Boric acid–phenolic relationships within the *Pinus echinata–Pisolithus tinctorius* ectomycorrhizal association

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Summary

At germination, container-grown shortleaf pine seedlings were inoculated with *Pisolithus tinctorius* (Pers.) Coker & Couch or left uninoculated, and both groups were fertilized semiweekly with a modified Hoagland's solution supplemented with 0 or 0.4 mM boric acid. After 12, 16 and 24 weeks, seedling root tissue was analyzed for ectomycorrhizal colonization, phenolic concentration and phenoloxidase activity. In addition, phenoloxidase activity was assayed in *P. tinctorius* that had been cultured in a liquid medium containing boric acid.

Inoculation with *P. tinctorius* increased the root phenolic concentration of 16- and 24-week-old seedlings, and increased root phenoloxidase activity in 12-, 16- and 24-week-old seedlings. Fertilization with boric acid reduced the phenolic concentration of *P. tinctorius* ectomycorrhizae after 24 weeks. Although boric acid fertilization did not affect the phenoloxidase activity of 12-, 16- and 24-week-old inoculated roots, or that of 16- and 24-week-old uninoculated roots, it increased the phenoloxidase activity of *P. tinctorius* grown *in vitro* and 12-week-old uninoculated roots. We conclude that boric acid fertilization influences the phenolic relations of the shortleaf pine–*P. tinctorius* ectomycorrhizal association, possibly through a boric acid-induced increase in phenoloxidase activity.

Keywords: ectomycorrhizal colonization, phenolic concentration, phenoloxidase activity, shortleaf pine.

Introduction

Ectomycorrhizal colonization can result in the deposition of phenolic compounds in peripheral cortex cells (Ling-Lee et al. 1977, Molina 1981, Molina and Trappe 1982, Malajczuk et al. 1982). A similar response has been recognized as one mode of plant defense against pathogenic infection (Ebel 1986). Furthermore, Lindeberg (1948) and Olsen et al. (1971) demonstrated inhibitory effects of phenolics on the growth of ectomycorrhizal fungi. The intensity of phenolic deposition in response to invasion by ectomycorrhizal-forming fungi and the ability of an ectomycorrhizal fungus to tolerate phenolics may be associated with the symbiotic compatibility of fungus and host associates (Molina 1981, Molina and Trappe 1982, Malajczuk et al. 1982).

Boric acid fertilization can enhance both endo- and ectomycorrhizal colonization. Lambert et al. (1980) found that boric acid fertilization of alfalfa (*Medicago sativa* L.) seedlings inoculated with *Glomus fasiculatus* (Thaxt. sensu Gerd.) Gerd. & Trappe increased endomycorrhizal colonization by 88%. Mitchell et al. (1987) reported boric acid fertilization increased the spread and the intensity of *Pisolithus*

tinctorius (Pers.) Coker & Couch ectomycorrhizal colonization of shortleaf pine (*Pinus echinata* Mill.) seedlings.

Boron is associated with the metabolism of plant phenolics (Lee and Arnoff 1967) and the phenolic content of plant tissues (Perkins and Arnoff 1956, Rajaratnam and Lowry 1974). Hydroxylation of phenolics has been associated with their toxicity to fungi (Christie 1965, Li et al. 1969, Larson and Bussard 1979). Modification of phenoloxidase activity could alter the hydroxylation, and therefore, the toxicity of root phenolics. Boron may regulate phenoloxidase activity (Gómez-Rodríguez et al. 1987).

We hypothesized that boric acid fertilization could reduce the inhibitory effect of fungitoxic phenolics produced by the host in response to colonization and thereby increase compatibility between an ectomycorrhizal fungus and its host. Improved compatibility may result in increased ectomycorrhizal colonization.

To examine the possible role of boron in regulating ectomycorrhizal colonization through modification of phenolic relations, we evaluated the effect of boric acid on the phenoloxidase activity of *P. tinctorius* grown *in vitro*. We also determined the effects of boric acid fertilization on ectomycorrhizal colonization, root phenolic concentrations and phenoloxidase activities of 12-, 16- and 24-week-old shortleaf pine seedlings inoculated with *P. tinctorius*.

Materials and methods

In vitro P. tinctorius phenoloxidase activity

Stock cultures of *P. tinctorius* were maintained on petri plates containing modified Melin-Norkrans (MMN) agar medium at 25 °C (Marx 1969). Our source of *P. tinctorius* was a reisolation of Isolate 246 collected by D.H. Marx, in Georgia, in 1979, from a sporocarp associated with *Pinus taeda* L. Stationary cultures of *P. tinctorius* were prepared in MMN liquid medium and incubated at 25 °C for approximately 14 days. Inoculum was prepared by harvesting and homogenizing the flask contents.

Eight Erlenmeyer flasks (250 ml) containing 50 ml of sterilized MMN liquid medium and 0, 0.25 or 0.50 mM H_3BO_3 were prepared. Five ml of suspended mycelial slurry was added to each flask. Cultures were incubated on a reciprocating shaker (100 excursions min^{-1}) at 28 °C and harvested after 14 days using suction filtration.

Fungal tissue, scraped from preweighed filter paper, was frozen in liquid nitrogen and crushed in a chilled mortar containing 0.1 g of soluble polyvinylpyrrolidone (PVP). The frozen fungal tissue-PVP powder was homogenized with 5.0 ml of chilled 0.05 M phosphate buffer (pH 6.8) containing 0.24 mM Triton X-100 in test tubes immersed in an ice bath. Homogenates were centrifuged at 20,000 g for 15 min at 4 °C. Supernatants were decanted and diluted to 10 ml with chilled 0.05 M phosphate buffer (pH 6.8). Pellets were resuspended and filtered onto preweighed filter paper. Residual fungal tissue, after suction filtration, and pellets were oven-

dried (62 °C, 24 h) and weighed.

Phenoloxidase activity (polyphenoloxidase: tyrosinase, E.C.1.14.18.1, catechol oxidase, E.C.1.10.3.2; laccase, E.C.1.10.3.1) was assayed by a modification of the procedure of Leonard (1971). Dihydroxyphenylalanine (DOPA) (9.0 mM) prepared in 0.05 M phosphate buffer (pH 6.8) was the enzyme substrate. Enzyme activity was determined based on a standard curve prepared from mushroom tyrosinase (Sigma Chemical Co., St. Louis, MO). An enzyme unit was defined as enzyme activity resulting in a change in A_{475nm} of 0.001 min^{-1} at 28 °C. Enzyme activity was expressed as units mg_{DW}^{-1} fungus. Also, dry weights of residual fungus tissue and fungus tissue pellets were added to obtain total fungus dry weights.

A completely random experimental design was based on three H_3BO_3 treatments (0, 0.25 and 0.50 mM H_3BO_3) and eight replications. Data were subjected to an analysis of variance and differences between treatment means were compared by the LSD test.

Shortleaf pine root analyses

Control-pollinated shortleaf pine seed was surface sterilized in 0.5% sodium hypochlorite (NaClO·5H$_2$O) for 15 s and cold stratified for 30 days at 4 °C. Tinus Spencer-LeMaire containers (500 ml) were filled with 1/1/2/2 (v/v) peat moss/vermiculite/sand/perlite sterilized with methyl bromide, and planted with sterilized seed. Using the *P. tinctorius* source previously described, half of the containers were inoculated, 1/7 (v/v), with vegetative inoculum (Mycorr Tech Inc., Pittsburgh, PA) and the remaining containers were left uninoculated.

Seedlings were grown in a 16-h photoperiod. Photosynthetically active radiation from high pressure sodium vapor lamps was approximately 544 µmol m^{-2} s^{-1}. Seedlings were watered semiweekly before initiation of fertilization. Fertilization began four weeks after 90% germination. Seedlings were fertilized semiweekly with 20 ml of modified Hoagland's nutrient solution (Bonner and Galston 1952). Six weeks after 90% germination, H_3BO_3 treatments consisting of 0 or 0.5 mg of H_3BO_3 were applied semiweekly to the soil. Following initiation of fertilization, seedlings were watered when the growth medium appeared dry.

A randomized complete block design with four blocks was established. Treatments consisted of no inoculation or inoculation with *P. tinctorius* and semiweekly fertilization with 0 or 0.5 mg H_3BO_3. At three harvest times (12, 16 and 24 weeks after 90% germination), 48 containers, three per treatment and block, were randomly harvested. Of these, 16 containers, one per treatment and block, were analyzed daily for three consecutive days. One set of containers was used for ectomycorrhizal colonization measurements, one for measurement of phenoloxidase activity and the third for analysis of root phenolic concentration. Root systems were washed free of growth medium with tap water. Following washing, seedlings used for analysis of root phenolic concentration and phenoloxidase activity were placed in plastic bags in an ice bath, and processed within two hours.

Seedling root systems were analyzed for the spread and intensity of ectomycorrhizal colonization by the methods of Mitchell et al. (1987). Spread was

expressed as the percentage of primary lateral roots colonized. This variable describes early colonization during radicle elongation and primary lateral root formation. Intensity was defined as the number of ectomycorrhizae per infected primary lateral root. This measurement describes colonization as fine root development progresses. In addition, the "percentage short roots colonized" provides information on both colonization intensity and the potential for further formation of ectomycorrhizae. Ectomycorrhizae were characterized by the presence of a fungus mantle or a swollen appearance when compared with uninfected short roots.

Approximately 250 mg of fine root tissue was gently hand-abraded from root systems and used for measurement of phenoloxidase activity. Root tissue was immersed in an ice bath and immediately homogenized for one minute in 5.0 ml of chilled 0.05 M 3-[N-Morpholino]-propane-sulfonic acid (MOPS) buffer (pH 6.8) containing 0.24 mM Triton X-100 and 0.1 g insoluble PVP. Homogenates were centrifuged at 20,000 g for 15 min at 4 °C. The supernatants were decanted, diluted to 10 ml with chilled 0.05 M MOPS buffer (pH 6.8) and phenoloxidase activity was assayed as described above. However, the substrate, DOPA (9.0 mM), was prepared in 0.05 M MOPS buffer (pH 6.8) and phenoloxidase activity was expressed as units per 100 mg_{FW}.

For analysis of phenolics, root tissue was lyophilized and stored at −20 °C until determination of phenolic concentrations. A modification of the insoluble polymer bonding procedure of Walter and Purcell (1979) was used for phenolic compound extraction and quantification. Approximately 0.1 g of lyophilized root tissue was homogenized in 5.0 ml of hot 95% ethanol. Homogenates were cooled to room temperature and centrifuged at 20,000 g for 30 min at 28 °C. Supernatants were decanted and evaporated to dryness in nitrogen at 28 °C. Ten ml aliquots of the extracts were agitated with 0.75 g of insoluble PVP for 30 min (160 excursions min^{-1}, 28 °C) and centrifuged at 20,000 g for 30 min at 28 °C. The difference between A_{323nm} measurements before and after PVP treatment represented absorbance by phenolic compounds. Phenolic concentrations were expressed as mg phenolics g_{DW}^{-1} and were determined from a standard curve prepared with a series of caffeic acid standards treated similarly to the root extracts. Data were subjected to an analysis of variance and differences between treatment means were compared by the LSD test.

Results

In vitro P. tinctorius phenoloxidase activity

The phenoloxidase activity of *P. tinctorius* grown in the presence of 0.25 mM H_3BO_3 was significantly greater than that of *P. tinctorius* grown in the absence of supplemental H_3BO_3 (Table 1). A similar, but nonsignificant response was also observed at 0.50 mM H_3BO_3. Dry weights of 14-day-old *P. tinctorius* were not significantly affected by 0.25 or 0.50 mM H_3BO_3.

Table 1. Mean phenoloxidase activity (units mg_{DW}^{-1}) and dry weight of *Pisolithus tinctorius* grown in liquid MMN medium containing 0, 0.25 or 0.50 mM H_3BO_3. Means, within a variable, followed by a different letter are significantly different at $P \leq 0.05$ by the LSD test.

Treatment	Phenoloxidase activity (units mg^{-1})	Dry weight (mg)
0 H_3BO_3	32.1 b	58.5 a
0.25 mM H_3BO_3	61.1 a	55.1 a
0.50 mM H_3BO_3	52.3 ab	56.0 a

Shortleaf pine root analyses

After the 12-, 16- and 24-week cultural periods, less than 2% of the short roots of uninoculated shortleaf pine seedlings were colonized by indigenous ectomycorrhizal fungi. The percentage of primary lateral roots colonized by *P. tinctorius* in inoculated plants was significantly increased by H_3BO_3 fertilization after 12 weeks (Table 2). Boric acid fertilization did not significantly affect ectomycorrhizal colonization at 16 weeks. After the 24-week cultural period, H_3BO_3 fertilization had significantly increased the percentage of short roots colonized by *P. tinctorius* compared to inoculated seedlings that were not fertilized with H_3BO_3.

The phenolic concentration of shortleaf pine roots was significantly increased by inoculation with *P. tinctorius* after 16 and 24 weeks, but not after 12 weeks (Table 3). Boric acid fertilization significantly reduced (32%) the phenolic concentration of ectomycorrhizal roots after 24 weeks. Although nonsignificant, fertilization with H_3BO_3 decreased the phenolic concentration of ectomycorrhizal roots by 22 and 24% after 12 and 16 weeks, respectively, and that of uninfected roots by 26, 17 and 12%

Table 2. Mean ectomycorrhizal colonization of 12-, 16- and 24-week-old container-grown shortleaf pine seedlings inoculated with *Pisolithus tinctorius* and fertilized semiweekly with 0 or 0.5 mg H_3BO_3.

Treatment	% Primary lateral infection[1]	Infection points per infected primary lateral	% Short roots colonized
12 Weeks post-germination			
0 H_3BO_3	77.8[2]	14.1	25.6
0.5 mg H_3BO_3	94.8	14.2	33.8
16 Weeks post-germination			
0 H_3BO_3	80.2	19.3	18.8
0.5 mg H_3BO_3	79.4	14.9	15.5
24 Weeks post-germination			
0 H_3BO_3	90.1	22.8	21.4[3]
0.5 mg H_3BO_3	90.9	19.2	31.7

[1] % Primary lateral infection = number of colonized primary lateral roots/total number of primary lateral roots.

[2] Means within a variable and a post-germination period are significantly different at $P \leq 0.10$ by the LSD test ($P = 0.074$).

[3] Means within a variable and a post-germination period are significantly different at $P \leq 0.10$ by the LSD test ($P = 0.090$).

Table 3. Mean phenolic concentration of nonmycorrhizal roots and *Pisolithus tinctorius* ectomycorrhizae of 12-, 16- and 24-week-old shortleaf pine seedlings fertilized semiweekly with 0 or 0.5 mg H_3BO_3.

Source	Nonmycorrhizal roots		Ectomycorrhizae		Significance[1]
	0 H_3BO_3	0.5 mg H_3BO_3	0 H_3BO_3	0.5 mg H_3BO_3	
12-Week-old culture	0.38	0.28	0.50	0.39	
Inoculation (I)					ns
H_3BO_3 (B)					ns
I x B					ns
16-Week-old culture	0.52	0.43	0.85	0.65	
Inoculation					**
H_3BO_3					ns
I x B					ns
24-Week-old culture	1.04 bc[2]	0.92 c	1.93 a	1.32 b	
Inoculation					*
H_3BO_3					***
I x B					****

[1] Probability of obtaining a calculated F-value larger than the tabular F-value (ns = not significant, * = $P \leq 0.07$, ** = $P \leq 0.05$, *** = $P \leq 0.01$ and **** = $P \leq 0.0003$).

[2] Means, within the 24-week cultural period, followed by a different letter are significantly different at $P \leq 0.05$ by the LSD test.

after 12, 16 and 24 weeks, respectively.

Inoculation with *P. tinctorius* significantly increased the root phenoloxidase activity of 12-, 16- and 24-week-old shortleaf pine seedlings (Figure 1). After 12 weeks, H_3BO_3 fertilization significantly increased (41%) the phenoloxidase activity of uninoculated shortleaf pine root tissue ($P = 0.0910$). However, fertilization with H_3BO_3 did not significantly affect phenoloxidase activities of uninoculated root tissue after 16 and 24 weeks, or inoculated root tissue after 12, 16 and 24 weeks.

Discussion

Phenolics have an inhibitory effect on the growth of ectomycorrhizal fungi
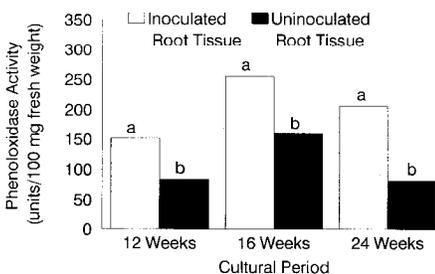
Figure 1. Root phenoloxidase activity of 12-, 16- and 24-week-old shortleaf pine seedlings uninoculated or inoculated with *Pisolithus tinctorius*. Means are of 0 and 0.5 mg H_3BO_3 treatments combined. Means within a cultural period are significantly different at $P \leq 0.001$ by the LSD test.

(Lindeberg 1948, Olsen et al. 1971). Deposition of phenolics in peripheral cortex cells, resulting in the formation of a "tannin layer," often accompanies the synthesis of ectomycorrhizae (Ling-Lee et al. 1977, Molina 1981, Molina and Trappe 1982, Malajczuk et al. 1982). Moreover, the synthesis of ectomycorrhizae may be dependent on the ability of the fungal associate to tolerate the host response to ectomycorrhizal colonization (Molina 1981, Molina and Trappe 1982). Failure of ectomycorrhizal synthesis because of incompatibility between fungus and host associates has been attributed to an increase in root phenolics (Molina 1981, Molina and Trappe 1982, Malajczuk et al. 1982).

Mitchell et al. (1987) found that boric acid fertilization resulted in a 111% increase in the number of *P. tinctorius* ectomycorrhizae per infected primary lateral root of 16-week-old shortleaf pine seedlings. In the present study, boric acid fertilization resulted in a significant increase in the percentage of primary lateral roots infected at Week 12 followed by a significant increase in the percentage of short roots colonized by *P. tinctorius* at Week 24. However, no significant responses were observed between Weeks 12 and 24.

At the termination of the 12-, 16- and 24-week cultural periods, boric acid fertilization resulted in 22, 24 and 32% decreases, respectively, in the phenolic concentration of *P. tinctorius* ectomycorrhizal roots. Although significance was only detected in the oldest seedlings, a trend of reduced ectomycorrhizal phenolic concentration in response to boric acid fertilization was apparent throughout the 24-week cultural period.

Boron deficiency is accompanied by an accumulation of phenolics in some plant tissues (Perkins and Arnoff 1956, Rajaratnam and Lowry 1974, Lewis 1980). Lee and Arnoff (1967) postulated that boron regulates the synthesis of phenolic compounds. A boric-acid-induced reduction in the rate of synthesis of phenolic compounds may be one explanation for the increased proliferation of ectomycorrhizae observed in our work and that of Mitchell et al. (1987).

Boric acid could also alter root phenolic concentrations by modifying fungus and plant phenoloxidase activities. Polyphenol oxidase, an intracellular phenoloxidase, catalyzes both the hydroxylation of monohydroxy phenolics to *ortho*-dihydroxy phenolics and the oxidation of *ortho*-dihydroxy phenolics to *ortho*-quinones (Butt 1980). Laccase, predominantly an extracellular phenoloxidase, catalyzes both the hydroxylation of monohydroxy phenolics to *para*- and *ortho*-dihydroxy phenolics and the oxidation of *para*- and *ortho*-dihydroxy phenolics to *para*- and *ortho*-quinones (Butt 1980).

Both increased and decreased plant phenoloxidase activities have been associated with supplemental boron nutrition (Shkol'nik 1984, Gómez-Rodríguez et al. 1987). Gómez-Rodríguez et al. (1987) hydroponically cultured sunflower seedlings in solutions containing various amounts of boric acid and found a positive correlation between the boron concentration and the polyphenol oxidase activity of foliar tissue. In contrast, Shkol'nik (1984) presented evidence suggesting that boron sufficiency was accompanied by lower plant polyphenol oxidase activities when compared to activities associated with boron deficiency. Marziah and Lam (1987) found no effect

of boron deficiency on the polyphenol oxidase activity of soybean (*Glycine max* L. Merrill).

Polyphenol oxidase has been reported to be both cytoplasmic and membrane-bound (Parish 1972, Ruis 1972, Vaughn and Duke 1984). Inconsistency in the phenoloxidase response to boron nutrition may be attributed to the cellular location of enzymes. The proposed role of boron in maintenance of cell membrane structure and function (Tanada 1974, Pollard et al. 1977, Tanada 1983, Schon et al. 1990), suggests boron deficiency may disrupt cellular membranes. This disruption could activate latent, membrane-bound polyphenol oxidase. However, in tissues with high extracellular or cytoplasmic phenoloxidase activity, boron may be stimulatory.

In the present study, membrane-bound fungus and root phenoloxidases were solubilized with a nonionic detergent. Consistent with other reports of phenoloxidase activity in ectomycorrhizal fungi (Lindeberg 1948, Giltrap 1982, Ramstedt and Söderhäll 1983), we observed phenoloxidase activity in *P. tinctorius* tissue grown *in vitro*. We also found that inoculation with *P. tinctorius* increased the phenoloxidase activity of shortleaf pine root tissue. In addition, the phenoloxidase activities of both *P. tinctorius* grown *in vitro* and 12-week-old uninoculated root tissue were enhanced by boric acid.

We did not observe an effect of boric acid fertilization on the phenoloxidase activity of ectomycorrhizal roots. Moreover, boric acid fertilization did not affect the phenoloxidase activity of uninoculated roots beyond 12 weeks. Phenolics and their oxidation products react with proteins (Butt 1980, Spencer et al. 1988). Thus, it is possible that as the root phenolic concentration increased with seedling age and ectomycorrhizal development, our extraction procedure became inadequate for preventing enzyme denaturation by phenolics and quinones.

Conclusions

Our results and those of Mitchell et al. (1987) suggest that boric acid fertilization brings about an increase in *P. tinctorius* colonization of shortleaf pine. We found a simultaneous decrease in the phenolic concentration of *P. tinctorius* ectomycorrhizae caused by boric acid fertilization, suggesting that a relationship may exist in shortleaf pine between boric acid fertilization, phenolic metabolism and *P. tinctorius* colonization. Phenoloxidase activities of *P. tinctorius* mycelium and the short roots of 12-week-old uninoculated shortleaf pine seedlings were stimulated by boric acid. Boric acid may modify phenolic metabolism through stimulation of phenoloxidase activity.

We conclude that boric acid may possess a regulatory role in the mechanism of ectomycorrhizal colonization through modification of root–fungus phenolic relations. Further research is needed to define the regulatory mechanisms that control ectomycorrhizal formation and fungus–host compatibility.

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