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Cellular Response of Loblolly Pine to Wound Inoculation with Bark Beetle-Associated Fungi and Chitosan

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Cellular Response of Loblolly Pine to Wound Inoculation with Bark Beetle-Associated Fungi and Chitosan

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Abstract

We inoculated loblolly pines with bark beetle-associated fungi and a fungal cell wall component, chitosan, known to induce responses in some pines and many other plants. Trees in Florida were inoculated with *Leptographium procerum, L. terebrantis, Ophiostoma minus,* or chitosan. Trees in Louisiana were inoculated with *O. minus, Entomocorticium* sp. A, or *Ceratocystiopsis ranaculosus*. In both Florida and Louisiana, mechanical wounds served as controls. Treatment responses were sampled after 3 weeks, and all produced uniform responses across trees. Inoculations with *E.* sp. A and *C. ranaculosus* appeared similar to controls. Inoculations with *L. procerum* produced slightly higher levels of host damage. Loblolly pine responded similarly to chitosan and pathogenic bark beetle-associated fungi (*O. minus* and *L. terebrantis*), producing high levels of phenolic compounds and cell hydrolysis in the callus. In addition, callus inoculated with *O. minus* appeared extremely disrupted and "stringy." Chitosan inoculations resulted in no hydrolysis, but produced extremely high levels of phenolics deposition, as well as noticeable periderm formation. Our results reveal possible morphological mechanisms for pine secondary response to these fungi and suggest that chitosan may have potential as a stable material for testing variability in this response.

Keywords: *Leptographium, Ophiostoma*, resin, resistance, southern pine beetle.

Introduction

An important component of defense in conifer interaction with pathogenic fungi and their insect vectors lies in an ability to recognize early signals that an invader is present. Chitosan is a small fragment, found in the cell walls of fungi and insects, that has been proposed as a general recognition signal in a variety of plant-pathogen systems (Constabel and others 1995, Ryan 1988, among others). Although the most mobile and active signaling molecules are relatively short chitosan oligomers [degree of polymerization (dp) = 6 to 11], researchers have elicited defensive responses in pine-bark beetle systems using longer chain (and readily available) chitosan preparations. Popp and others (1997) used acid deacetylated crab shell chitosan (Sigma-Aldrich, St. Louis, MO USA) (the dp of which was not noted, but was likely much higher than 6 to 11). They observed increased ethylene production and pronounced increases in precursors of lignin deposition in loblolly pine (*Pinus taeda* L.) tissue culture cells. Miller and others (1986) used a similarly treated and likely similar chain-size preparation of shrimp shell chitosan (Sigma) (as described in Hadwiger and Beckman 1980) to elicit defensive responses in lodgepole pine (*P. contorta* Dougl. ex Loud.). They noted elevated total monoterpene concentrations, similar to those associated with inoculation of trees with the bark beetle-associated fungus *Ophiostoma clavigerum* (Robinson-Jeffrey and Davidson). In a histological study, three western pine species reacted similarly to chitosan and a bark beetle-associated fungus (Lieutier and Berryman 1988). Both fungus and chitosan (of unspecified, though likely high, dp) inoculations were associated with resin soaking in parenchyma cells in the phloem, and in the rays of the phloem and sapwood (Lieutier and Berryman 1988).

In this experiment, we shared with earlier investigators a desire for "a more reliable material with more stable properties than a fungal culture" to test the "intensity of the secondary defensive response . . . as a test of tree vigor or its ability to resist bark beetle attacks" (Lieutier and Berryman 1988). We also sought to thoroughly examine the histological response of loblolly pine to chitosan and the bluestain fungus primarily associated with the southern pine beetle, *O. minus* [(Hedgcock) H. and P. Sydow].

Materials and Methods

In July and September of 1996, we conducted inoculation experiments in loblolly pine stands in Florida and Louisiana, respectively. We selected 10 codominant loblolly pines (approximately 40 years old in Florida, 15 years old in Louisiana) at stands in Florida (Ruth Springs Tract, Water Management District, Lafayette County, FL) and Louisiana (Johnson Tract, Kisatchie National Forest, Rapides Parish, LA). We used a 1-cm diameter cork borer to remove a disk of the outer bark and phloem from 10 loblolly pine trees at each of the 2 sites. We wounded each tree five times and placed one of five treatments in each of the wounds. At the Florida site, trees were inoculated with a 0.5-cm disk of malt extract agar colonized by either *Leptographium procerum* [(Kend.) Wingf., *L. terebrantis* Barras and Perry] [both isolated from *Hylobius pales* (Hbst.) root weevils collected in Louisiana], or *O. minus* (isolated from southern pine

beetle adults collected in Louisiana). In Louisiana, trees were inoculated with a 0.5-cm disk of malt extract agar colonized by either *O. minus*, *Entomocorticium* sp. A, or *Ceratocystiopsis ranaculosus* (all isolated from southern pine beetle adults collected in Louisiana). We also applied chitosan and mechanical wound treatments to each tree at both sites. In all cases each tree received every treatment at that site. Treatments were applied at breast height on each tree, equally spaced around the tree circumference. Chitosan-inoculated trees were inoculated with 0.1 mL of a 1-percent solution (in acetic acid) of chitosan (dp = 3,100, Vanson, Redmond, WA). Mechanical wounds (uninoculated cork borer wounds) served as controls at both sites. In all treatments we taped the bark/phloem disk back in place over the wound.

We sampled all treatments at both sites 3 weeks after inoculation. At each wound/inoculation site we used a chisel to carefully remove a small (approximately 1- by 1-cm) subsample of phloem from the upper and lower edges of each circular wound. We then immediately placed each of these subsamples into formalin:acetic acid:ethyl alcohol. After 2 weeks, we rinsed the samples and stored them in 70-percent ethyl alcohol. We cut the fixed tissues into several thin sections (7 to 10 µm) per sample. We mounted the sections and stained them with hematoxylin and eosin, Papanicolaou's stain or periodic acid-schift (Horbin and Bancroft 1998).

We did not measure resinous lesion formation in trees inoculated with *O. minus*, *E.* sp. A., or *C. ranaculosus* because this aspect of the virulence of southern pine beetle fungi has been well studied (Cook and Hain 1985, 1986, 1987a, 1987b, 1988; Nevill and others 1995; Paine and Stephen 1987a, 1987b; Paine and others 1988; Ross and others 1992). In Florida we measured the extent of resinosis [extent of tree response is correlated with the extent of fungal growth (Paine and others 1997, Raffa and Berryman 1983)] resulting from each treatment. We did this because currently there are few data (but see Nevill and others 1995) on the virulence (as measured by extent of resinous lesion formation) of *L. procerum* and *L. terebrantis* in mature loblolly pine. We used drawknives to carefully shave the outer bark and expose the phloem surrounding the wound. Using transparency film we traced the full extent of resinous lesion associated with each treatment. At the laboratory we used a digital planimeter to trace the areas on the transparency film, and recorded the resinous area per wound.

We selected 36 subsamples from the Florida trees (9 trees per treatment) and mounted 9 thin sections from each of the trees. We selected 16 subsamples from the Louisiana trees (4 trees per treatment) and made 9 thin sections from each tree. For each of the sections, we observed, recorded, and (where appropriate) quantified the following parameters: number of starch grains per cell, number of ergastic cells per section, number of cortical rows per section, percent of section margin reflecting polarized light, percent clumping of callus observed per section, percent hydrolysis of callus observed per section, and number of cells per ray. For the Florida samples we selected one set of nine sections per treatment [to give an n = 9 per treatment for analysis of variance (ANOVA) and mean separations]. For analytical purposes we averaged the values of these parameters for each of the four trees per treatment in Louisiana. The ANOVAs and mean separations were, thus, calculated from the mean values for four trees per treatment in Louisiana (n = 4 per treatment). All data were analyzed using ANOVA, and mean separations calculated using Fisher's Protected LSD, within Statview (SAS Institute 1998).

Results

Variation by Treatment

All treatments produced qualitatively uniform responses across trees, but tree reaction varied substantially by treatment (table 1). Control (mechanically wounded only) tissues appeared very normal and (but for the actual wound damage) healthy. Inoculations with *E.* sp. A and *C. ranaculosus* appeared similar to controls. Callus cells in those treatments appeared normal, exhibited little or no hydrolysis, and there was little sign of the accumulation of phenolic compounds (figs. 1a, 1b, 2a, and 2c). Inoculations with *L. procerum* produced slightly higher levels of host damage. Cells challenged with this fungus exhibited mild hydrolysis and larger areas of apparent phenolic deposition than mechanical wound, *E.* sp. A, or *C. ranaculosus* treatments. However, the number of cells per ray within this treatment fell within the normal range (fig. 2b). Loblolly pine responded similarly to chitosan and pathogenic bark beetle-associated fungi (*O. minus* and *L. terebrantis*). Tissues challenged with these fungal pathogens produced high levels of phenolic compounds and cell hydrolysis in the callus (figs. 1c and 3a). In addition, callus inoculated with *O. minus* appeared extremely disrupted and "stringy" (fig. 1d). Chitosan inoculations resulted in no hydrolysis but were characterized by extremely large areas of phenolic deposition, as well as noticeable periderm formation (figs. 2d, 3b, 3c, and 3d).

Table 1—Comparison of loblolly pine cellular reactions to wound-inoculation treatments

	Observed reactions	
Treatment	Callus	Parent tissues/rays/ fibers
Mechanical wound	Normal	Normal to low phenolic accumulation
Entomocortocium sp. A.	Normal, mild hydrolysis	Normal to low phenolic accumulation
Ceratocystiopis ranaculosus	Normal, no hydrolysis	Low to moderate phenolic accumulation
Leptographium procerum	Mild hydrolysis	Increased phenolics
L. terebrantis	Severe hydrolysis	High phenolic accumulation
Ophiostoma minus	Severe hydrolysis,'stringy'	Moderate to high phenolic accumulation
Chitosan	No hydrolysis, periderm formation	Very high phenolic accumulation, periderm formation

Starch grains—The number of starch grains per cell (indicating overall cell health, with more grains equaling greater cell vigor) was significantly affected by treatment at both the Florida ($F_{4,40} = 37.69$, $p < 0.0001$) and Louisiana ($F_{4,15} = 8.43$, $p < 0.0009$, degrees of freedom = 4) sites. Mean number of starch grains was higher in mechanically wounded treatments than in all other treatments, except *E.* sp. A. in Louisiana, at both sites (table 2). At the Florida site none of the other treatments differed significantly from one another in this parameter. In Louisiana the number of starch grains in cells responding to *O. minus* vs. chitosan did not differ significantly ($p < 0.07$), although *O. minus*-inoculated tissues contained significantly fewer starch grains than all other treatments.

Ergastic cells—The number of ergastic cells (those exhibiting signs of nonliving materials and/or inclusions) was not significantly affected by treatment at the Florida site ($F_{4,40} = 0.37$, $p < 0.83$). Chitosan-treated tissues in the Florida trees contained numbers of ergastic cells equivalent to the other treatments. In Louisiana, treatment significantly affected the number of ergastic cells ($F_{4,15} = 8.02$, $p < 0.001$). Tissues inoculated with chitosan and *O. minus*-inoculated tissues had significantly higher numbers of ergastic cells than did any other treatment (table 2).

Cortical rows—The number of cortical rows of callus (indicative of growth rate during the experiment) did not differ significantly by treatment at either the Florida ($F_{4,40} = 0.59$, $p < 0.68$) or Louisiana ($F_{4,15} = 2.68$, $p < 0.07$) sites.

Percent margin polarized—The percentage of sections with margins reflecting polarized light (indicative of the presence of the cellulose-lignin complex) was significantly affected by treatment at the Louisiana site ($F_{4,15} = 3.20$, $p < 0.05$). There was a significantly higher degree of polarization along the margin in inoculated tissues than in the chitosan treatment (table 2). Although data could not be analyzed for the Florida site as they were for Louisiana, similar trends appeared to occur there.

Percent clumping—At the Louisiana site the percentage of sections showing clumping (abnormal cytoplasm, indicative of probable cell death) was significantly affected by treatment ($F_{4,15} = 6.12$, $p < 0.04$). Clumping occurred significantly more frequently in *O. minus*- and *E.* sp. A-inoculated tissues than in the mechanical or chitosan treatments at the Louisiana site (table 2). Although data could not be analyzed for the Florida site as they were for the Louisiana site, similar trends appeared to occur there.

Percent hydrolysis—The percentage of sections showing cell hydrolysis (disruption and rupture of cells and, therefore, cell death) was significantly affected by treatment at the Louisiana site ($F_{4,15} = 41.80$, $p < 0.0001$). Cell hydrolysis was more prevalent in the *L. terebrantis* treatment in Florida than in any other treatment, and significantly more prevalent in *O. minus*-inoculated tissues than in any other treatment at the Louisiana site.

Ray cells—The number of cells per ray did not differ significantly by treatment at the Louisiana site ($F_{4,15} = 3.00$, $p < 0.06$). Although data could not be analyzed for the Florida site as they were for Louisiana, similar trends appeared to occur there.

Resinous lesions—Lesion size was significantly affected by treatment ($F_{4,94} = 37.07$, $p < 0.0001$). Lesions formed in

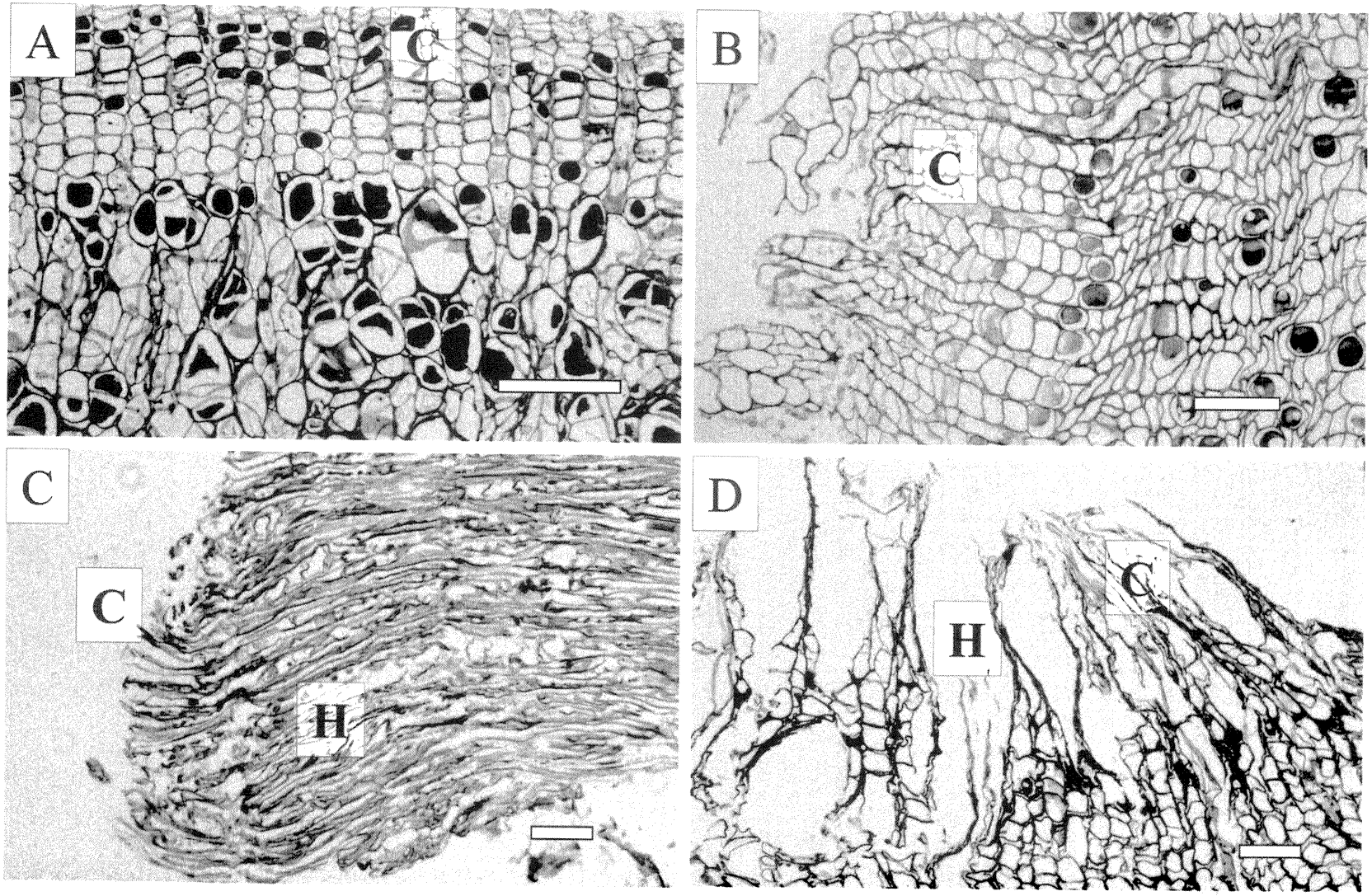

Figure 1—Variation in appearance of loblolly pine callus border in response to wounding and inoculation treatments. (A) Normal growth and morphology (mechanical wound); (B) Normal growth with hypertrophy of border callus (*Ceratocystiopsis ranaculosus*); (C) Hydrolysis (h) with intercellular spaces between rows of abnormal callus (*Leptographium terebrantis*); (D) Extensive hydrolysis (h), complete destruction of cellular integrity (*Ophiostoma minus*).

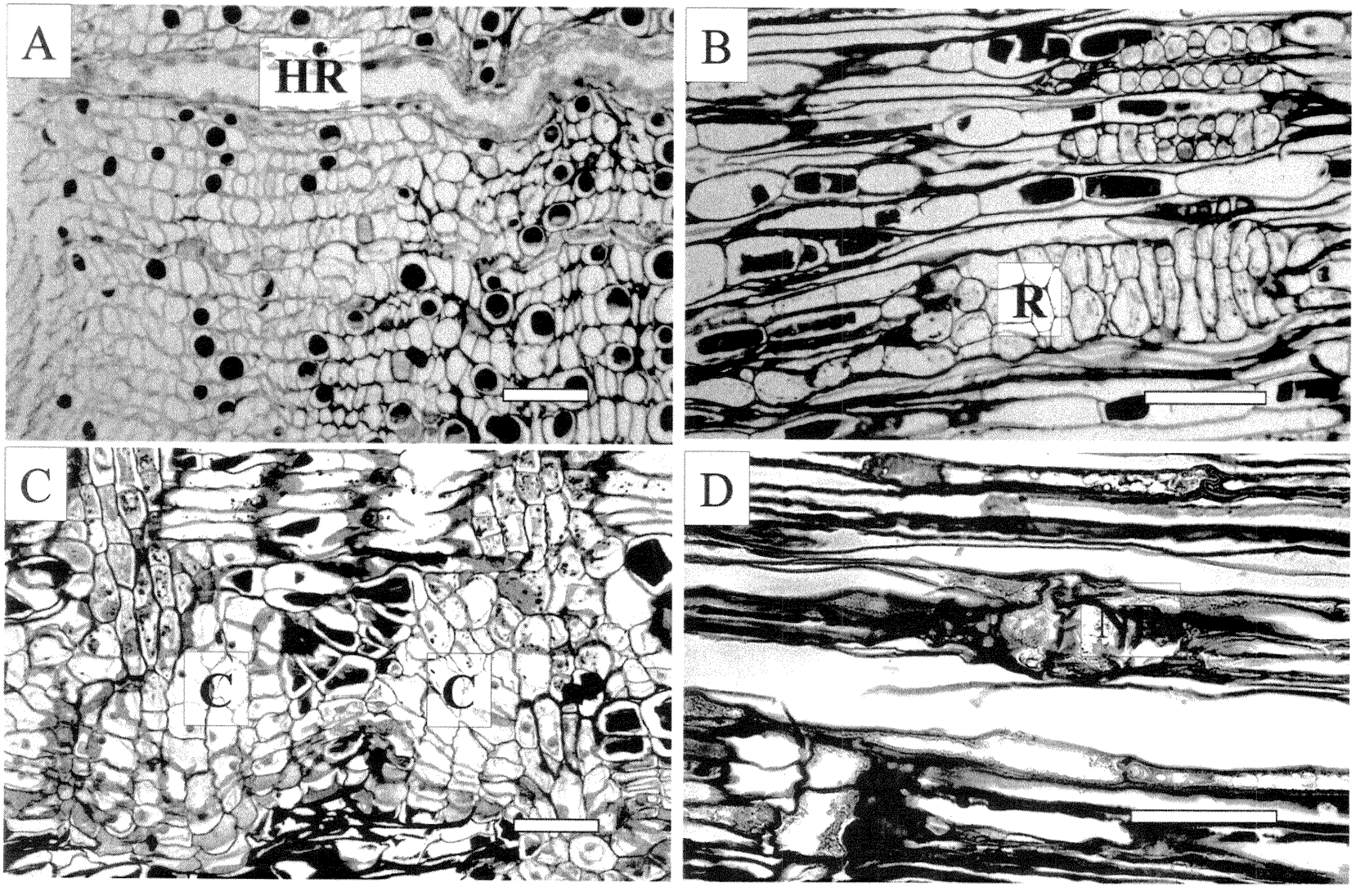

Figure 2—Variation in appearance of ray cells in respone to wounding and inoculation treatments. (A) Normal ray extending from parent tissue to edge of callus (*Entomocorticium* sp. A); (B) Range in number of ray cells that are in normal rays (*Leptographium procerum*); (C) Rays as source of normal callus cells (cc) (mechanical wound); (D) Dead ray cells with phenolic compounds (p) (chitosan).

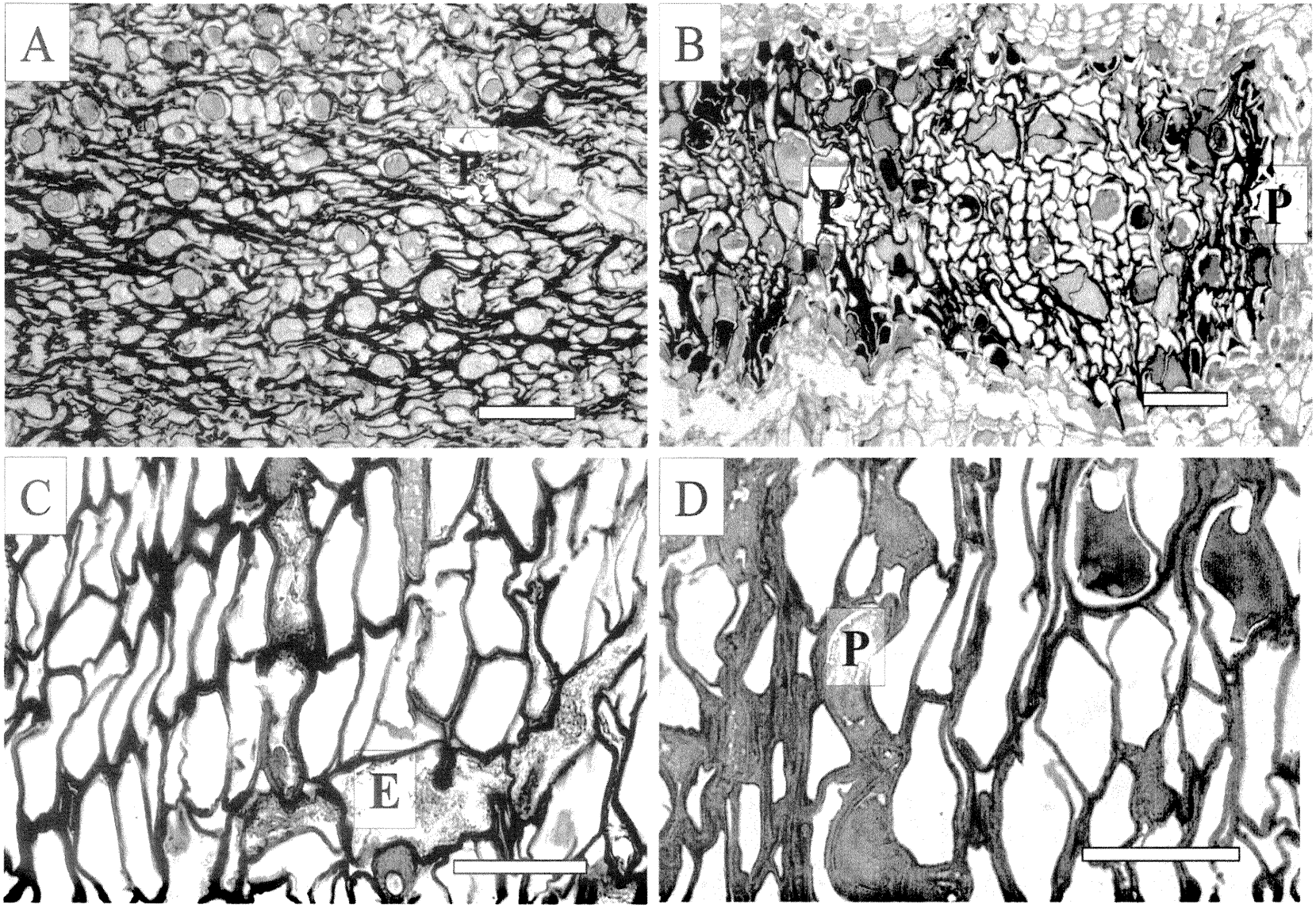

Figure 3—Variation in deposition of phenolic compounds in response to wounding and inoculation treatments. (A) Small area of phenolic (p) deposition in callus and parent cells (*Leptographium terebrantis*); (B) Large area of phenolic (p) deposition, with dying cells that are isolated from callus (chitosan); (C) Early stage of phenolic deposition (chitosan); (D) Late stage of phenolic deposition (chitosan).

Table 2—Significant parameters of the response of loblolly pine to wounding and/or inoculation at sites in Florida and Louisiana[a]

Florida

Treatment	Starch grains	Lesion
Mechanical	7.7 (0.8) a	1.5 (0.2) ab
Leptographium procerum	2.6 (0.6) b	3.8 (0.5) b
L. terebrantis	3.2 (0.6) b	8.7 (0.8) c
Ophiostoma minus	3.2 (0.5) b	9.0 (0.9) c
Chitosan	3.8 (0.7) b	1.5 (0.3) a

Louisiana

Treatment	Starch grains	Ergastic cells	Polarized	Clumping
			-------*Percent*-------	
Mechanical	9.5 (0.5) a	3.0 (0.7) a	49.3 (14.2) ab	0 (0) a
Entomocortocium sp. A	7.5 (0.6) ab	5.2 (0.2) a	79.3 (12.5) a	51.8 (11.6) b
Ceratocystiopsis ranaculosus	6.3 (0.3) b	4.4 (0.8) a	72.5 (7.5) a	30.0 (12.9) ab
O. minus	4.2 (1.0) c	13.3 (0.2) b	66.8 (6.7) a	56.0 (17.1) b
Chitosan	6.1 (0.5) bc	15.1 (4.2) b	33.3 (9.4) b	0 (0) a

[a] Means (standard error) followed by same letter within a column, are not significantly different at $p < 0.05$ as determined by Fisher's Protected LSD.

response to *L. terebrantis* and *O. minus* were significantly larger ($p < 0.0001$) than those formed in response to any other treatment (table 2). However, these two fungi did not differ from one another in their ability to grow and cause resinosis within trees ($p < 0.69$). The chitosan treatment resulted in limited resinous lesions that did not differ significantly ($p < 0.98$) in extent from those seen in response to mechanical wounding only. Although we did not quantify the lesions from inoculations in Louisiana, they appeared consistent with previously reported observations. Inoculations with *O. minus* produced large, resinous lesions. Inoculations with *C. ranaculosus* and *E.* sp. A. produced small lesions, similar in size and resinosis to those from mechanical wounding alone.

Discussion

This study provides a way to express and describe one component of pine-pathogen interactions in quantitative, cellular terms. The number of starch grains per cell was higher in the mechanically wounded treatments, indicating greater cell health in the control treatment. The higher levels of ergastic cells in the *O. minus* and chitosan treatments were indicative of the strong defensive response elicited by these treatments. A similar reaction was noted to *L. terebrantis* as well. That the number of cortical rows formed did not differ among treatments indicates that the trees continued to grow during the experiment, regardless of treatment. The higher levels of light polarization within tissues inoculated with fungi (relative to wound only and chitosan) indicated deposition of lignin and cellulose in response to these infectious organisms. The high levels of clumped cytoplasm within fungal inoculated tissues (vs. wounded only and chitosan-inoculated tissues) is also indicative of a defensive response on the part of the host. Extensive hydrolysis occurred only in response to *O. minus* and *L. terebrantis*, the most virulent pathogens we tested.

Our results are similar to those reported earlier (Lieutier and Berryman 1988) in that chitosan elicited similar reactions in host phloem tissue to inoculation with pathogenic fungi. A noteworthy difference, however, is the relative lack of disruption seen in response to the chitosan treatment vs. the substantial disruption caused by *O. minus* and *L. terebrantis*. This study furthers the cases that the secondary defensive

response of conifers to bark beetle-associated fungi originates in the parenchyma cells (Berryman 1969, Franceschi and others 1998, Nagy and others 2000, Reid and others 1967). We noted, as have others (Lieutier and Berryman 1988, Wong and Berryman 1977), the accumulation of phenolic compounds [which have been implicated as allelochemicals against bark beetle-associated fungi (Klepzig and others 1996a, among others)] in these affected tissues. The abundance of these phenol-accumulating cells in the zone of inoculation may form a "potent protective structure" capable of inhibiting the further spread of pathogenic organisms (Nagy and others 2000).

A likely scenario for the action of chitosan in inducing host defenses involves the entry of chitosan into cell nuclei, and subsequent activation of genes that activate a phenol-propanoid pathway (Lieutier and Berryman 1988). This process would continue as long as the fungus keeps growing within the host, involving progressively more parenchyma cells and an ever expanding reaction zone. Once fungal growth stops, however, wound compartmentalization and healing are initiated and the reaction zone ceases expansion. Following this scenario, a larger lesion in the host indicates a more virulent pathogen (Cook and Hain 1985, 1986, 1987a, 1987b, 1988; Harrington and Cobb 1983; Klepzig and others 1996b; Krokene and Solheim 1997; Nevill and others 1995; Raffa and Smalley 1988; Wallin and Raffa 2001). This explains the relatively large lesions formed in pines by *O. minus* and *L. terebrantis*, and the relatively small lesions formed by *L. procerum*, *E.* sp. A., and *C. ranaculosus*. The less virulent fungi likely could not tolerate the defensive chemistry of pines, did not grow within host tissues, and did not elicit as large or disrupted a host response.

This correlation between the degree of host response and the level of virulence of the invading agent is evident at the histological level. In these experiments, the reaction of loblolly pine to a signal (chitosan) that an actively growing, and, therefore, potentially pathogenic fungus was present was very similar to tree reaction to pathogenic fungi, *O. minus,* and *L. terebrantis*. In contrast, the fungi that were much less, if at all, able to grow within the tree, especially *E.* sp. A., but also *C. ranaculosus* and *L. procerum*, elicited reactions similar to those formed in response to mere mechanical wounding. Future studies will concentrate on the time sequence of, and effects of host vigor on, the cellular reaction to chitosan and bark beetle-associated fungi.

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