Effects of a Commercial Chitosan Formulation on Bark Beetle (Coleoptera: Curculionidae) Resistance Parameters in Loblolly Pine[1]

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Abstract A commercially available chitosan product, Beyond™, was evaluated for its effects on loblolly pine, *Pinus taeda* L., responses believed related to bark beetle resistance. Treatments were applied 4 times at approx. 6-wk intervals between May and November 2008. Five treatments were evaluated: ground application (soil drench), foliar application, ground and foliar applications combined, water-only (soil drench), and untreated. Two response variables were measured: yield of oleoresin accumulated at 48 h from wounds inflicted at breast height (5 sample periods) and area of lesion formed in response to inoculation with *Ophiostoma minus* (Hedgcock) Sydow & P. Sydow (2 sample periods). One treatment, ground application of Beyond, resulted in a significant increase in oleoresin yield (about 40% experiment-wide). Foliar treatment alone, or combined with ground application, did not result in a significant change in oleoresin yield. None of the treatments resulted in a difference in lesion area produced in response to inoculation with *O. minus*. In summary, application of a commercially-prepared chitosan formulation to loblolly pine produced inconsistent responses in tree parameters associated with bark beetle resistance, suggesting that exogenously applied chitosan preparations have limited utility for managing bark beetles.

Key Words induced response, southern pine beetle, blue stain, chitin, *Ophiostoma minus*

In general, rapid recognition and signaling of an invading pathogen or phytophagous insect are keys to successfully initiating the defensive systems of plants. Chitosan, an analog of chitin and a component of fungal cell walls and insect exoskeletons, is one such early signaler. Chitin behaves as a pathogen-associated molecular pattern signal in a number of plant-pathogen systems and may have similar functions in plant-insect interactions (Ryan 1988, Constabel et al.1995, Kaku et al. 2006, among others). Small fragments of chitin, found in the cell walls of fungi and insects, are known to generate general recognition signals in a number of plant-pathogen systems (Ryan 1988, Constabel et al.1995, among others). A variety of defensive responses in pine-bark beetle systems have been elicited with readily available chitosan preparations (Hadwiger and Beckman 1980, Miller et al. 1986, Lieutier and Berryman 1988, Popp et al. 1997) and related methyl jasmonate applications (e.g., Erbilgin et al. 2006, Zeneli et al. 2006). Recently, working with loblolly pine, Klepzig and Walkinshaw (2003) found localized cellular disruption due to injection with small quantities of chitosan, but did not note any systemic effects on pine defenses. Neither the effects of larger doses of

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chitosan preparations nor the exogenous application of such preparations have yet been tested with the southern pines.

Two categories of physiological resistance are recognized in pines against attacking bark beetles: oleoresin flow and induced lesion formation. Interactions between certain species of bark beetles and host trees are primarily mitigated by one, the other or their combination. For example, tree mortality is believed more dependent on oleoresin quantity in the southern pine-southern pine beetle system (e.g., Blanche et al. 1983, Reeve et al. 1995, Strom et al. 2002), whereas in the lodgepole pine-mountain pine beetle system, host lesions at beetle attack sites are considered of primary importance (e.g., Raffa and Berryman 1982). Both categories of resistance are considered relevant and methods for their assessment are widely used (e.g., Strom et al. 2002, Klepzig et al. 2005). The objective of this experiment was to evaluate the commercial chitosan product, Beyond™ (now ODC Colloidal Chitosan [active ingredient 0.25% chitosan], Agrihouse, Inc., Berthoud, CO; EPA reg. no. 83,729 - 1), for its effects on loblolly pine (*Pinus taeda* L.) responses believed to be related to bark beetle resistance. More specifically, we sought to determine whether chitosan application would stimulate defenses in loblolly pine to an extent that might be useful in managing pine bark beetles.

Materials and Methods

Standard methods were used to assess tree resistance (Karsky et al. 2004, Klepzig et al. 2005): oleoresin yield in response to mechanical injury, and lesion formation in response to inoculation with *Ophiostoma minus* (Hedgcock) Sydow & P. Sydow. Oleoresin flow is typically measured as yield, and lesion formation is typically measured as length or area; both categories of responses are evaluated over time, commonly 1 - 7 days for yield and about a month for lesion size (Klepzig et al. 2005). Oleoresin yield is considered to be positively correlated with resistance (more resin provides greater resistance) whereas lesion area is the opposite (smaller lesions depict greater resistance). In this experiment we measured the oleoresin yield that had accumulated at 48 h after wounding, while lesion areas were measured approx. 1 month after inoculation with fungi (see below).

Site and stand conditions. Forty individual loblolly pines (planted in 1993) were selected along a transect extending for about 700 m into a single stand (plantation) near Winnfield, LA. Trees were selected by size, relative location and canopy position. We selected trees that were approx. 12 m tall to facilitate spraying their entire crowns, trees sufficiently spaced (a minimum of 25 m apart) to assure no contamination via spray drift, and trees with dominant or codominant crowns. Trees were measured for diameter at breast height (dbh), total height and crown height (Table 1) prior to implementation of treatments. Pretreatment yields of resin also were measured for each tree.

Treatments and responses. Treatments consisted of combinations of 3 factors: dosage of Beyond, application technique and dosage of applied water (Table 2). Dosage of Beyond was 1x, 2x or 0. Individual trees receiving only foliar or ground applications received a 1x dosage. The 1x dose was delivered with the following pattern: 1 ml Beyond on 12 May 2008, 1 ml on 18 June 2008, 80 ml on 28 July 2008 and 80 ml on 23 September 2008. For each tree receiving the 1x dose, Beyond was delivered in 38 L of water at each application time. Each tree treated with the combined foliar and ground application of Beyond received a 2x dose. Application times were the same as

Table 1. Diameter at breast height (dbh), total height and live crown ratio of loblolly pine trees growing near Winnfield, LA, and used to evaluate effects of Beyond™ chitosan product on tree responses related to defense against bark beetles. Values are means followed by one standard error.

Treatment	n	dbh (cm)	tree height (m)	live crown ratio	treatment application site
Untreated	5	13.8 ± 0.1	12.1 ± 0.7	0.462 ± 0.041	None
Water	10	15.0 ± 0.3	13.0 ± 0.3	0.478 ± 0.021	Ground
Beyond1x	10	15.0 ± 0.4	12.0 ± 0.4	0.505 ± 0.029	Ground
Beyond 2x	10	14.4 ± 0.3	12.1 ± 0.3	0.464 ± 0.018	Ground + Foliar
Beyond Foliar	5	14.7 ± 0.2	12.0 ± 0.3	0.507 ± 0.021	Foliar

above, but each quantity was doubled (2, 2, 160, 160 ml, respectively) and 57 L of water was used to apply the product at each time (19 L ground and 38 L foliar spray).

Ground application (soil drench) was delivered in 38 L of tap water in a raked area (duff cleared to mineral soil) about 1 m radius around each tree stem. The foliar treatment consisted of the 1x Beyond dosage, carried in 38 L of water, applied to the crown using a Hypro® diaphragm pump (Model no. 9910-D30GRG1, Hypro Corp., New Brighton, MN) and a John Bean spray gun (Model JBS 785, John Bean Sprayers, Hogansville, GA). Initial testing showed that 38 L was a convenient amount to assure that the entire dosage of the treatment, included only in the first 19 L, made it through the sprayer and hoses to the target tree (i.e., the second 19 L were applied to the tree and used to flush the sprayer and hoses). The 2x treatment consisted of double the Beyond dosage, delivered half to the foliage (in 38 L of water) and half on the ground, as in the 1x treatment, but using 19 L of water. Water-only treatment received 38 L of water as a soil drench, and the dry treatment (untreated) did not receive Beyond or water.

Table 2. Description of treatments applied to loblolly pines growing near Winnfield, LA to evaluate effects of Beyond™ chitosan product on tree responses related to defense against bark beetles.

Treatment	Relative Beyond dosage	Beyond application rate (ml) during periods 1 - 2*	Beyond application periods rate during 3 - 4	Water application rate (liters)
Ground	1x	1	80	38
Ground + Foliar	2x	2	160	57
Foliar	1x	1	80	38
Water	0	0	0	38
Dry	0	0	0	0

* Volume of Beyond applied during sample periods 3 and 4 was 80x greater than that applied during periods 1 and 2.

Sampling for oleoresin yield followed established protocols (e.g., Karsky et al. 2004, Klepzig et al. 2005). For each tree at each sampling time, two 1.27 cm round holes were punched through the bark to the sapwood face on opposite sides at breast height. Oleoresin was collected into 15-ml polyethylene centrifuge tubes via samplers developed for this purpose (Karsky et al. 2004). Wounds were made just prior to treatment on the first day (12 May), collections made 24 and 48 h after each treatment, and sample mass determined (grams); the sum of these 2 measurements was considered total yield for each wound.

Trees were inoculated with *O. minus* on 12 June 2008 and 26 August 2008 using standard protocols (e.g., Klepzig et al. 2005). Briefly, a 0.5-cm round plug of *O. minus* growing on malt agar was placed onto the face of the sapwood in each 1.27-cm diam. wound. Each inoculation period consisted of 2 (12 June 2008) or 4 (26 August 2008) inoculation points per tree. In each case, the bark plug was replaced following inoculation to promote more natural conditions for the fungi and to avoid desiccation. Resulting lesions were measured about 1 month after inoculation: 15 July 2008 (33 d postinoculation; 2 per tree) and 1 October 2008 (36 d postinoculation; 4 per tree). Measurements were made by scraping away bark until lesions were wholly visible, at which point their outline was traced onto transparency film (overhead projector sheets). Tracings were returned to the laboratory and lesion area (cm^2) determined by tracing with a digital planimeter (Lasico model no. 1281 - 12). Mean lesion area per tree, transformed as indicated below, was used as the response variable in analyses.

Statistical analyses. Data on yields of oleoresin (mean of square-root transformed values for each tree [two samples per tree] and time period) were subjected to mixed-model repeated measures analysis of covariance (ANCOVA; split-plot analysis with time as a subplot factor) following determination that square-root transformed values met the condition of sphericity ($P > 0.20$; JMP V. 7.0.2, SAS Institute, Cary, NC). The initial (pretreatment) mean resin yield for each tree was used as the covariate. The interaction between treatment and time was included in the model but other interactions (i.e., covariate*treatment) were removed if $P > 0.20$ and models rerun. Treatment means, adjusted for the covariate, were subjected to Tukey's HSD following a significant ($P < 0.05$) F-test. Effects of treatments on lesion area were evaluated using a similar split-plot model but without a covariate (ANOVA) because there was no pretreatment measurement of lesion area. Data were transformed (square-root[y] for oleoresin yield and ln[y] for lesion area) to better meet the assumptions of parametric statistics.

For oleoresin yield, 2 treatment contrasts were also of interest. Trees that received Beyond treatment (1x, 2x and foliar treatments, $n = 25$) were compared with those that did not (water and dry controls, $n = 15$), and trees that received foliar treatment of Beyond (foliar and 2x treatments, $n = 15$) were compared with those trees that received no Beyond treatment (water and dry controls, $n = 15$). In all analyses, $P < 0.05$ was used to determine significance.

Results

Mean oleoresin yields for each treatment and sample period are shown in Fig. 1. The ANCOVA model resulted in a significant overall treatment effect ($P < 0.0153$; Table 3). Mean separation by Tukey's HSD resulted in only the ground treatment (soil drench) being significantly different from the other treatments (Table 5A). This treatment produced resin yields that were significantly greater than the dry or water

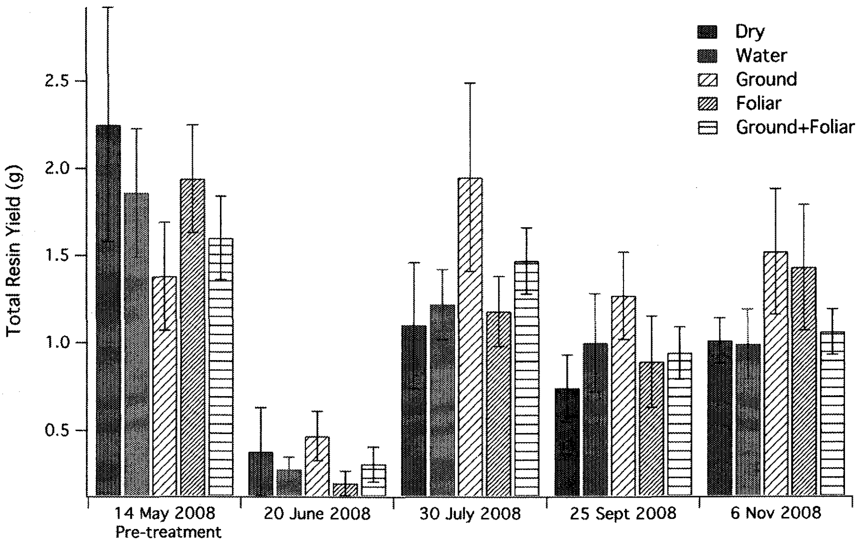

Fig. 1. Mean untransformed resin yields (total g at 48 h) for each treatment and date. Complete treatment descriptions appear in Table 2 and the text. Brief descriptions are: 1x = ground; 2x = ground + foliar; Foliar = foliar; Dry = dry control; Water = water control. Error bars show one standard error of the associated untransformed mean. Transformed yields (sqrt[y]) were subjected to ANCOVA and Tukey's HSD to determine significance of effects.

(control) treatments, though yields did not differ significantly from other Beyond treatments, and the other Beyond treatments (foliar and 2x) did not differ significantly from control treatments (Table 5A). Additional evaluations were conducted by developing contrasts to compare oleoresin yields from trees treated with Beyond or not. This contrast produced a significant difference ($F = 5.41$, df = 1, 34, $P = 0.026$), indicating that Beyond treated trees had increased oleoresin yields relative to controls. A second contrast was used to compare trees receiving no treatment of Beyond with those

Table 3. Analysis of covariance (ANCOVA) table showing oleoresin yield results from loblolly pine trees growing near Winnfield, LA and treated with Beyond™ chitosan product on tree responses related to defense against bark beetles. Treatments are described in the text and Table 1.

Source	df num	df den	*F* ratio	*P*-value
Treatment	4	34	3.5830	0.0153
Sample Period	3	105	43.1883	< 0.0001
Covariate*	1	34	22.5069	< 0.0001
Treatment X Sample Period	12	105	0.3415	0.9794

* pretreatment 48 h oleoresin yield for each tree.

that received foliar treatment. This comparison resulted in no difference between means ($F = 1.18$, df = 1, 34, $P = 0.29$).

Mean lesion areas for each treatment and sample time are given in Fig. 2. Treatment with Beyond produced no effect on lesion area formed in response to *O. minus* inoculation ($F = 1.13$, df = 4, 35, $P = 0.36$; Tables 4 and 5B). Lesions made in response to June inoculations were much larger (mean = 26.0, SEM = 1.6 cm^2) than were those made in response to August inoculations (mean = 15.0, SEM = 0.7 cm^2), resulting in the effect of sampling period being highly significant ($F = 55.6$, df = 1, 35, $P < 0.0001$; Table 4).

Discussion

Effective and useful tree protection products must have at least 4 characteristics: (1) their magnitude of impact against the target pest must be sufficient to produce the desired result; (2) their effect(s) must be consistent; (3) they must be cost-effective; and, (4) they must be safe when used properly. The chitosan product we tested is labeled for use by the US-EPA, being considered safe when label procedures are followed. Its cost, ignoring application costs, was considerably higher than the most commonly used synthetic insecticides for southern pine bark beetles. For example, the product cost for the synthetic insecticide Onyx® is about $2.60 per tree for a 7.6 L application, compared with a per tree cost for Beyond of about $82.00 - 164.00 in this

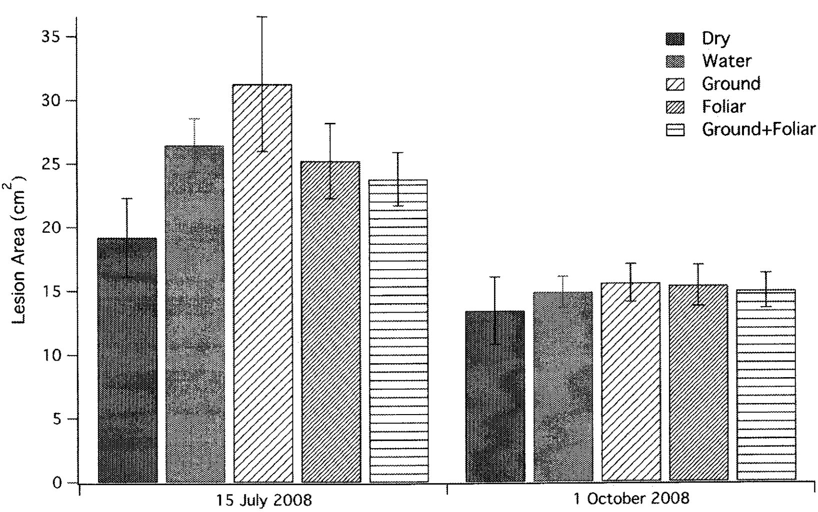

Fig. 2. Mean untransformed lesion areas (square cm) following bole inoculations of experimental loblolly pines with *Ophiostoma minus*. Dates at bottom are the dates lesions were observed. Fungal inoculations were made on 12 June 2008 (two per tree, read on 15 July, 33 d) and 26 July (four per tree, read on 1 October, 36 d). Treatments with Beyond began on 12 May 2008 and were repeated on a six-week schedule (see text). Treatments as in Figure 1. Error bars show one standard error of the associated untransformed mean. Transformed values (ln[y]) were subjected to ANOVA to determine significance of effects.

Table 4. Analysis of variance (ANOVA) table showing lesion area results from loblolly pine trees growing near Winnfield, LA and treated with Beyond™ chitosan product on tree responses related to defense against bark beetles. Treatments are described in the text and Table 1.

Source	df num	df den	*F* ratio	*P*-value
Treatment	4	35	1.1296	0.3585
Sample Period	1	35	55.5637	< 0.0001
Treatment X Sample Period	4	35	0.3218	0.8614

experiment (see below). The magnitude of Beyond's effects on loblolly pine defenses was moderate for oleoresin yield and insignificant for response to blue-stain fungi. Its effects on oleoresin yield in this experiment were inconsistent (Fig. 3). Averaged over dates, effects of treatment with Beyond on oleoresin yield of loblolly pine were modest but significant (ground application increased back-transformed yields from 0.71 g for water controls to 1.2 for ground-applied Beyond) or not significant (ground + foliar or foliar applications). Individual treatment contrasts detected a significant effect of treatment with Beyond compared with untreated trees for resin yield. There was no significant effect of foliar treatment compared with no Beyond treatment. That is, the treatment effect was only observed in trees receiving ground application by itself; it was not observed in trees subjected to the other application treatments, even when ground treatment was a component of another treatment.

The impact of this level of increase in resin yield for promoting tree protection against bark beetle attack is uncertain. To be successful, individual tree protection requires greater increases in resin yield than does area-wide suppression of populations; the latter does not require that preselected individual trees survive because impacts may, at least theoretically, be realized through decreased beetle success despite tree mortality. The levels of increased yield observed in this study are modest compared with other factors known to impact resistance, such as lightning strikes, fungal inoculation, mass wounding and fire injury. Blanche et al. (1985) observed that an individual lightning-struck loblolly pine had nearly zero resin yield for at least 11 d following the strike. By day 21 yield had increased to 230% of the level observed pre-strike. Strom et al. (2002) found that progeny of trees that escaped mortality from southern pine beetle (*Dendroctonus frontalis* Zimmermann) produced an average of 165% of the oleoresin yield of unselected trees. Mass inoculation with *O. minus* generated 200 - 300% of the resin yield seen in untreated trees, and this from 15 d to at least 105 d after treatment (Klepzig et al. 2005). Fire and mechanical injuries to pine stems are also known to increase resin yields substantially, e.g., to about 200% of that seen in controls 55 d post treatment in red pine, *Pinus resinosa* Aiton (Lombardero et al. 2006).

The question also remains as to the impact that such a resin elevation would have on individual beetles. In a study evaluating drought effects on loblolly pine, Reeve et al. (1995) determined that attack success, defined as eggs per southern pine beetle attack, were estimated by the equation $Y = 7.88 (X^{\wedge}-0.780)$, where Y is number of eggs laid and X is resin flow (g/wound/day). Using this equation, and our 48 h results, the mean resin yield differences observed in this study with the 1x Beyond soil drench (back-transformed experiment-wide mean = 1.2 g resin) compared

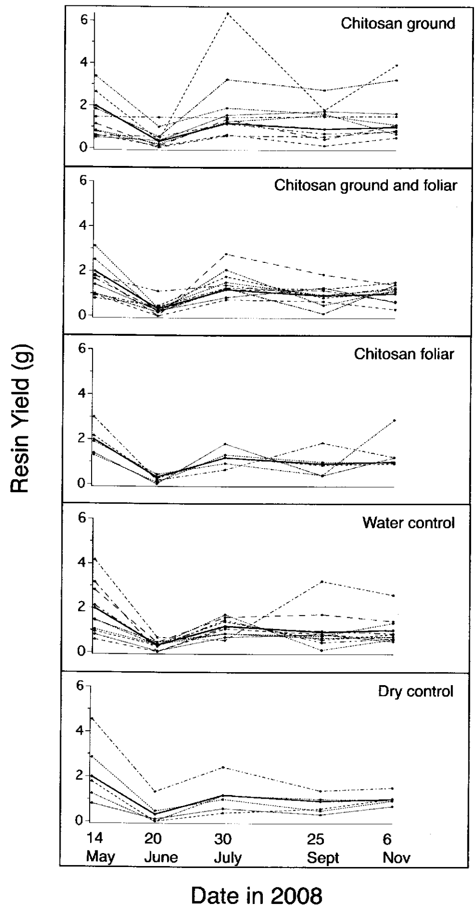

Fig. 3. Resin yields (untransformed) for each loblolly pine in this experiment showing the observed variability in tree responses. Each symbol represents a tree mean (of two samples collected on opposite sides of the bole) from each sampling period. For reference, a solid bold line is included in each panel to indicate the overall mean of experimental trees. This line is the same in each panel. Treatment populations consisted of 10 trees (Chitosan ground, Chitosan ground and foliar, Water control) or 5 trees (Chitosan foliar, Dry control), totaling 40 experimental trees. In the Chitosan ground panel, the resin yield in two trees increased notably following treatment on 30 July 2008, however, increases were not consistent (see other dates).

with all untreated control trees (mean = 0.67 g resin) would reduce egg production by about 37% (10.8 - 6.8 eggs per attack). Note that this is the maximum possible effect that could be predicted from this study, as it compares only the best Beyond treatment with the combined control treatments (see Table 5A). It is also important to consider that, despite any reductions in egg production, the trees would likely still be attacked and killed by the bark beetles.

Treatment with Beyond did not affect the lesion area formed in loblolly pines when inoculated with *O. minus*. Lesions, however, were significantly larger in July than in October. This lack of response may limit interest in this treatment to those bark beetle-tree systems in which resin yield is believed to be the dominant factor in tree resistance (e.g., southern pine beetle), and away from those in which response to fungal invasion is considered most important (e.g., mountain pine beetle in lodgepole pine).

The chitosan product Beyond costs between $13.89 and $24.99 USD per oz, about $0.50 per ml at the lower cost (http://www.agrihouse.com, accessed 14 July 2010). Trees receiving our 1x dosage received a total of 162 ml Beyond. The 2x dosage consisted of 324 ml. Thus, using the lower cost of $0.47 per ml, the per tree cost of the Beyond product used in this experiment was: 1x (ground or foliar) = 162 ml = 162*0.47 = $76.14 per tree (total for the duration of expt).; 2x (ground and foliage) = 324 ml = 324*0.47 = $162.28 per tree. The overall costs, along with the labor and feasibility of transporting sufficient water to target trees, are additional factors to consider when evaluating the practicality of using this product.

Three results from this study suggest that our chitosan treatments were not sufficient for providing a targeted and effective management option for pine bark beetles. First, increases in resin yield were inconsistent, being limited to one treatment application method. To be useful as an individual tree protectant against bark beetles, increases in resin yield need to be consistent and likely quite high. For example, Strom et al. (2002) observed a resin yield increase of 1.65 times when comparing progeny of escape trees to a population of unselected trees. Using the concepts of heritability

Table 5. Experimentwide least-squares means (± 1 SEM) of resin yield (A; square-root transformed) and lesion area (B; ln transformed) by treatment.

Treatment	n	mean (transformed(y)) *
(A) Resin Yield		
ground	10	1.095 ± 0.059 A
foliar	5	0.842 ± 0.083 AB
ground + foliar	10	0.915 ± 0.058 AB
water control	10	0.845 ± 0.058 B
dry control	5	0.758 ± 0.083 B
(B) Lesion Area		
ground	10	2.99 ± 0.079 A
foliar	5	2.96 ± 0.112 A
ground + foliar	10	2.90 ± 0.079 A
water control	10	2.96 ± 0.079 A
dry control	5	2.72 ± 0.112 A

* Values followed by different letters are significantly different by Tukey's HSD within each response variable ($P < 0.05$). Prior to transformations, oleoresin yield values were grams and lesion area values were square centimeters.

and gain (gain = [selection differential] × heritability; Falconer 1989) one can predict the difference in mean resin yield between the trees that actually escaped southern pine beetle infestation (i.e., the parent trees from Strom et al. [2002]) and trees from their unselected population. Considering a heritability for resin yield of about 0.5 for loblolly growing in a managed plantation (Roberds et al. 2003), and an experiment-wide mean difference of 3.27 g of resin between loblolly pine escapes and the unselected population (Strom et al. 2002), the predicted resin yield value for the escape parents (i.e., the trees that actually survived southern pine beetle pressure) is 11.6 g. The equivalent value for unselected trees in the same experiment is 5.1 g. The difference of 6.5 g is large, and is probably itself conservative because the heritability value of 0.5 was generated from an old-field loblolly plantation, so is likely somewhat high (Roberds et al. 2003). A lower heritability would increase the estimate of parental resin yield (see above). This suggests that resin yield must be very high to influence tree survival. Indeed, resin yield may not, by itself, be a sufficient mechanism to achieve this goal with a high probability in many management scenarios (e.g., those with high-value individual trees), but the moderate levels of increase that we observed in this study are unlikely to be enough.

Second, there was no impact of chitosan treatments on tree lesion response to blue-stain fungi. A tree's ability to limit lesion size in response to blue-stain invasion is considered an important mechanism of resistance to bark beetles and is particularly important for tree survival in some pine-bark beetle systems (e.g., Raffa and Berryman 1982). The lack of impact of chitosan treatments on this tree resistance mechanism reduces its potential utility. Third, impacts on resin yield were limited to soil drench applications. Widespread use of chitosan as a plant amendment to increase pine resistance to bark beetles is most likely limited to 2 primary scenarios: application to selected, high-value trees to provide immunity from bark beetle attack, or application to forests to reduce bark beetle infestation growth. In the first scenario, soil drench is a reasonable method for treatment, whereas in the latter, aerial application is likely to be preferred. Our results do not support the effectiveness of chitosan treatments in either of these scenarios.

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