Single and Mixed Inoculations of Ponderosa Pine with Fungal Associates of *Dendroctonus* spp.

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ABSTRACT

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Small ponderosa pines (10–25 cm in diameter at breast height) were inoculated with single and paired isolates of *Leptographium terebrantis* and *Ceratocystis ips* from *Dendroctonus valens* and single and paired isolates of *C. minor* and *C. nigrocarpa* from *D. brevicomis*. The radial depth of sapwood occlusion at sites of inoculation was assayed by standing 25- to 35-cm-long stem sections in solutions of Fast Green dye and observing patterns of nonconduction after 24 hr. The average radial depths of radial sapwood occlusion resulting from inoculation with either isolate of *L. terebrantis* alone were significantly greater than depths obtained when these isolates were coinoculated with isolates of *C. ips*. The amount of sapwood occlusion varied within and among treatments and, in the case of fungi carried by *D. valens*, was reduced in trees inoculated with isolates of *L. terebrantis* and *C. ips* together. Sapwood occlusion preceded blue staining and provided an early assay of sapwood colonization and dysfunction.

Because of the possible implications of fungi carried by bark beetles in affecting hosts and host-beetle interactions in conifers, the pathogenicity of these fungi has been a subject of recent interest (7,9,12,13,20,22,24–26,29,33). The roles that fungi carried by conifer bark beetles (*Dendroctonus* spp.) might play in bark beetle biology are yet uncertain, but evidence suggests that interference with water transport in attacked trees is involved (1,3,10,13,16,18,26). Various inoculation methods and water conduction measurements have been used to assay effects of fungi on hosts and host water relations (2–4,7,13,16–21,26,27,29,34). Both injecting dye solutions into trees at point locations (30,31) and standing whole trees in vessels of dye solution (3,4,11,16,18) have proven useful in determining patterns of conduction in colonized stems.

We report here studies designed to determine if 1) interference of one fungus with another, which occurs in coinoculated seedlings (20), also occurs in trees large enough to be susceptible to attack by *Dendroctonus* spp., 2) these fungi affect the likelihood of beetle attack on trees inoculated with them, and 3) fungal effects on host water transport provide a useful assay of pathogenicity or virulence.

MATERIALS AND METHODS

The isolates used were also employed in seedling inoculation studies by Owen et al (20). Isolates 1 and 2 of *Ceratocystis minor* (Hedgc.) Hunt were from the bodies of adult *D. brevicomis* LeConte (western pine beetle) collected in Yosemite Valley, Mariposa County, California. Isolate 1 of *C. ips* (Rumb.) C. Moreau was obtained from a gallery of *D. valens* LeConte (red turpentine beetle) in ponderosa pine (*Pinus ponderosa* Laws.) collected at Boggs Mountain, Lake County, California; isolate 2 was from phloem surrounding a gallery of *D. valens* in ponderosa pine collected on the Georgetown Divide, El Dorado County, California. An isolate of *C. nigrocarpa* Davids. was obtained from an adult *D. brevicomis* collected on the Georgetown Divide. Isolate 1 of *Leptographium terebrantis* Barras & Perry was obtained from the phloem surrounding a gallery of *D. valens* in ponderosa pine on the Georgetown Divide; isolate 2 was from the body surface of an adult *D. valens* collected at Boggs Mountain. All isolates were maintained on potato-dextrose agar in a refrigerator.

Inoculum was prepared with dental cotton rolls (about 4 cm long and 1 cm in diameter) cut into 1-cm segments, soaked in 2% malt extract, and then autoclaved in screw-capped jars. Colonized agar blocks approximately 3 × 3 mm were cut from 2-wk-old cultures on potato-dextrose agar, placed in 10 cm^3 of sterile water in test tubes, and held against a vibrator for about 1 min. The resultant suspension was poured over the sterile cotton rolls, and the jars were incubated at room temperature for 2 wk or more.

For inoculation, ponderosa pine trees with a 10- to 25-cm dbh (diameter at breast height) were selected in two stands, one in the Miwok District (elevation 1,550 m) and one in the Summit District (elevation 1,600 m) of the Stanislaus National Forest (Tuolumne County, California), in the central Sierra Nevada. In each stand, 140 trees were tagged, and the dbh, height, and crown class (dominant, codominant, intermediate, or suppressed) were recorded for each. Treatments were then randomly assigned to the trees.

Each tree was inoculated at three sites with the same treatment and at a control site or, in the case of control trees, at four control sites. The treatments were inoculation with each of the seven isolates singly; inoculation with *L. terebrantis* isolate 1 plus *C. ips* isolate 1, *L. terebrantis* isolate 2 plus *C. ips* isolate 2, and *C. minor* isolate 1 plus *C. nigrocarpa*; and sterile controls. Five trees (replicates) received the same treatment in each stand in each of two seasons, to give 20 trees per treatment, except that 40 trees (10 per stand on each date) received control treatments. Another treatment with *C. nigrocarpa* was dropped from the study after it did not make adequate growth for spring inoculations.

The inoculation sites were prepared by smoothing the bark with a drawknife or a machete at four places approximately equidistant around the stem and about 1.2–1.5 m above the ground. These sites and a drill bit 1.6 cm in diameter were sprayed

with 95% ethanol, and a hole was drilled to the xylem at each site. A colonized cotton roll or an uncolonized control cotton roll was aseptically placed in the hole with ethanol-dipped forceps (two rolls were inserted for mixed inoculations), and the hole was covered immediately with a square of duct tape. A strip of brown duct tape was wrapped around the stem to cover the four wound sites. Brown tape was used to preclude the possibility that beetles might be attracted to or repelled by silver or white tape.

To evaluate possible seasonal effects (15,21), the first series of inoculations was made on 4 and 5 May 1987, when new shoots of ponderosa pine were about 10–15 cm long; the second series was made on 17 and 18 August, after the trees had completed shoot growth.

The trees in each series were cut about 8 wk after inoculation. They were felled and examined for insect activity, and their stems were cut approximately 5 cm below and 20–30 cm above the inoculation wounds. The resultant stem sections were placed upright for 20–24 hr in buckets containing an approximately 3-cm-deep solution of Fast Green dye (Fast Green FCF, Sigma Chemical Co., St. Louis, MO) in water (about 0.25 g/L). They were then removed and cross-sectioned horizontally through the inoculation points. The section diameter, depth of conducting sapwood, and depth and width of nonconducting sectors were recorded. For the August inoculations, the extent of injury above the point of inoculation was also measured, after removal of the bark to expose any necrotic lesions in the cambium and phloem.

The colonization of sapwood was confirmed either by aseptically removing 2- to 3-mm^3 chips of wood and plating them on water agar or by incubating cross sections in polyethylene bags for 1–2 wk to induce sporulation.

For statistical analysis, the mean response of the three inoculation sites per tree was used, and thus each tree contributed a single treatment observation. The statistical tests employed were Student's one-sample *t*-test for assessing individual treatment effects above the control, Student's two-sample *t*-test for comparing stands, one-way analysis of variance with the Student-Newman-Keuls procedure for overall treatment effects, and three-way analysis of variance for evaluating the simultaneous influence of treatment, season, and stand and their interactions. All statistical computations were performed with SPSS/PC+ (SPSS, Inc., Chicago, IL).

RESULTS

Beetle attack. Bark beetles attacked 21 (9%) of the 200 treated and 40 control trees. Most trees had only one or a few attacks; only three trees were heavily attacked. None of the trees exhibited fading foliage. The numbers of trees attacked in the seven general treatments were as follows: five control trees, five inoculated with *C. ips*, three with *C. minor*, three with *L. terebrantis* plus *C. ips*, three with *C. minor* plus *C. nigrocarpa*, two with *L. terebrantis*, and none with *C. nigrocarpa*. Although the trees were not debarked to identify the species, the pattern of attack and character of pitch tubes indicated that *D. valens* had attacked at least 12 of the trees. Five trees inoculated in May and 16 inoculated in August were attacked. Five trees in the Miwok District and 16 in the Summit District were attacked. The 5:16 proportion for both factors is coincidental, not coordinant.

Dye flow in inoculated trees. The dye test provided excellent differentiation of conducting and occluded areas of sapwood (Fig. 1) 5 cm above the immersed cut and adequate differentiation to approximately 10–15 cm above it. Beyond this, the dye intensity was often inadequate to show occlusion patterns. It was therefore important to keep the cuts within 3–5 cm of the points of inoculation for the best definition. Four stem sections inadvertently placed upside down failed to take up dye when they were turned right side up a day later.

A few data also were lost when the four wound sites were not in the same horizontal plane on a tree stem. If a crosscut missed any one of the points of inoculation, the datum for that point was not included in the analysis; hence, analyses sometimes involved fewer than the 80 treatment sites or the 20 trees per treatment.

The width of occluded sectors varied somewhat, but the range of variation was inadequate to provide useful data for treatment comparisons. The depth of penetration provided a better range for analysis, but this measurement gave a conservative comparison, because in many instances the fungi penetrated the entire depth of the sapwood and might have penetrated farther had there been more sapwood. The percentages of inoculations that resulted in penetration of the full sapwood depth were 43.5% for *L. terebrantis*, 12.5% for *C. minor*, 7.5% for *C. ips*, and 0% for *C. nigrocarpa*. Comparable percentages for combinations were 22.5% for *L. terebrantis* plus *C. ips* and 3.5% for *C. minor* plus *C. nigrocarpa*.

A comparison of the average depths of penetration (Table 1)

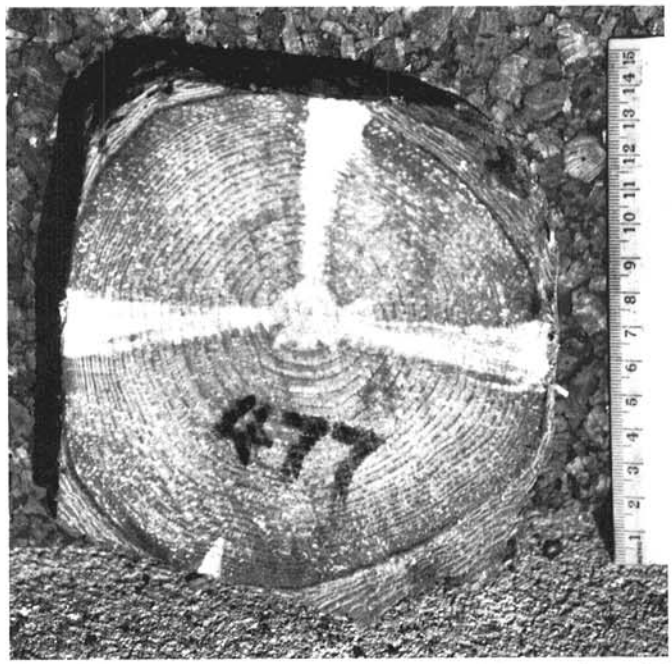

Fig. 1. Cross section showing three sites of inoculation with *Leptographium terebrantis* (isolate 2) and one control site (bottom). Colonized sectors show neither dye conduction nor stain.

TABLE 1. Depth of sapwood penetration in different inoculation treatments

Inoculum[x]	No. of trees[y]	Mean penetration[z] (cm)	Standard deviation
Leptographium terebrantis 2	18	4.80 a	1.36
L. terebrantis 2 + *Ceratocystis ips* 2	19	3.30 b	1.15
L. terebrantis 1	19	3.09 b	1.05
L. terebrantis 1 + *C. ips* 1	16	2.47 c	0.83
C. minor 1	17	2.20 c	0.75
C. ips 2	19	2.18 c	0.84
C. ips 1	18	1.97 c	0.45
C. minor 2	19	2.09 c	0.65
C. minor 1 + *C. nigrocarpa*	20	1.86 c	0.38
C. nigrocarpa	19	1.48	0.29
Control	184	1.43	0.18

[x] Numerals refer to isolates.

[y] In each treatment, 20 trees were inoculated, but inadequate dye uptake or uncentered crosscuts precluded the use of some trees. Each treated tree had one control wound.

[z] Values followed by different letters are significantly different ($P < 0.05$) by the Student-Newman-Keuls procedure for ad hoc comparison of means. Values for inoculation with *C. nigrocarpa* alone and for the control were not included in the comparisons because they represent only the depth of the wound from the drill bit.

shows that penetration in most inoculations (except inoculation with *C. nigrocarpa*) was significantly different from that in the control treatments. "Penetration" in the controls resulted from penetration by the drill bit (Fig. 2).

The average depths of penetration (Table 1) also show significant differences between inoculation with *L. terebrantis* isolate 2 and inoculation with that isolate plus *C. ips* isolate 2; similarly, there were significant differences between inoculation with *L. terebrantis* isolate 1 and inoculation with that isolate plus *C. ips* isolate 1. The first three of these treatments were also significantly different from all other treatments. The differences between treatments with the remaining fungi and fungus combinations were not statistically significant.

An analysis of the vertical length of necrotic lesions (Fig. 3) above the points of inoculation (in the August inoculations only) was performed separately for each stand (Table 2). The mean vertical length of necrotic lesions was significantly greater in all treatments except inoculation with *C. nigrocarpa* than in the control, in both stands. It was significantly greater in inoculation with *L. terebrantis* isolate 2 than in all other treatments in the Summit stand, and it was significantly greater in inoculation with *L. terebrantis* isolate 2 or isolate 1 than in all other treatments in the Miwok stand ($P < 0.0001$). There was a strong overall correlation ($r = 0.60$; $P < 0.001$) between the vertical length of lesions and the depth of sapwood penetration (based on 118 trees from the two stands). The correlations in the two stands were

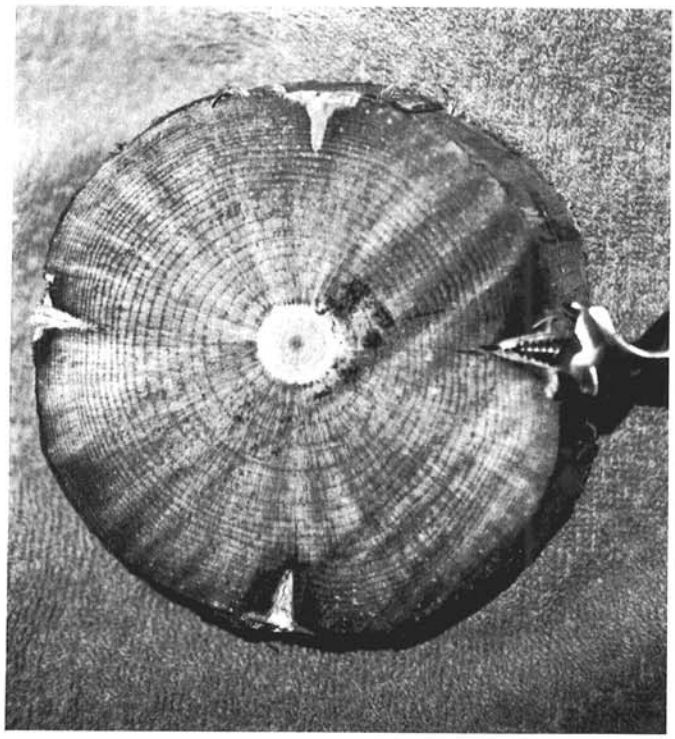

Fig. 2. Stem section showing four uninoculated controls and the relationship of the drill bit (1.6 cm) to the pattern of occlusion in the controls.

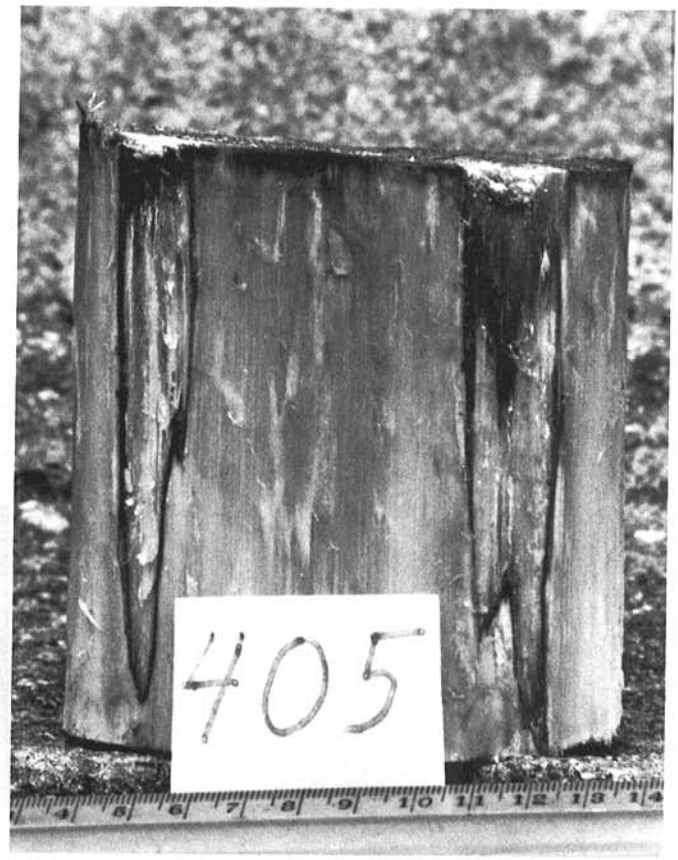

Fig. 3. Pine stem section cut at points of inoculation and debarked, showing necrotic lesions above mixed inoculations of *Leptographium terebrantis* (isolate 1) plus *Ceratocystis ips* (isolate 1). The stem section is inverted in the photograph.

TABLE 2. Vertical length of lesions in different inoculation treatments, by stand

Inoculum[x]	Summit District			Miwok District		
	No. of trees[y]	Mean length[z] (cm)	Standard deviation	No. of trees[y]	Mean length[z] (cm)	Standard deviation
Leptographium terebrantis 2	5	16.12 a	8.59	4	5.50 a	2.67
L. terebrantis 2 + *Ceratocystis ips* 2	5	5.06 b	3.35	5	3.40 b	1.47
L. terebrantis 1	5	9.76 b	3.34	4	5.70 a	2.77
L. terebrantis 1 + *C. ips* 1	5	5.54 b	0.75	4	2.88 b	0.69
C. minor 1	5	3.62 b	2.70	5	2.38 b	0.44
C. ips 2	5	3.48 b	1.45	4	1.18 b	0.75
C. ips 1	5	4.10 b	1.63	5	2.82 b	1.58
C. minor 1 + *C. nigrocarpa*	5	2.50 b	0.93	5	2.10 b	0.35
C. nigrocarpa	4	0.23	0.29	5	0.02	0.04
Control	48	0.29	1.45	46	0.23	0.59

[x]Numerals refer to isolates.

[y]In each stand, five trees were inoculated for each treatment, but uncentered crosscuts precluded the use of some trees. Each treated tree had one control wound.

[z]Values in the same column followed by different letters are significantly different ($P < 0.05$) by the Student-Newman-Keuls procedure for ad hoc comparison of means. Values for inoculation with *C. nigrocarpa* alone and for the control were not included in the comparisons because they represent only the extent of the wound from the drill bit.

similar: for the Summit stand, $r = 0.59$; for the Miwok stand, $r = 0.64$. Vertical lesions in trees inoculated with *C. nigrocarpa* were not significantly different from those of the controls in either stand.

In a separate analysis, we found that the average vertical length of lesions (caused by all fungi) in Summit trees (4.77 cm) was significantly different ($P = 0.001$) from that in Miwok trees (2.36 cm). A sample of ages and of cumulative radial growth over the last 10 yr for 25 trees from each stand gave an average age of 42 yr (a range of 21–57 yr) and growth of 0.71 cm (a range of 0.25–2.20 cm) for Summit trees and 18 yr (a range of 11–30 yr) and 4.48 cm (a range of 2.25–7.40 cm) for Miwok trees. These means were significantly different ($P < 0.001$). Because vertical lesions were measured only in the summer series, seasonal effects could not be evaluated.

Reisolations. Reisolations were attempted from seven to 14 inoculation sites for each fungus, combination of fungi, and control. *L. terebrantis, C. minor*, and *C. ips* were isolated readily from most inoculation sites (13 of 14, 13 of 13, and nine of 13 sites, respectively) and at all depths within occluded xylem. *C. nigrocarpa* was obtained from only three of eight inoculation sites. From mixed inoculations, the faster-growing fungus was most easily isolated; i.e., *L. terebrantis* (seven of eight sites) was isolated more easily than *C. ips* (five of eight sites), and *C. minor* (five of seven sites) more easily than *C. nigrocarpa* (two of seven sites). Of 60 chips from 10 control sites, 44 were negative, and 16 yielded miscellaneous fungi. Because equipment was not sterilized during harvest and reisolations could not be made until the materials were taken to the laboratory (4–6 days after harvest), it is likely that some controls were contaminated at harvest. Contamination did not materially affect the results, because only two of the 380 control inoculations produced occlusion beyond the depth of penetration of the drill bit. Incubation of disks provided a simpler measure of colonization than did isolation.

Incubation of disks provided evidence of the relationship of fungal invasion and the blockage of water transport. It also provided a convenient method to confirm inoculations. Freshly cut disks showed almost no discoloration in occluded areas (Fig. 1), but incubation for 1–2 wk under humid conditions resulted in the development of dark mycelium, generally over the surface of the occluded area. Examination with a dissecting microscope often permitted the identification of *C. ips, C. minor*, or *L. terebrantis*, because each produced characteristic conidiophores and conidia within 7–14 days. In addition, *C. ips* and *C. minor* produced perithecia on some disks. *C. nigrocarpa* produced no identifiable structures and was identified only by isolation.

Early examination, after about 1 wk of incubation, was necessary to observe the general correspondence of mycelium with sapwood occlusion. As the incubation time increased, mycelium often spread across the disk, although the heaviest development remained in the occluded area. Often the central area of an occluded sector was heavily impregnated with resin, and less mycelium was produced and less blue staining occurred there than at the periphery of the sector.

DISCUSSION

Because bark beetle attack was relatively rare (occurring in 21 of 240 trees) and distributed rather evenly among treatments (including controls), neither the attraction of beetles nor the predisposition of trees to attack by beetles was indicated. Apparently, colonization by these fungi to the extent produced in these tests did not affect beetle activity more than the control wounding did.

Standing short stem sections in dye solution for 24 hr was simpler than using whole trees, provided excellent delineation of conducting and occluded xylem in cross sections at the point of inoculation, and provided a convenient assay to compare the effects of different fungi or combinations of fungi. The successful use of stem sections required that the cut surface immersed in the dye be no more than 3–5 cm from the sites of inoculation, for the best definition of occlusion. Preliminary studies (unpublished) showed that dye uptake at 6 hr was inadequate to provide good staining at 5 cm and that uptake at 48 hr was not sufficiently greater to justify the added time.

The comparison of fungal species and isolates by this method indicates that, under the conditions of this study, the two isolates of *L. terebrantis* were much more virulent than the isolates of *C. ips* and *C. minor*. This corresponds to results obtained with seedling inoculations (20). In addition, one isolate of *L. terebrantis* was more virulent than the other. Data on vertical lesion extension support these comparisons. These data suggest that an evaluation of variation within fungal species may be important in comparing the activities of the fungal associates of various beetles.

Other studies have shown that *C. minor* is inhibited when combined with other beetle-associated fungi (6). Whitney and Cobb (32) stated that *C. minor* and other beetle-associated nonstaining fungi appear to be mutually exclusive. Our studies support the conclusion drawn from seedling studies (20) that *C. ips* in some way interferes with *L. terebrantis*. The average sapwood penetration by either isolate of *L. terebrantis* alone was greater than that of the isolate combined with *C. ips*. Data on vertical lesion extension also indicate significant differences between inoculation with *L. terebrantis* and other treatments. Whereas penetration by the *C. minor* isolates was greater alone than with *C. nigrocarpa*, the differences were statistically not quite significant. Failure to confirm seedling results with *C. minor* and *C. nigrocarpa* may rest with the fact that *C. minor* is a "weaker" pathogen and may therefore be more affected by host variability. This is suggested by the lesser average penetration (2.15 cm) by *C. minor* than by *L. terebrantis* (3.95 cm) and by the lower frequency with which *C. minor* penetrated the entire sapwood depth (12.5%), compared with *L. terebrantis* (43.5%).

Patterns of dye flow indicate that water moves readily through the entire depth of uncolonized sapwood. When outer rings were occluded, the dye solution moved through the deeper rings. Thus, unless the entire depth of the sapwood was colonized, the flow of the dye solution was not completely obstructed. This suggests that the capacity to penetrate and occlude the entire depth of the sapwood provides a measure of virulence and of interference with host water transport.

With the same fungus isolate, trees varied in their capacity to resist deep penetration of the sapwood. Even the most virulent isolate, *L. terebrantis* isolate 2, penetrated the entire depth of the sapwood in only 54% of inoculations. Generally, results were similar within trees but varied markedly among trees. If, as evidence increasingly indicates, sapwood occlusion associated with fungus colonization plays a role in the death of trees attacked by bark beetles, then the susceptibility of a tree to deep sapwood penetration by various fungi may be one measure of liability to death from beetle attack.

Christiansen (8) presented evidence that the development of stain in *Picea abies* attacked by *Ips typographus* did not proceed until the number of attacks per square meter reached at least 120. Inoculation of trees with a 20-cm dbh with *C. polonica* did not result in death until at least 75 inoculations were made in a 40- to 60-cm-wide strip around a tree at about breast height. The depth of sapwood penetration was not reported. Kaufmann and Stevens (14) found that ponderosa pines surviving attacks by *D. ponderosae* had only partial sapwood staining.

It is likely that both the depth of sapwood penetration and the number of infections per unit of area of the stem are important. Neither numerous shallow infections nor sparse deep infections would reduce water movement sufficiently to kill a tree. When trees were sectioned 8 wk after inoculation, occluded sapwood showed no stain or discoloration. The extent of fungal colonization and vascular occlusion could be observed only by assaying dye flow patterns. Bridges et al (5) and Rudinsky (28) reported tree killing by bark beetles in the absence of blue stain. Our results suggest that sapwood can be colonized and occluded before stain is evident and that occlusion, rather than fungal staining, may provide a better measure of fungal colonization during the early weeks of bark beetle attack.

Several workers have evaluated lengths of lesions above or below points of inoculation as assays of virulence or host response (9,13,16,17,21–23,33,34). Since the radial penetration of sapwood or phloem is limited by depth, whereas vertical extension is essentially unrestricted, the vertical length of lesions may be a statistically more sensitive measure of fungus activity, but further study of the relationship of lesion length to depth of occlusion is needed.

Other studies (3,7,16,23) have demonstrated that low-vigor trees tend to be less resistant to fungal invasion. In general, trees in the Miwok District were young and fast-growing, whereas trees in the Summit District were older and slower-growing. These differences did not result in statistically significant differences in the depth of penetration of trees by the fungi; however, between-tree variability may have masked such differences. The overall average vertical length of lesions was significantly greater in Summit trees than in Miwok trees.

Seasonal differences in host reaction, which have been reported (15,21), were not detected by our study design, but further investigation is needed.

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