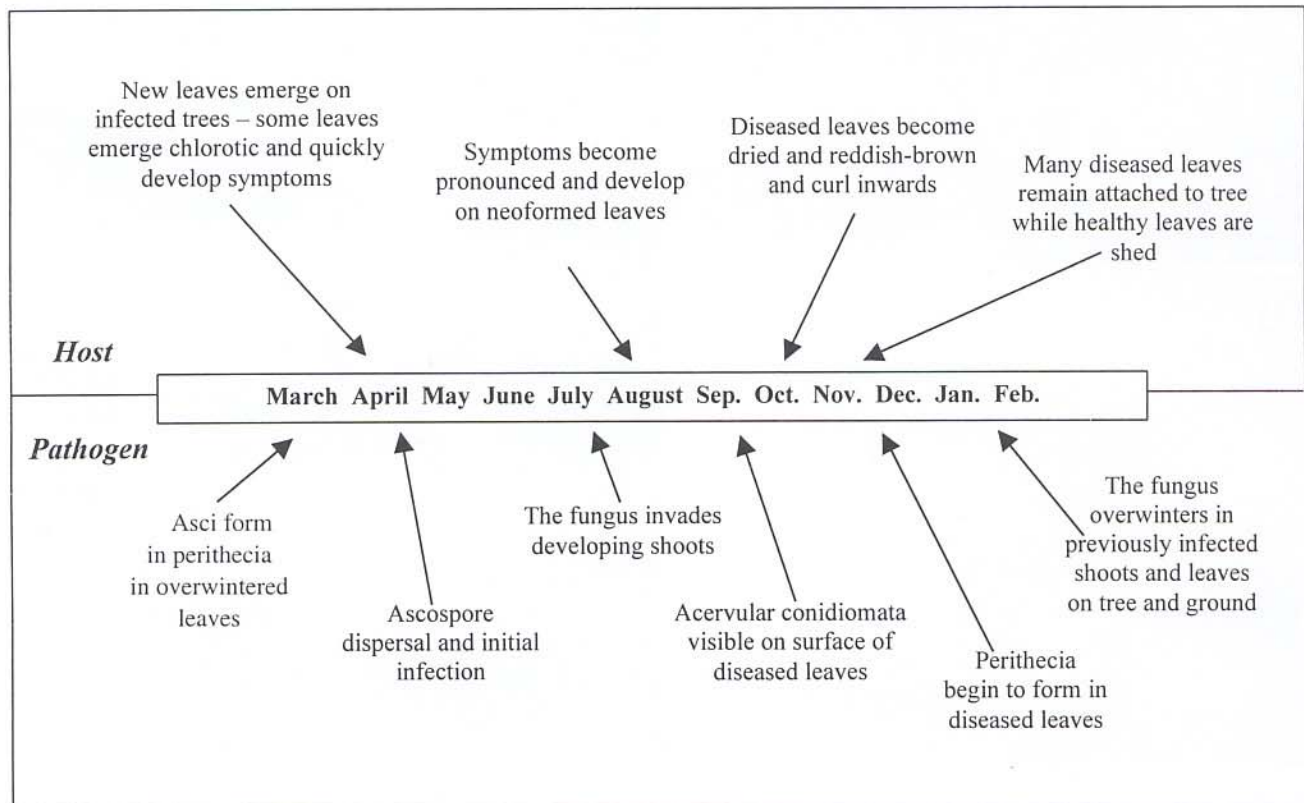


**Fig. 3.** Bronze leaf disease timeline in east-central Minnesota representing disease development in the host (top) and fungal development of *Apioplagiostoma populi*, the causal agent (bottom).

Bronze leaf disease has had considerable impact on plantations of susceptible clones (4,11). Several potentially valuable hybrids have been eliminated from use in commercial plantings because of their susceptibility to the disease. For example, *P. grandidentata* has exhibited some resistance to Entoleuca (Hypoxylon) cankers caused by *Entoleuca mammata* (Wahlenb.) J. D. Rogers and J. M. Ju; as a result, it has gained favor in breeding programs aimed at breeding for resistance to this limiting canker disease. The hybrids *P. alba* × *grandidentata*, *P. grandidentata* × *canescens*, and *P. grandidentata* × *davidiana* exhibit hybrid vigor, disease resistance, and adaptability to poor soils (11). Unfortunately, these crosses are susceptible to bronze leaf disease (11). As a result, the disease is a deterrent in breeding programs and hybrid aspen silviculture in general.

In addition to impacting forestry, the disease also has been observed on the ornamental clones of *P.* × *canescens* 'Tower' and *P. tremula* 'Erecta' (known as "Tower poplar" and "Swedish columnar aspen" in the nursery industry). These columnar clones are valuable because they provide an alternative to the disease-prone Lombardy poplar (*P. nigra* 'Italica'), which exhibits the same extreme fastigiate growth habit. Both 'Tower' and 'Erecta' are popular in the horticulture industry, especially in Canada, but there have been no reports of bronze leaf disease on these clones there.

Bronze leaf disease has potential to become more common in the landscape. The movement of the pathogen on propagative material, in addition to the ability of the pathogen to be transmitted via ascospores, makes pathogen dispersal very efficient. The studies reported here provide the basics of the disease cycle and verify that *A. populi* is the causal agent. Further studies are needed to better understand the relationship between *A. populi* and the other fungal species associated with the disease, including identification of the anamorph of *A. populi*.

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