Received: 25 August 2015 | Accepted: 26 May 2016

DOI: 10.1111/efp.12293

ORIGINAL ARTICLE

WILEY Forest Pathology

Disease biology and histopathology of bronze leaf disease: a threat to cultivated aspens, white poplars and their hybrids

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Editor: J. Roux

Summary

Bronze leaf disease (BLD) of species and hybrids of aspens, grey and white poplars (Genus *Populus*, section Populus) is a systemic disease incited by the fungus *Apioplagiostoma populi* (E.K. Cash & A.M. Waterman) M.E. Barr that results in a characteristic bronze to dark brown pigmentation of infected leaves in late summer and early fall. Branches on affected trees die over a period of several years, disfiguring trees and eventually leading to mortality of susceptible trees planted in landscapes. In this study, we describe the histopathology of the disease and fungus development relative to external symptoms on affected trees. *A. populi* hyphae were observed in leaf vessels, lateral veins and xylem vessels of stems and roots of affected trees. Hyphae were also observed in veins and vessels of asymptomatic leaves. Xylem vessels of growth rings on dead branches were completely occluded with *A. populi* near the vascular cambium and together with the ray parenchyma were stained a bronze colour. Evidence of putative toxin damage to mesophyll cells, not colonized by hyphae, was observed and may contribute to the characteristic tissue staining observed. The development of the fungus was consistent with previous reports, but in this study, we report additional details including perithecial development and ascospore release from predominantly abaxial leaf surfaces. We also report for the first time the isolation and growth of the pathogen in pure culture and systemic infection of roots.

1 | INTRODUCTION

Since the mid-1950s, a little known disease, bronze leaf disease (BLD), caused by the fungus *Apioplagiostoma populi* (E.K. Cash & A.M. Waterman) M.E. Barr (Barr, 1978; Cash & Waterman, 1957), has damaged and killed aspen, grey and white poplar cultivars, and hybrids in section Populus of the genus *Populus* growing in plantations and landscape plantings in the north central and north-eastern United States, as well as the Canadian Provinces of Ontario, Quebec, Saskatchewan, Alberta and Manitoba (Dance, 1957; Heimburger, 1940; Kawchuk et al., 2010; Northover & Desjardins, 2003; Robbins, Ostry, & Prey, 1973; Smith, Blanchette, Ostry, & Anderson, 2002). The Swedish columnar aspen (*Populus tremula* L. "Erecta") and Tower poplar, *P.* × *canescens* Sm. "Tower" (*Populus alba* × *P. tremula* "Erecta") have been severely affected in urban and suburban landscapes (Schroeder, Soolanayakanahally, & Lindquist, 2013). These two clones are in widespread use in the Prairie Provinces of Canada, where losses to BLD are contributing to significant changes in urban forest composition (Schroeder et al., 2013). Other promising aspen hybrids (Benson, 1972) have since been eliminated from future planting after many years of testing and use in industrial and ornamental plantings because of their susceptibility to BLD.

The disease also is common among naturally occurring hybrid trees from seed. Aspen hybrids and aspen-white poplar hybrids can occur on disturbed sites such as roadsides, railways, power line right-of-ways, old fields and other disturbed sites with exposed mineral soil where seedlings establish and survive (Barnes, 1961; Pauley, 1956). These hybrid trees are very frequently infected by *A. populi*, thereby serving

as a source of inoculum in the landscape. *Populus alba* × *P. grandidentata* "Crandon" is a clone of natural hybrid origin that was selected and has been widely used in experimental phytoremediation and biomass plantings and has been used for genetic modification studies (Donahue et al., 1994). "Crandon" is extremely susceptible to BLD (Smith et al., 2002) and may have contributed to the widespread distribution of BLD.

Although there have been a few reports of pure native North American aspen species *P. tremuloides* Mich. and *P. grandidentata* Mich. being affected by BLD (Heimburger, 1966; Robbins et al., 1973), to our knowledge molecular analyses to definitively identify these affected trees as non-hybrids were not carried out. Similarly, certain putative clones of pure *P. alba* L. susceptible to BLD were characterized as atypical in various morphological traits by the authors (Heimburger, 1966; Smith et al., 2002). The only confirmed report of BLD affecting a non-hybrid species has been with *P. tremula*, and this has only been reported for the male clone "Erecta" (Smith et al., 2002), which originated in Sweden (Schroeder et al., 2013).

Within their range, hybrids between bigtooth (*P. grandidentata*) and trembling (*P. tremuloides*) aspens (*P.* × *smithii* Boivin) are known to be locally common (Barnes, 1961; Barnes & Pregitzer, 1985; Henry & Barnes, 1977), and although bigtooth aspen generally flowers 10–14 days later than trembling aspen, temperature inversions (Pauley, 1956) and late-flowering catkins (Einspahr & Joranson, 1960) can overcome the differences in time of flowering of the two species resulting in natural hybrids. The European white poplar (*P. alba*) was introduced into North America almost entirely as female populations and can readily hybridize with bigtooth and trembling aspen (Barnes, 1961; McComb & Hansen, 1954; Spies & Barnes, 1982). In early hybridization trials, it was reported that many of these hybrids were highly susceptible to dieback caused by stem and leaf diseases (Heimburger, 1940) and later Heimburger (1966) described a serious unknown disease of *P. alba* hybrids with bigtooth and trembling aspen that he suggested was a "genetic barrier" to hybrid aspen species. The disease he described is now known as BLD (Heimburger, 1966).

The disease takes its name from the distinctive colour of affected leaves (Fig. 1a–c). Leaves progress in colour in midsummer from yellow-orange-red (Fig. 1b) to bronze-chocolate brown in late summer and early fall (Fig. 1c). Diseased leaves characteristically tightly curl inwards from leaf margins towards the midrib (Fig. 1c), and after senescence either fall or remain attached throughout winter (marcescence). The fungus produces ascospores that are released during periods of wet weather in the spring and infect developing leaves

FIGURE 1 Foliar symptoms of bronze leaf disease (BLD) on aspens. (a) Swedish columnar aspen (*Populus tremula* "Erecta" affected by bronze leaf disease. (b) Characteristic symptomatic tree foliage of a systemically infected hybrid aspen (August), Rosemount, MN. (c) Symptoms present on preformed (curled, brown) and neoformed (yellow-red) leaves of a hybrid aspen affected by BLD (September), Rosemount, MN

scattered throughout the crown (Smith et al., 2002). Movement of the fungus from these infected leaves into twigs and branches leads to systemic infection of leaves on new branch growth in following years (Smith et al., 2002), resulting in portions of, or entire crowns becoming symptomatic. The fungus remains systemic within infected trees and after a few years affected branches exhibit dieback leading to the decline in vigour and eventual death of the trees.

The only known control for BLD is through sanitation and avoidance. In the early stages of the disease, pruning out symptomatic branches may protect affected trees if these branches are removed before the fungus grows into adjacent branches or the main stem. However, infected trees located in the surrounding area may provide fungal inoculum for reinfection. There has also been concern (especially in Canada) that trees are becoming infected in nurseries (Kawchuk et al., 2010; J.A. Smith, unpublished), often surrounded by windbreaks of susceptible clones such as *P.* × *canescens* "Tower" and *P. tremula* "Erecta." These infected trees are then being shipped to areas where BLD was absent previously, leading to expansion of the disease in ornamental plantings of these two clones.

Although some aspects of the life cycle of BLD have been reported (Ostry, Jacobs, Moore, & Anderson, 2013; Ostry, Moore, & Anderson, 2012; Smith et al., 2002), the histopathology of BLD and fungus development within hosts were unknown until this study. Previous to this study, *A. populi* had not been grown successfully on artificial media, greatly complicating its study. However, the pathogen's identity was confirmed by its spermatia (Ostry et al., 2012), characteristic ascospore stage (Cash & Waterman, 1957) and sequencing of the internal transcribed spacer (ITS) regions 1 and 2 along with the 5.8S region of the nuclear rDNA (Kawchuk et al., 2010; Ostry et al., 2012). In this paper, we report the results of a 3-year field and laboratory study of the histopathology and fungus development of this disease. The objective was to describe the development of the fungus within diseased tissues relative to visible symptoms and signs of the disease throughout a calendar year.

2 | MATERIALS AND METHODS

2.1 | Field study and tree parentage

A total of 40 ramets, 5–23 years old, of *P. alba* × *P. sieboldii* Miquel affected by BLD growing in a research plantation at the University of Minnesota Rosemount Research and Outreach Center near Rosemount, MN, were used for study. Observations on disease symptomatology were recorded weekly from April to October during 2012–14 and compared to records of disease development on a *P. grandidentata* × *P. tremuloides* (*P.* × *smithii* Boivin) population from a previous study at the same location (Smith et al., 2002).

2.2 | Histopathology study

Leaf, branch and root samples from 5 to 15 trees each week were collected and transported to the laboratory on ice where they were prepared for microscopic examination and fungal isolation. Samples for microscopic examination were fixed in formalin–acetic acid–alcohol (FAA), dehydrated in a tert-butyl alcohol series and embedded in paraffin using standard methods (Ruzin, 1999) or prepared for examination when fresh.

Thin sections (15 μm) were prepared for light microscopy using a rotary microtome and stained with safranin-fast green (SFG) or periodic acid–Schiff (PAS) to observe host responses and to visualize fungal structures present within leaves. Fresh leaf and branch sections were prepared using a Vibratome 1000 (TPI, Inc. St. Louis, MO, USA) or hand sectioned and examined as is or stained with phloxine, Bismarck brown or methyl violet. Infected leaves were also cleared (Tisdall & Donnelly, 1988) and examined for evidence of the pathogen in leaf veins and to detect when host cells were injured or killed.

2.3 | Pathogen development study

Leaf samples collected in the fall and spring were placed in moisture chambers and examined for the development of spermatogonia and perithecia and release of spermatia and ascospores, respectively, from these structures. Leaf pieces with mature perithecia were attached to Petri dish covers with petroleum jelly, and ascospores were cast onto various nutrient media in light or dark incubators at 18–22°C based on spore trapping performed by Smith et al. (2002) to induce spore germination in an attempt to obtain cultures of the fungus.

2.4 | Pathogen isolation and DNA sequencing

Symptomatic leaf, stem and root pieces were surface sterilized in a 1.0% sodium hypochlorite solution, rinsed in sterile water, and placed onto either malt agar (MA, Difco, Detroit, MI, USA), acidified PDA (Fisher Scientific, Atlanta, GA, USA), ½ strength PDA, Czapek Dox agar (Sigma Aldrich, St. Louis, MO, USA), V8 agar (800 ml H_2O, 200 ml V8 juice, 3 g $CaCo_3$) or woody plant media (WPM, McCown's Woody Plant Medium, Research Products International Corp., Mt. Prospect, IL, USA) amended with 100 mg L^{-1} penicillin–streptomycin solution (Medatech Inc., Manassas, VA, USA) and incubated in the dark at 20°C in attempts to culture *A. populi*.

After incubation and subculture onto fresh media, fungal cultures were identified microscopically and total genomic DNA from selected isolates was extracted using the protocol of Arenz and Blanchette (2011). The internal transcribed spacer (ITS) region of rDNA was PCR amplified and sequenced with primers ITS1F and ITS4 (White, Bruns, Lee, & Taylor, 1990). Contigs of forward and reverse sequences were constructed and compared to the NCBI GenBank database using a BLASTn search. Final edited sequences were deposited into GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and were assigned the following accession numbers (in parentheses): Isolate MNBLDE2 (KR081253), Isolate MNDLDD2 (KR081252), Isolate MNBLDC (KR081251), Isolate MNBLD3 (KR081250) and Isolate MNBLD16 (KR081249).

3 | RESULTS

3.1 | Field study and tree parentage

The progression of disease symptoms and development of the fungus within diseased trees throughout the year are summarized in Fig. 2. In May, pale green infected preformed leaves (on previous year's severely affected branches) remained undersized and became chlorotic. By mid-June, these leaves had wilted and many of the affected branches died. In July, infected preformed leaves became yellow-orange, progressing to red within weeks, usually from the margins inward beginning at the base of affected leaves. Petioles and veins of affected leaves remained green-yellow. Diseased preformed leaves began to curl inward in late July and early August. By late August and early September, preformed leaves were bronze to dark chocolate brown and tightly curled inward with petioles and veins becoming necrotic (Fig. 1c).

Neoformed leaves (on current year's infected branches) became yellow-orange progressing to red beginning in August (Fig. 1b), curling and turning bronze to dark chocolate brown in late September and October (Fig. 1c). Many of these leaves remained attached to branches through early winter.

3.2 | Histopathology study

Histological examination of non-symptomatic leaves on affected branches revealed *A. populi* hyphae in leaf vessels and lateral veins. Hyphae of *A. populi* also were present in xylem vessels of stems and roots of affected trees (Fig. 3a, b). Xylem vessels of growth rings on dead branches were completely occluded with *A. populi* near the vascular cambium and together with the ray parenchyma were stained a bronze colour.

The rapid colour change of leaves and death of the palisade cells observed in histological sections were suggestive of a toxic metabolite moving within and outward from the veins. Similarly, sections of cleared leaves observed under the microscope revealed discoloured veins and surrounding tissues perhaps resulting from a putative toxin leaking from the veins and killing leaf cells. Microscopic evidence of the pathogen in leaves was only detected within the veins (Fig. 3c, d).

In late July and early August, palisade cells were degraded and the majority of the mesophyll cells were dead. Hyphae of the pathogen were present in intercellular spaces, occluded the mesophyll cells and contributed to palisade cell degradation. The upper and lower epidermal cells were heavily stained with the characteristic bronze pigmentation.

3.3 | Pathogen development study

Ascospores of *A. populi* were produced in perithecia (Fig. 3e) within the mesophyll cells and released from the abaxial surface of the previous year's fallen, infected leaves in late April and early May. Presumably, these ascospores can spread the pathogen throughout tree crowns by infecting leaves on branches and trees not systemically infected.

Diseased preformed leaves began to curl inward in late July and early August and numerous spermatogonia (Ostry et al., 2012) began to develop uniformly across leaves ($\bar{x}$ = 209/cm^2) between the upper epidermis and cuticle (Fig. 3f). Spermatia were released beginning in late summer (Fig. 3g), but the specific weather conditions that trigger their development and release are not known.

After spermatia were released, perithecia (Fig. 3h) began to develop within the mesophyll tissues in September and October. The life cycle of the fungus was completed by production and release of ascospores from ostioles of the perithecia on the abaxial leaf surface the following spring shortly after leaf flush.

3.4 | Pathogen isolation and DNA sequencing

Isolations from leaves throughout the season and directly from perithecia in the spring failed to yield cultures of *A. populi*. Ascospores cast onto media in the spring readily germinated, but germ tubes did not elongate and form colonies on any of the media tested. In contrast, isolations from 1- to 4-year-old branches and 3- to 5-year-old roots of symptomatic trees after 1 month yielded a slow-growing yellowish-orange fungus on nutrient medium rarely exceeding 5 mm beyond host tissues. After subculturing onto fresh V8 agar, good colony growth was obtained in 2 months.

BLASTn searches revealed that sequences from a total of 18 independent samples of this fungus (from five symptomatic trees over a

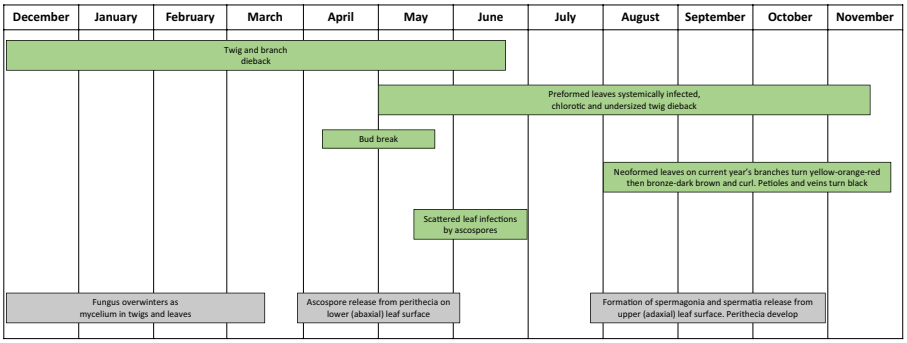

FIGURE 2 Seasonal hybrid aspen host and bronze leaf disease development, Rosemount, MN, 2012–2014

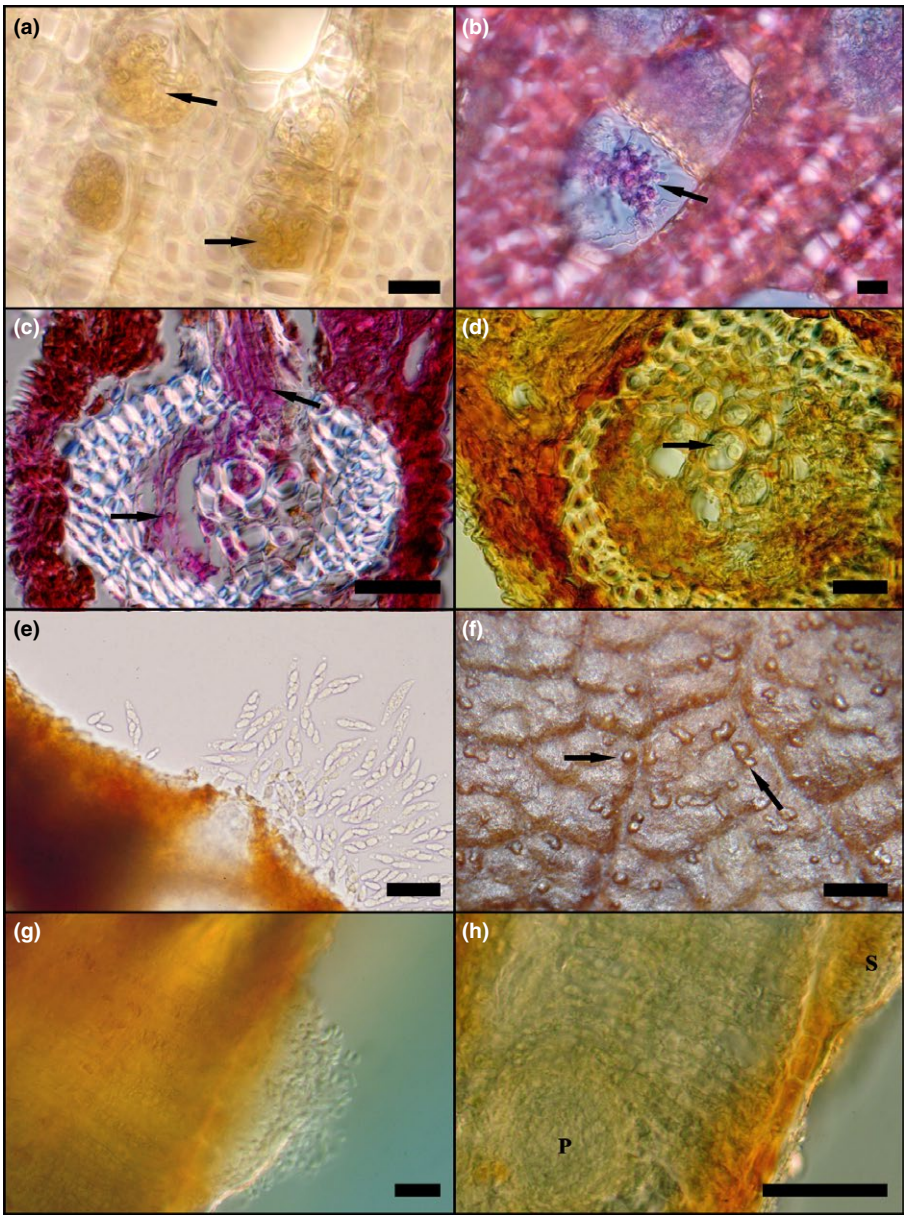

FIGURE 3 Microscopic symptoms of bronze leaf disease (BLD) caused by *Apioplagiostoma populi*. (a) Transverse section of 1-year-old branch affected by BLD with hyphae (arrows) in vessels (May). (b) Transverse section of 4-year-old root from tree affected by BLD with hyphae (phloxine stain, arrow) in xylem vessels (September). (c) *A. populi* hyphae (periodic acid–Schiff stain, arrows) in leaf vascular tissue (September). (d) *A. populi* hyphae (arrow) within vascular bundles of infected leaf (September). (e) *A. populi* perithecium releasing ascospores from abaxial leaf surface (May). (f) *A. populi* spermatia (arrows) on adaxial surface of infected leaf (July). (g) *A. populi* spermatia releasing gametes (August). (h) *A. populi* spermatia (S) and perithecia (P) within affected leaf. Note bronze staining of epidermal cells (September). Scale bars: A–E—50 μm; F—300μm; G—25 μm; H—100 μm

two-year period) were homologous (99% identity, mean 328 of 330 nucleotides) to *A.populi* from isolated perithecia (GenBank Accession No. GU205341.1).

4 | DISCUSSION

This is the first report of isolation of *A. populi* in pure culture from BLD-infected trees and provides additional evidence to that provided by Smith et al. (2002) that the pathogen systemically colonizes all parts of the host, including the roots. Now that pure cultures are available, additional studies to clarify infection and disease epidemiology questions could be addressed. In addition, population genetic structure of the pathogen could be examined.

The unique tight inward curl of diseased leaves may be the result of the extensive exclusive colonization of the upper (adaxial) leaf surface by the fungus and facilitates fertilization of compatible cells by spermatia released from spermatogonia in wet weather. In addition, in the following spring, release of ascospores from resulting perithecia is almost exclusively through the exposed lower leaf surface of the

FIGURE 4 Trembling aspen (*Populus tremuloides*) in southern Manitoba, Canada, displaying symptoms of BLD

curled leaves. The cause of the characteristic bronze colour observed in symptomatic tissues is unknown, and although suspected as having a role in the disease, a toxin has not been isolated.

Near-complete occlusion of xylem vessels by hyphae of *A. populi* in annual growth rings explains the observed disease symptoms such as wilting of leaves early in the growing season, the dieback of affected branches and the gradual decline and eventual death of diseased trees after several years.

Although a systematic evaluation of disease transmission to suckers was not performed, we did monitor a non-symptomatic sucker some 1 m from an infected sapling during the 3 years. The sucker never exhibited any leaf symptoms until the last year when the lower, inner leaves on branches proximal to the sampled root connected to the sapling became uniformly symptomatic. Hyphae were found within the connecting root along the entire length (1 m). A similar pattern of infection was observed on many other small suckers strongly suggesting root transmission rather than spore infection.

Several aspects of BLD present challenges in developing strategies for minimizing its damage. First, it is not known whether the fungus is native or was inadvertently introduced; however, there are no reports of its presence outside of North America. Second, once the disease becomes systemic, control by pruning and sanitation is of questionable long-term effectiveness. Transmission through roots could result in spread of the fungus into adjacent trees and sucker regeneration (although this needs to be proven experimentally). Lastly, the difficulty in isolating the fungus and the slow growth on artificial media hinders the development of an easy resistance screening technique of new poplar hybrids using artificial inoculations under controlled conditions. Prior observations indicate that it may take many years, if not decades for the disease to become evident in field trials and landscape plantings of new hybrids.

The disease limits our continued use of several valuable hybrid trees that were developed for industrial and ornamental plantings. Currently, finding suitable replacements for susceptible clones has been achieved by selecting material from other sections of the genus (i.e. Aigeiros, Tacamahaca). For example, the clone *P.* × *canadensis* "Sundancer" has been selected as an alternative to *P. tremula* "Erecta" and *P.* × *canescens* "Tower" (Schroeder et al., 2013). Until a reliable technique for resistance testing is developed, caution is advised in breeding, propagating and planting aspen hybrids in section Populus.

Ecologically, this pathogen has been described as a "genetic barrier" (Heimburger, 1966) to natural hybridization of the species in section Populus. The progeny of natural hybridization among several native and introduced aspen and poplar species are severely impacted, and although this preserves the pure species in nature, it is an obstacle to breeding reliable new aspen hybrids. While there is the potential for BLD to affect natural aspen forests within North America, the risk is primarily a concern for *P. tremula*, a species known to be susceptible (at least certain cultivated clones), which has a very large geographical range in Europe and Asia. Its introduction into these regions should be a concern to forest biosecurity efforts there, but so far, the disease has never been reported outside of North America. The authors have observed what appear to be symptomatic *P. grandidentata* and *P. tremuloides* on occasion (Fig. 4), but additional studies would be needed to be certain of the identities as pure species and not hybrids (as introgression is common in *Populus*).

In the Niobrara River valley of northern Nebraska, *P.* × *smithii* apparently persists along with other relictual species and forms unique forests, not found elsewhere in the Great Plains (Johnsgard, 2001). There are efforts to conserve these populations, and several agencies are involved in identifying threats as well as propagating these populations (http://unlgardens.unl.edu/populus-x-smithii). Given the observed susceptibility of this hybrid in the lake states, BLD should be considered a major threat to these natural hybrid populations.

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