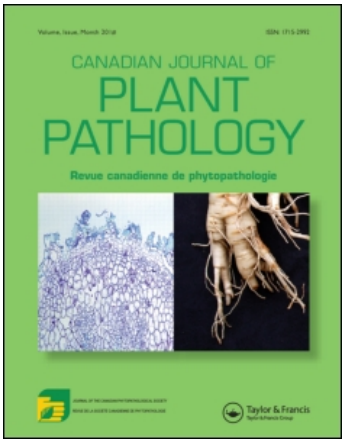

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Response of *Alnus tenuifolia* to inoculation with *Valsa melanodiscus*

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Abstract: *Valsa melanodiscus* (anamorph *Cytospora umbrina*) has been associated with cankers, dieback, and death of alder (*Alnus*) stems in western North America. To determine the ability of this fungus to induce these symptoms, the responses of thinleaf alder (*Alnus tenuifolia*) stems to inoculation with *V. melanodiscus* were studied in field locations in Alaska and on plants in a greenhouse. In the field, woody stems were wounded to expose both inner bark and sapwood and inoculated in early May 2007 or September 2007 by placing a colonized agar plug over the wound. Sunken, elongated cankers that developed on inoculated stems in the field closely resembled those attributed to natural infection of thinleaf alders by *V. melanodiscus*. In contrast, wounded control stems exhibited strong callus production and wound closure. In the greenhouse, actively growing lateral shoots were inoculated by placing a colonized agar plug over a fresh leaf scar. Inoculation in the greenhouse resulted in development of cankers, and severity of symptoms was affected by the maturity of the shoot at the point of inoculation. These results support the conclusion that *V. melanodiscus* is a cause of alder dieback in western North America.

Keywords: *Alnus tenuifolia*, canker, *Cytospora umbrina*, thinleaf alder, *Valsa melanodiscus*

Résumé: *Valsa melanodiscus* (anamorphe *Cytospora umbrina*) a été associé à la formation de chancre, au dépérissement des rameaux et à la mort des tiges de l'aulne (*Alnus*) dans l'ouest de l'Amérique du Nord. Afin de déterminer la capacité de ce champignon à produire ces symptômes, nous avons étudié, sur le terrain en Alaska et sur des plantes en serre, les réactions de l'aulne à feuilles minces (*Alnus tenuifolia*) inoculé avec *V. melanodiscus*. Sur le terrain, les tiges ligneuses ont été blessées de façon à exposer l'écorce interne et l'aubier. Ces tiges ont été inoculées au début de mai 2007 ainsi qu'en septembre 2007 en plaçant un disque d'agar colonisé sur la blessure. Les chancres enfoncés et allongés qui se sont développés sur les tiges inoculées d'aulne à feuilles minces étaient semblables à ceux découlant d'une infection naturelle causée par *V. melanodiscus*. Par contre, sur les tiges témoins blessées on observait de nombreux cals et des blessures en voie de cicatrisation. En serre, des pousses latérales vigoureuses ont été inoculées en plaçant un disque d'agar infecté sur la cicatrice fraîche d'une feuille. Cette inoculation a produit des chancres et la gravité des symptômes était influencée par la maturité de la pousse au point d'inoculation. Ces résultats confirment que *V. melanodiscus* est une cause du dépérissement des rameaux de l'aulne dans l'ouest de l'Amérique du Nord.

Mots clés: *Alnus tenuifolia*, chancre, *Cytospora umbrina*, aulne à feuilles minces, *Valsa melanodiscus*

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Introduction

Thinleaf alder (*Alnus tenuifolia* Nutt. = *Alnus incana* (L.) Moench subsp. *tenuifolia* (Nutt.) Breitung) is a shrub or small tree distributed widely in western North America from the southern Rocky Mountains to the Arctic. It is common in moist sites, including wetlands and riparian areas, where it can predominate. In addition to reproducing by seed, thinleaf alders also may vigorously produce stump sprouts and root suckers. Although not valued for timber products, the great importance of thinleaf alders lies with their role as fast-growing pioneers of disturbed or newly deposited soils and their relationships with nitrogen-fixing bacteria. For example, during the first 15–30 years of vegetation development along the Tanana River in Alaska, nitrogen fixation by *A. tenuifolia* accounted for nearly all of the nitrogen accumulated during 150 years of forest succession (Uliassi & Ruess, 2002). Stabilization and alteration of soils by thinleaf alder allows utilization by associated plant species and those which follow in the pattern of ecological succession of sites it occupies. The long-term effect of *Alnus* on ecosystem function has been evidenced by enhanced levels of nitrogen cycling and ecosystem primary productivity that accompanied the expansion of *Alnus* during the mid-Holocene (Hu *et al.*, 2001).

Deterioration of the health of thinleaf alder has occurred over large areas of western North America. Ruess *et al.* (2009) reported results of a survey for canker incidence in 2005 in three study areas of Alaska located from the Kenai Peninsula to south of Fairbanks. The percentage of living stems (ramets) with cankers at these three sites ranged from 29% to 66%, and the percentage of all stems (living or dead) with cankers ranged from 54% to 76%. Elongated, sunken cankers were attributed to *Valsa melanodiscus* G. H. Otth (anamorph *Cytospora umbrina* Bonord. (Sacc.)) based on presence of perithecia or pycnidia of this fungus. A resurvey of thinleaf alder in 2008 revealed that 74% of stems > 4 cm in diameter that had a canker three years earlier had since died. Twenty-five per cent of stems that were canker-free in 2005 were cankered in 2008. Worrall (2009) conducted an extensive survey of the condition of trees from northern New Mexico to southern Wyoming in 2004. Sixty-six per cent of 6503 stems examined exhibited dieback or were dead, with cankers on both stems and upper branches. Fruiting bodies of *V. melanodiscus* occurred on the surfaces of cankers, and the fungus was isolated from canker margins.

Despite association of *V. melanodiscus* with cankers and dieback of thinleaf alders, proof of the ability of this fungus to induce these symptoms, especially in nature, is lacking. Other recent attempts to induce canker development by inoculation of thinleaf alder stems have failed (Worrall *et al.*, 2010). Our objective, therefore, was to determine the potential for well-characterized isolates of *V. melanodiscus* to induce cankers on woody stems of thinleaf alders under field conditions. While there was no intent to study the effects of site characteristics or season of inoculation on host response, field studies included multiple locations and two seasons of inoculation. Because symptoms attributed to *V. melanodiscus* in the field include dieback of shoot tips, the responses of young shoots to inoculation were also conducted in a greenhouse experiment.

Materials and methods

Field inoculations

In early May 2007, a location in the Matanuska-Susitna Valley of south-central Alaska with naturally occurring thinleaf alders was selected for the first inoculation experiment. This was the Eagle River Boat Launch (ERBL; 61°16′50″ N, 149°24′37″ W). To provide a location for a repeat of this spring experiment, a second location, Palmer Hay Flats State Game Refuge (PHF; 61°32′40″ N, 149°15′20″ W) was also selected. The elevation at each of these two locations is less than 50 m. Vegetation at PHF is a closed tall shrub swamp with a *Betula papyrifera* Marsh.–*A. tenuifolia–Calamagrostis canadensis* (Michx). P. Beauv. community (Viereck *et al.*, 1992). Soil is poorly drained, with hummocky microrelief and depressions containing standing water at times. Other common woody species include wild roses (*Rosa acicularis* Lindl.) and highbush cranberries (*Viburnum edule* (Michx.) Raf.). Consistent with the wet conditions, *Equisetum* (L.) spp. are present. Vegetation at ERBL is a closed needleleaf forest with a *Picea glauca* (Moench) Voss–*A. tenuifolia–Hylocomium splendens* (Hedw.) Shimp. in B.S.G. community (Viereck *et al.*, 1992). The later successional stage at this site also is characterized by a thick moss layer in places, lower light, and coarse woody debris. Common understorey species include dwarf dogwood (*Cornus canadensis* L.) and twinflower (*Linnaea borealis* L.).

A second inoculation experiment was initiated in early September 2007 at ERBL, again repeated at PHF. A third location was also chosen for yet another repeat of this autumn experiment. This was Eagle River Nature Center (ERNC; 61°14′23″ N, 149°16′14″ W), also within the Matanuska-Susitna Valley and located at an elevation of approximately 150 m. Vegetation at ERNC is an open tall scrub swamp with an *A. tenuifolia–Calamogrostis canadensis* community (Viereck *et al.*, 1992). This site is more open and riparian than PHF, with small waterways resulting from beaver activity. *Betula papyrifera* saplings are frequently associated with clumps of alder, with *P.*

glauca and *Populus tremuloides* Michx. present but not dominant. *Equisetum* L. sp. is also present.

The climate in this region is subarctic. Thirty-year mean daily temperatures (and mean minimum and maximum) recorded at a weather station near the PHF study location in January are −9.9 °C (−14.2 °C, −5.7 °C) and in July are 14.5 °C (9.6 °C, 19.4 °C), and 30-year mean annual precipitation at this station is 400 mm. Respective data from a weather station near both the ERBL and ERNC study locations in January are −10.7 °C (−15.1 °C, −6.3 °C) and in July are 14.6 °C (8.7 °C, 20.3 °C), and 438 mm of precipitation (National Climatic Data Center).

Inoculum was produced using two isolates (MSC392492 and MSC392499) of *V. melanodiscus* that had been obtained from cankers on thinleaf alders in Alaska. Each isolate was grown on 20 mL of Difco PDA (Difco potato dextrose agar, Beckton, Dickinson and Company, Sparks, MD) in Petri plates 84 mm in diameter. Inoculum consisted of 1-cm diameter plugs bearing mycelium cut from the margins of six- to eight-day-old cultures.

Stems to be inoculated ranged from 11–32 mm in diameter, were apparently healthy, and were arbitrarily selected among thinleaf alder sprout clumps (genets) at intervals of at least 3 m along a transect. Ages of stems at the height of inoculation, determined (after later evaluation described below) by counting growth rings, ranged from 6–23 years. In each of the two experiments, eight or nine stems per isolate in each location were inoculated approximately 50 cm above the soil line. The site of inoculation was wiped with 95% ethanol and then a scalpel was used to make a longitudinal wound approximately 1 cm long and 0.5 cm wide that exposed outer sapwood and surrounding inner bark. Plugs were placed mycelium side toward the wound. Additionally in each experiment, four or five stems at each location were used as controls by applying a sterile agar plug onto the wound. The treatment (isolate used or control) for each stem was determined randomly. Plugs were held in place by Parafilm (Pechiney Plastic Packaging, Menasha, WI) which was removed approximately four weeks after inoculation.

Responses of stems were evaluated in early September 2007 (four months after inoculation) and early September 2008 (12 months after inoculation) for the experiments initiated in May and September 2007, respectively. All stems from the first experiment were used, but some stems from the second experiment were missing or could not be relocated due to missing labels in September 2008. A segment approximately 600 mm long and centred on the wound site was excised, brought to the laboratory, and then refrigerated at approximately 5 °C until processing. Based on visual external examination, the length of necrotic bark was measured to the nearest mm and the percentage of stem circumference that was necrotic (girdle) was estimated to the nearest 5%. If necrotic outer bark was not present surrounding wound site, canker dimensions were recorded as the length and percentage girdle of exposed sapwood.

Culture of *V. melanodiscus* from inoculated stems and wounded, non-inoculated control stems was attempted. The outer bark was surface-disinfested by blotting generously with 70% ethanol and then five chips of bark and/or underlying wood were excised from the lower margin (toward the stem base) of the necrotic tissue surrounding the inoculation point. These chips were placed approximately equidistant on PDA amended with 100 mg L^{-1} streptomycin sulphate in an 84-mm diameter plastic Petri plate. This process was repeated for five chips from the upper margin (toward the stem apex) and five chips from the lateral margin of the necrotic tissue. Chips were similarly obtained and placed on the same medium from respective locations on wounded, non-inoculated control stems. Plates were incubated for up to two weeks in the dark at ambient laboratory temperatures. During this period plates were periodically examined and the number of chips from which colonies identified as *V. melanodiscus* (based on morphological characteristics and production of red pigment) developed was recorded. Subcultures were made to PDA in an attempt to isolate *V. melanodiscus* in pure culture once from each inoculated stem.

Data for each field experiment (autumn and spring) were analyzed separately using Minitab release 14 (Minitab, Inc., State College, PA) for all analyses. Possible effects of the sites and treatments (control and each of the isolates) on canker length and percentage of stem circumference girdled were initially examined using analysis of variance. Length data were log transformed before analyses of variance. Percentage (girdle) data were converted to proportions and transformed as the arcsine of the square root before these analyses.

For wounded and inoculated stems only (i.e. omitting data from wounded control stems), isolate and site effects were further examined using analyses of variance. Because results were similar for the two isolates, data were then pooled and analyzed for effect of site using analysis of variance and Tukey's multiple comparisons to examine differences among means. Additionally, because a relatively small number of unusual data (i.e. long cankers or high percentages of girdle) strongly affected the means, possible effects of site on canker length and percentages of stem circumference girdled were analyzed using the Kruskal–Wallis test for comparison of medians.

Finally, the possible effect of chip position (lower, lateral or upper margin of canker from which chips were removed for culturing) on the number of positive chips per Petri plate was also examined. An analysis of variance

of these data for each experiment was conducted after pooling data which were similar for isolates and locations.

Greenhouse inoculations

Greenhouse inoculations were made using thinleaf alders that had been propagated by rooting cuttings made from branches collected from dormant trees at a site where dieback was apparent in Gunnison County, Colorado (38°17′37″ N, 107°13′6″ W). In trial 1, eight trees (replicates) were maintained in a greenhouse at the University of Wisconsin-Madison. The medium was a 50:50 mixture of silt loam field soil and Fafard Mix 2 (Conrad Fafard, Inc., Agawam, MA) in 10 litre pots. In summer 2009, trees were cut back to leave a 15–30 cm stub. All but one stem that subsequently developed on this stub were pruned off to allow a single stem to grow and develop side branches for approximately three months under ambient light. During this period 71 g of Osmocote Plus slow release fertilizer (15–9–12) with micronutrients (The Scotts Company LLC, Marysville, OH) was applied once to each pot and trees were watered to field capacity every other day. An additional eight trees were used in a repeat of the experiment, using the same methods.

Inoculum of the same two isolates as used for the field experiments was prepared as described above. Four-millimetre diameter plugs bearing mycelium were cut from the margins of four-day-old cultures. Each of three lateral shoots per tree was wounded by removal of the third fully expanded leaf and a mycelial plug of one of the two isolates (or a sterile agar plug for controls) was placed on the leaf scar. This wound position is subsequently referred to as the distal site of inoculation, at which the lateral shoot was relatively juvenile. Each of three additional lateral shoots per tree was wounded by removal of an eighth through twelfth fully expanded leaf and then a mycelial plug of one of the two isolates (or a sterile agar plug for controls) was placed on the leaf scar. This wound position is subsequently referred to as the proximal site of inoculation, at which the lateral shoot was more mature. Plugs were placed mycelium side toward the wounds and held in place by Parafilm which was removed one week after inoculation.

Using the same methods, a second trial (repeat) was conducted by inoculation of eight additional trees one week after inoculation of trees in the first trial. Side windows and vents of the greenhouse (which was not air conditioned) were open during the entire period of both trials. Greenhouse temperatures were not recorded, but are assumed to parallel mean ambient daily temperatures (and mean ambient minimum and maximum) recorded for Madison, WI that were 14.6 °C (6.8 °C, 22.0 °C) and 17.8 °C (8.9 °C, 26.7 °C), for the first week of trials 1 and 2, respectively, and 16.9 °C (8.4 °C, 25.6 °C) and 17.9 °C (10.1 °C, 26.3 °C), for the entire three weeks of trials 1 and 2, respectively (National Climatic Data Center).

Responses of shoots were evaluated and culture of *V. melanodiscus* was attempted three weeks after inoculation. Inoculated and control lateral shoots were excised from the main stem and brought to the laboratory where they were processed the same day. Based on visual external examination, the length of necrosis of the shoot was measured to the nearest mm. A 5-cm long segment including the furthest proximal extent of the canker (or the point of inoculation on control shoots) was then excised and surface disinfested by immersion in 95% ethanol for 10 s and then 1.05% NaClO with 2 drops Tween 80 L^{-1} for 1 min. This segment then was placed on PDA amended with 100 mg L^{-1} streptomycin sulphate. Plates were incubated for one week at 20 °C under continuous fluorescent light. Colonies were identified as *V. melanodiscus* based on morphological characteristics and production of red pigment.

Data from the greenhouse experiment also were analyzed using Minitab release 14 (Minitab, Inc., State College, PA). Possible effects of trial, treatment (control and each of the isolates), and site of inoculation (distal or proximal) on canker length were initially examined using analysis of variance. Length data were log transformed before analyses of variance. Because of significant effect of trial, subsequent analyses were conducted using data for each trial separately.

For wounded and inoculated stems only (i.e. omitting data from wounded control stems), isolate and site of inoculation effects were further examined using analyses of variance. Because results were similar for the two isolates, data were then pooled and analyzed for effect of site of inoculation using analysis of variance and Tukey's multiple comparisons to examine differences among means. Additionally, because a relatively small number of unusual data (i.e. long cankers or high percentages of girdle) strongly affected the means, possible effects of site of inoculation on canker length and percentages of stem circumference girdled were analyzed using the Kruskal–Wallis test for comparison of medians.

Results

Field inoculations

Cankers developed on inoculated stems regardless of the season of inoculation, location or isolate used, and their appearance was consistent with those attributed to natural infection of thinleaf alders by *V. melanodiscus*,

and illustrated by Worrall (2009). The initial symptoms of discolouration and necrosis of bark immediately surrounding and spreading from the wound were visible on some May-inoculated stems within six weeks. Little change in appearance of the September-inoculated stems was observed until the following growing season. In both cases, as cankers did develop, they elongated longitudinally with lateral expansion limited by callus, but external appearance of cankers varied depending on bark characteristics. On stems with smooth green outer bark, the affected bark discoloured dark green to orange-brown and became sunken. On these stems, distinct margins between healthy and diseased bark were often apparent. On stems with thicker, grey bark, canker margins sometimes were less apparent. Inner bark on cankers was discoloured orange-brown to black, and underlying sapwood was orange to grey. Bark on cankers harvested one year after inoculation was sometimes cracked longitudinally. Pycnidia with conidia consistent with those of the anamorph of *V. melanodiscus* were only occasionally observed emerging from necrotic bark of the cankers, although when present these were sometimes abundant.

Responses of the wounded control stems contrasted with the cankers of the wounded and inoculated stems. Typical cankers did not develop on wounded, non-inoculated control stems on which necrosis usually was limited to a few mm of outer bark immediately adjacent to the edges of the wound. On these stems, strong, healthy callus often surrounded or closed the wound site. As noted above, canker length and girdle were recorded for these wounded, non-inoculated control trees and were, respectively, the longitudinal and circumferential distances between margins of any necrotic outer bark tissue surrounding the wound site (even if healthy callus intervened), or the length and percentage girdle represented by exposed sapwood. Means for these data were < 12 mm and $< 5\%$, for experiment 1, and < 17 mm and $< 13\%$ for experiment 2. Analyses of variance indicated effect of treatment (wounded, non-inoculated control vs. wounded and inoculated) on canker length and percentage of stem circumference girdled in each experiment (values of $P \leq 0.014$).

Because of the negligible necrosis of the wounded, non-inoculated control stems, data from these stems were omitted from later analyses to determine effects of isolate and field location on canker dimensions. Also omitted were data from two control stems that died from unknown causes (*V. melanodiscus* was not cultured from either of these two stems). Analysis of variance did not provide strong support for effect of isolate on either canker length or girdle for either field experiment (values of $P \geq 0.12$). Therefore, for each experiment separately, means (and medians) presented in Table 1 are those obtained using data for these two isolates pooled, and these pooled data were used for the further comparisons.

The range of canker lengths for wounded and inoculated stems varied greatly, from as little as 10 mm (the length of the wound made for inoculation) to 600 mm (the entire length of the evaluated stem segment). Means for canker length also varied greatly, although mean (and median) canker lengths differed between sites only for experiment 1 (values of $P \leq 0.05$). The effect on means of the small number of relatively long cankers is seen in the differences between some mean canker lengths and the corresponding medians.

The range in percentages of stem circumference girdled for wounded and inoculated stems varied greatly, from as little as 5% to 100%. Means for girdle also varied, from 11% to 37%, although mean (and median) girdle percentages differed significantly between field locations only for experiment 2 (values of $P \leq 0.05$). The effect on means of the small number of cankers that

Table 1. Responses of *Alnus tenuifolia* (thinleaf alder) stems to wounding and inoculation with isolates of *Valsa melanodiscus* in two separate field experiments in Alaska.

Inoculation date/ harvest date	Location[a]	No. stems	Canker length[b] (mm) mean (median) range	Canker girdle[b] (%) mean (median) range	Culture[c] (stems positive/ stems tested)
Experiment 1	ERBL	16	74*A* (67*a*) 22–201	22*A* (23*a*) 5–40	16/16
May 2007/ September 2007	PHF	16	45*B* (28*b*) 20–156	20*A* (20*a*) 10–30	16/16
Experiment 2	ERBL	18	111*Z* (34*z*) 15–600	37*Y* (20*y*) 5–100	17/18
September 2007/	PHF	11	144*Z* (20*z*) 16–600	31*YZ* (10*z*) 5–100	10/11
September 2008	ERNC	16	41*Z* (24*z*) 10–152	11*Z* (10*z*) 5–20	16/16

Notes: [a]ERBL, Eagle River Boat Landing; PHF, Palmer Hay Flats State Game Refuge; ERNC, Eagle River Nature Center.
[b]For each experiment considered separately, means for canker length and canker girdle, respectively, followed by different upper case letters differ significantly at $P \leq 0.05$, and medians for canker length and girdle, respectively, followed by the different lower case letters differ significantly at $P \leq 0.05$.
[c]The pathogen was cultured from one of 24 wounded, non-inoculated control stems.

mostly or completely girdled stems is seen in the differences between some mean percentages of girdle and the corresponding medians.

Valsa melanodiscus was cultured from all 32 stems inoculated with the fungus in May 2007 (evaluated in September 2007). It was also cultured from 43 of the 45 stems inoculated with the fungus in September 2007 (evaluated in September 2008). Additionally, subculturing allowed isolation of *V. melanodiscus* in pure culture from all of the stems from which the fungus was cultured in each field experiment. Of the 24 wounded, non-inoculated control stems used, only one (wounded in September 2007 and evaluated in September 2008) yielded the fungus. Analysis of variance did not indicate strong support in either experiment for an effect of position (lower, upper, or lateral margin of the canker from which chips were removed for culturing) on the number of chips per Petri plate that yielded *V. melanodiscus*.

Greenhouse inoculations

Responses of the lateral shoots to treatments varied greatly. On control shoots, response was limited to leaf scar discolouration and sometimes callus on the surface of the leaf scar. Inoculation of lateral shoots, however, resulted in development of cankers. Analyses of variance indicated effect of treatment on canker length ($P < 0.001$). Results also indicated an effect of trial on canker length ($P = 0.032$), and therefore, subsequent analyses were conducted using data for each trial separately.

Because of the negligible response of the wounded control shoots, data from these shoots were omitted from analyses to determine effects of isolate and site of inoculation on canker dimensions. Analysis of variance did not provide strong support for effect of isolate on canker length for either greenhouse trial (values of $P \geq 0.12$). Therefore, for each trial separately, means (and medians) presented in Table 2 are those obtained using data for the two isolates pooled.

The effect of inoculation of lateral shoots differed depending on the site of inoculation (Table 2). Relatively small, elongated cankers did develop on lateral shoots inoculated at the proximal (i.e. more mature) site of inoculation, with green outer bark discoloured dark green to brown. Symptoms were more severe on lateral shoots inoculated at the distal site. Cankers, also discoloured orange to brown, were much longer and often girdled stems on which leaves wilted. The effect of site of inoculation on means (and medians) for canker length was significant for each trial (values of $P \leq 0.001$).

Valsa melanodiscus was cultured from all wounded and inoculated shoots in each trial. It was never cultured from wounded control shoots.

Table 2. Responses of *Alnus tenuifolia* (thinleaf alder) lateral shoots to wounding and inoculation with isolates of *Valsa melanodiscus* in two greenhouse trials.

Trial	Site of inoculation	Canker length[a] (mm) mean (median) range	Culture[b] (shoots positive/ shoots tested)
1	proximal	10*A* (8*a*) 4 – 44	16/16
	distal	61*B* (44*b*) 5 – 161	16/16
2	proximal	11*Y* (9*y*) 7 – 18	16/16
	distal	102*Z* (116*z*) 9 – 165	16/16

Notes: [a]For each trial considered separately, means for canker length followed by different upper case letters differ significantly at values of $P \leq 0.001$, and medians for canker length followed by the different lower case letters differ significantly at values of $P \leq 0.001$.
[b]The pathogen was not cultured from wounded, non-inoculated control shoots.

Discussion

Completion of the third of Koch's postulates, as applied by plant pathologists to identify a pathogen as responsible for a particular disease, requires that the pathogen from pure culture be inoculated on healthy plants to produce the 'same disease' (Agrios, 1997). Previously, Filip *et al*. (1992) studied the ability of one isolate identified to the genus *Cytospora* Ehrenb. (based on colony morphology and colour) to induce cankers of thinleaf alder in the field in Oregon. Use of an inoculation procedure similar to that used in our field experiments in Alaska resulted in development of disease and the inoculated fungus was reisolated. However, wounded non-inoculated stems were also infected. Further, the means for area of stem affected on wounded non-inoculated and wounded inoculated trees after two growing seasons were not statistically significantly different, apparently in part due to large variances. Another previous study involved inoculation of drought-stressed, two- to four-year-old, potted thinleaf alder seedlings with an isolate identified as *C. umbrina* (Kepley & Jacobi, 2000). Responses were evaluated just four weeks after inoculation. There was a statistically significant difference between the cankers of inoculated trees and those of the wounded non-inoculated trees in only one of three experiments. And although horizontal and vertical dimensions of the cankers were measured, the appearance of the cankers was not described. Our experiments, including field inoculations in Alaska and greenhouse inoculations of plant material from Colorado, have provided definitive evidence that *V. melanodiscus* has the ability to induce cankers typical of those with which it has been associated in the areas affected by alder dieback in western North America.

Although the relatively severe inoculation method used in this study is common to many studies of canker pathogens of woody stems and did result in disease

development, the natural infection court and means of penetration by *V. melanodiscus* is unknown. Worrall (2009) observed that cankers, even those on large stems, were never associated with visible wounds. It was also noted that cankers sometimes originated from shoot tips, leading to dieback and development of branch and stem cankers as these shoot tip cankers expanded proximally. The susceptibility of young shoots is demonstrated by the rapid development of relatively severe symptoms on lateral shoots inoculated after wounding by removal of the third fully expanded leaf in our greenhouse experiment. This suggests that such juvenile shoots might be an appropriate organ to challenge, at least initially, in future studies using conidia or ascospores as inocula to examine the potential for infection of non-wounded stems.

Lack of clear location, isolate, and season of inoculation effects on incidence and severity of disease in the field experiments do not detract from the new knowledge of the ability of *V. melanodiscus* to induce symptoms attributed to it. Rather, these results confirm the potential to cause disease regardless of these factors at least within their range during this study. Additional research is necessary to determine the manner in which these factors might influence disease development. In addition, variation in individual plant responses that could result from differences in host genotype or microsite conditions may be important to consider. The relatively large variation in canker sizes obtained in this study, however, indicates that large numbers of stems might be necessary to clearly determine such effects.

Season has often been reported to influence host susceptibility to infection and severity of disease caused by canker pathogens of trees, including *Cytospora* species. Temperature is one seasonal factor and warmer ambient (and we assume greenhouse) temperatures during the second greenhouse trial may have resulted in the greater mean canker length observed. Seasonal effects can vary among even related canker pathogens, however. More rapid growth of cankers induced by *C. leucostoma* (Pers.) Sacc. on French prune trees (*Prunus domestica* L. 'French') in California orchards reportedly occurred during warmer months and more rapid growth of cankers induced by *C. cinta* Sacc. occurred in cooler months (Bertrand & English, 1976). These results were consistent with those reported for disease development by these same two pathogens on peach (*P. persica* (L.) Batsch) in both New York and Ontario (Hildebrand, 1947; Wensley, 1964). Our experiments were not designed to examine the potential importance of seasonally related factors on disease development. However, results from Alaska do demonstrate that canker development can result from either spring or early fall inoculation. Thus, *V. melanodiscus* can overcome potential resistance responses during the growing season and also survive in the dormant host under the harsh conditions of the Alaskan winter, and initiate or resume growth and canker expansion the following spring. Further research may reveal the potential influence of seasonally related factors on both activity of *V. melanodiscus* and susceptibility or resistance of the thinleaf alders.

The large variation in sizes of cankers induced by *V. melanodiscus* in both field and greenhouse experiments, not attributable to differences in aggressiveness of the isolates, indicates that inherent host characteristics or host condition affect the severity of symptom expression. Canker disease severity, observed in response to natural infection or artificial inoculation can be largely genetically influenced. Responses of poplar hybrids to *Septoria musiva* Peck, for example, are strongly genetically controlled, differing greatly among clones, even among sibling progeny from the same crosses (Dickman, 1983). In the study by Worrall (2009), approximately one-third of stems were categorized as healthy, one-third living but with dieback, and one-third dead. The ability to successfully inoculate clones (or progeny from controlled crosses) and quantitatively evaluate responses offers the potential for examining if such observed differences have a genetic basis. Alternatively, severe canker disease development, including disease caused by *Cytospora* species, has frequently been attributed to moisture stress and other abiotic environmental factors (Bloomberg, 1962; Bertrand *et al*., 1976; Guyon *et al*., 1996; McIntyre *et al*., 1996; Kepley & Jacobi, 2000). Ruess *et al*. (2009) proposed that an unusually hot and dry summer climate throughout Alaska during the past decade may have contributed to the recent increase in incidence and severity of canker disease on thinleaf alder there. Worrall (2009) suggested that drought alone may not explain incidence or severity of Cytospora canker of thinleaf alder in the southern Rocky Mountain areas of the USA. He noted that alder canker and dieback were observed prior to recent droughts in that region and damage has continued in spite of the apparent end of drought conditions. In addition, affected trees can be rooted in banks of streams that did not dry up even during the recent droughts, and active disease is common even on trees rooted along full streams. High temperatures during or independent of drought events, however, may be an important factor (Worrall *et al*., 2010).

The results of this study contrast with the lack of canker development following thinleaf alder field inoculation trials in each of two years in Colorado (Worrall *et al*., 2010). As mentioned above, outcomes of such experiments can be affected by differences in host condition due

to abiotic environmental factors both at the time of and following inoculation, and the genotypes of both hosts and pathogens. In addition, inoculation methods might influence the ability of a pathogen to become established in the host and subsequently induce cankers. In the field inoculations described by Worrall *et al*. (2010), stems were wounded by either a needle stab or use of a cork borer to remove a 4-mm diameter bark plug. In those trials, inoculum consisted of a 4-mm diameter disk of mycelium on malt-extract agar. We made larger wounds (approximately 1 cm long by 0.5 cm wide). Our larger inoculum plug (1-cm diameter) on PDA also may have provided the *V. melanodiscus* with greater inoculum potential, which might have enabled the pathogen to overcome host resistance responses. Obviously more study is needed to fully understand how abiotic environmental factors affecting host condition, host and pathogen genotype, and processes involved in natural infection and induction of cankers influence the widespread and severe damage to thinleaf alder attributed to *V. melanodiscus*.

As noted by Ruess *et al*. (2009), the observed deterioration of this nitrogen-fixing foundation species has potentially important consequences for ecosystem function and landscape change. Given proof that *V. melanodiscus* can cause the symptoms attributed to it in these regions, we agree with the statement of Worrall (2009) that it can be considered a proximate cause of dieback and mortality of thinleaf alder stems. But the roles and relative importance of all of the factors that may be involved in the widespread dieback and mortality of this host in the southern Rocky Mountains and also in Alaska are not yet clear. Additional research, including both long-term field studies and designed experiments under controlled conditions, are necessary to clarify the possible effects of and interrelationships among the multiple biotic agents and abiotic factors that may be involved in development of this epidemic.

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