

BORON FERTILIZATION AND THE ROOT MORPHOLOGY OF
SHORTLEAF PINE SEEDLINGS
INOCULATED WITH *PISOLITHUS TINCTORIUS*¹

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Abstract. Benefits associated with ectomycorrhizal infection are well established. However, exploitation of this symbiosis has been hindered, in part, by the inability to obtain consistently high infection rates. Past research in our laboratory has identified a twofold increase in *Pisolithus tinctorius* [(Pers.) Coker and Couch] ectomycorrhizal infection of shortleaf pine (*Pinus echinata* Mill.) seedlings in response to boric acid fertilization. This response was accompanied by a decrease in root indole-3-acetic acid (IAA) content. Other research investigations have associated the physiological function of boron in plants with root growth. An experiment has been conducted to assess the root system morphology of shortleaf pine seedlings inoculated or noninoculated with *P. tinctorius* and fertilized with none or 25 µg/ml boric acid. Results suggest that boric acid fertilization may enhance lateral root branching and elongation of shortleaf pine seedlings inoculated with *P. tinctorius*. This response, attributed to interaction between ectomycorrhizal root IAA and boric acid, may have resulted in increased ectomycorrhizal colonization.

Introduction

In the regeneration environment, survival of conifer seedlings is dependent upon efficient root growth immediately following planting. Both root morphology and elongation rate contribute to efficient root growth and are responsible for interfacing between root and soil. Past research has indicated that physiological processes within the root system may be manipulated by exogenous stimuli such that root

morphology and elongation rate are altered (Hess 1969, Torrey 1986). Perhaps, exogenous stimuli could be used to modify the morphology and elongation rate of nursery- and greenhouse-grown conifer seedling root systems resulting in increased interfacing between root and soil. Direct advantages of this response would include increased exposure to indigenous mycorrhizal propagules and subsequent enhancement of mineral nutrient and water absorption. Finally, survival following out-planting, would improve.

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The micronutrient, boron, has been identified as necessary for emergence of root primordia (Cohen and Lepper 1977; Jarvis et al., 1984; Ali and Jarvis 1988). Early work of Warington (1923) identified boron as essential for plant growth and

indicated that it has a strong effect on root system morphology. Further research has indicated that boron is necessary for root elongation (Whittington 1959, Albert 1965, Cohen and Lepper 1977, Tang and dela Fuente 1986).

Synergism between auxin compounds, produced by ectomycorrhizal fungi, and boron may result in both increased root primordia development and increased root primordia elongation such that root system morphology is improved. Promotion of root growth by boron has been attributed to an interaction between auxins and boron (Jarvis et al., 1984; Ali and Jarvis 1988). Ali and Jarvis (1988) found that an optimum exogenous concentration of either indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) and boric acid resulted in maximum adventitious lateral root formation in mung bean (*Phaseolus aureus* Roxb. cv. Berlin) cuttings. Interaction between IAA and boron has also been reported by Mitchell et al. (1986) who found that ectomycorrhizal inoculation of shortleaf pine (*Pinus echinata* Mill.) seedlings with *Pisolithus tinctorius* [(Pers.) Coker & Couch] resulted in elevated root IAA content. However, boric acid fertilization of these ectomycorrhizal seedlings resulted in a dramatic reduction in IAA level. In addition, boric acid fertilization has resulted in increased ectomycorrhizal colonization of shortleaf pine by *P. tinctorius* (Mitchell et al., 1987).

The objectives of this experiment were to evaluate root system morphology and ectomycorrhizal colonization of 12-week old greenhouse-grown shortleaf pine seedlings inoculated or not inoculated with *P. tinctorius* and fertilized with 0 or 25 µg/ml boric acid. Specifically, lateral root hierarchy, short roots and root primordia were quantified. Subsequently, the relationship between root system morphology and ectomycorrhizal colonization by *P. tinctorius* was assessed.

Materials And Methods

Full-sib shortleaf pine seed (improved source: USDA Forest Service, Arkansas) was surface sterilized in 0.5 percent NaClO (10 percent Cloroxtm) for 15 seconds and cold stratified for 30 days at 4°C (USDA 1974). The growth medium was 1:1:2:2 (v/v/v/v) peat moss-vermiculite-sand-perlite which was sterilized with methyl bromide. Stratified seed were sown, four seed per cavity, in 0.5 L Tinus Spencer-LeMaire root trainer containers. One-half of the containers were inoculated, 1:7, with vegetative inoculum of *P. tinctorius* isolate 306 (Mycorr Tech Inc., Pittsburgh, PA). Inoculation was done by thoroughly mixing 0.25 L of vegetative inoculum with 1.75 L of growth medium and pouring the 2.0-L volume into the four cavities of individual containers. Following germination, seedlings were thinned to one per cavity.

After 90 percent germination, a 16-h photoperiod was implemented using high-pressure sodium vapor lamps. Throughout this photoperiod, photosynthetically active radiation was approximately 544 µE m⁻² sec⁻¹. Seedlings were watered semiweekly throughout the initial 4 weeks of the cultural period. Four weeks following 90 percent germination, fertilization began.

Seedlings were fertilized semiweekly with 20 ml of modified Hoagland's nutrient solution (Mitchell 1984). Six weeks following 90 percent germination, boric acid fertilization treatments were initiated. With the exception of 0.006 μg boric acid in 20 ml volumes of the modified Hoagland's nutrient solution, seedlings received none or 0.5 mg of boric acid semiweekly. Following initiation of fertilization, seedlings were watered when the growth medium appeared dry.

This experiment utilized a randomized complete block design with four blocks. Treatments were no inoculation or inoculation with *P. tinctorius* and fertilization with 0 or 25 $\mu\text{g}/\text{ml}$ boric acid applied to the soil. Twelve weeks following 90 percent germination, 16 containers, one per treatment and block, were randomly harvested. Dependent upon analysis, data represent the mean of two or four seedlings per container. As a result, means represent measurements of 32 or 64 seedlings.

Root systems were washed free of growth medium using tap water. Growth parameters measured on four seedlings per container included shoot length, root collar diameter, number of branches, shoot and root dry weights (72 hr, 65°C), and root system length. Shoot length was defined as the distance from root collar to tip of stem. Root collar diameter was defined as the stem diameter at the root collar. Following assessment of ectomycorrhizal colonization and root system morphology, root system length was measured photoelectronically using the line intersect method of Rowse and Phillips (1974).

Two of four seedlings per container were randomly selected for quantification of ectomycorrhizal colonization and root system morphology. The remaining two seedlings were used for analysis of new lateral root growth.

Ectomycorrhizal colonization was determined using the method of Mitchell et al. (1987). The diffusion of ectomycorrhizal colonization throughout the root system was measured as the percentage of primary lateral roots colonized. Colonization was expressed as presence of at least one mycorrhizae on primary lateral roots. The intensity of ectomycorrhizal colonization was quantified as the number of mycorrhizae per infected primary lateral root. Mycorrhizae were characterized by the presence of a fungus mantle or swollen appearance when compared to uninfected short roots.

Following identification of primary lateral roots, secondary and tertiary lateral roots and short roots were quantified using a stereoscope. Criteria used for identification of lateral roots and their position in root system hierarchy were a visual diameter less than that of adjacent short roots, emergence of one or more short roots and branching from an immediately lower order lateral root. Short roots were defined as roots which were less than or equal to 0.5 cm in length and which lacked any emerging roots .

Following identification of lateral root hierarchy and quantification of short roots, 0.25 g of root tissue was randomly subsampled and stained for quantification of root primordia. Using a modification of the procedure of Wilcox (1968), root tissue was cleared in 0.5 percent NaClO (10

percent CloroxTM) for 1 hour, rinsed three times with distilled water, submersed in distilled water for 1 hour, and then submersed in 0.001 percent aqueous safranin. Following 24 hours, meristematic areas of root primordia were visible through root cortical tissue and were counted using a stereoscope.

New lateral root growth was excised from two seedlings per container. New roots were counted and their lengths measured. Criteria used for identification of new roots were lightness in color when compared with remaining root system and a length greater than or equal to 0.5 cm.

Data were subjected to an analysis of variance. Differences between treatment means were compared using the LSD test at $P \leq 0.05$ and $P \leq 0.10$.

Results

Shoot and root growth of shortleaf pine seedlings inoculated with *P. tinctorius* were significantly greater than those of noninoculated seedlings (Table 1). Six weeks following initiation of semiweekly boric acid applications, shoot growth of 12-week-old seedlings was not significantly affected by boric acid treatment. However, boric acid fertilization treatment resulted in a significant increase in the root system length of seedlings inoculated with *P. tinctorius* when compared with those inoculated but not receiving boric acid fertilization treatment. No boric acid fertilization treatment effect was observed in either the root dry weights of inoculated or noninoculated seedlings or the root system length of noninoculated seedlings.

Table 1. Growth of 12-week-old shortleaf pine seedlings inoculated or not inoculated with *Pisolithus tinctorius* and fertilized with 0 (-B) or 25 $\mu\text{g/ml}$ (+B) boric acid.

Variable (units)	Noninoculated		Inoculated	
	-B	+B	-B	+B
Shoot length (cm)	4.3 a*	4.6 a	4.8 a	5.0 a
Root collar dia. (cm)	0.16b	0.15b	0.19a	0.19a
Number of branches	3.6 b	4.0 b	5.5 a	5.6 a
Shoot dry weight (g)	0.18b	0.18b	0.34a	0.38a
Root dry weight (g)	0.09b	0.08b	0.15a	0.15a
Root system length (cm)	249 bBC	247 bC	289 abB	339aA

* Means within a variable followed by the same lower or uppercase letter are not significantly different at $P \leq 0.05$ or $P \leq 0.10$, respectively, using the LSD test.

Quantification of lateral root hierarchy indicated that, although no significant differences were found. Boric acid fertilization treatment appeared to increase lateral root branching of seedlings inoculated with *P. tinctorius* (Table 2). The number of primary lateral roots of 12-week-old shortleaf pine seedlings was unaffected by ectomycorrhizal inoculation and boric acid fertilization treatments. However, the numbers of secondary and tertiary lateral roots on root systems of seedlings inoculated with *P. tinctorius* were increased 41 and 74 percent, respectively, due to boric acid fertilization treatment.

Table 2. Quantification of lateral root hierarchy associated with 12-week-old shortleaf pine seedlings inoculated or not inoculated with *Pisolithus tinctorius* and fertilized with 0 (-B) or 25 $\mu\text{g/ml}$ (+B) boric acid.

Variable (units)	Noninoculated		Inoculated	
	-B	+B	-B	+B
Primary lateral roots (#)	20.1*	20.2	19.9	20.1
Secondary lateral roots (#)	16.8	18.2	18.2	25.7
Tertiary lateral roots (#)	0.7	2.5	2.3	4.0

* No significant differences were detected at $P < 0.05$ or $P < 0.10$ using the LSD test.

New root growth of 12-week old seedlings was significantly affected by both inoculation and boric acid fertilization treatments (Table 3). The number of actively elongating lateral roots was unaffected by inoculation and boric acid fertilization treatments. However, the length of this actively elongating lateral root tissue was significantly greater in inoculated seedlings when compared to that of noninoculated seedlings. Moreover, boric acid fertilization treatment significantly increased new lateral root length of noninoculated seedlings, relative to entire root systems. Although not significant, boric acid fertilization treatment also resulted in a 15 percent increase in the percentage of new lateral root length in inoculated seedlings.

Of shortleaf pine seedlings inoculated with *P. tinctorius*, those fertilized with boric acid had significantly fewer root primordia when compared with inoculated seedlings not receiving boric acid fertilization treatment (Table 4). Seedlings inoculated with *P. tinctorius* had a significantly greater quantity of root primordia when compared to those that were not inoculated. In contrast, inoculated and noninoculated seedlings that were fertilized with boric acid had similar quantities of root primordia.

Inoculation with *P. tinctorius* significantly reduced the number of short roots per length-of root system when compared to noninoculated seed-

lings (Table 4). Boric acid fertilization treatment had no significant effect on the quantity of short roots on 12-week-old seedling root systems. However, seedlings inoculated with P. tinctorius and fertilized with boric acid did have 16 percent more short roots when compared to inoculated seedlings not receiving boric acid fertilization. This response can be attributed to the boric acid-induced increase in root system length of seedlings inoculated with P. tinctorius, as previously reported.

Table 3. New lateral root growth of **12-week-old** shortleaf pine seedlings inoculated or not inoculated with Pisolithus tinctorius and fertilized with 0 (-B) or 25 $\mu\text{g/ml}$ (+B) boric acid.

Variable (units)	Noninoculated		Inoculated	
	-B	+B	-B	+B
Number of new roots *	14.0 a**	14.6 a	16.1 a	18.9 a
Length of new roots (cm)	12.6 c	18.2 bc	23.5 ab	30.4 a
Percent new root length/ total root length	3.9 b	6.1 a	6.6 a	7.6 a

* New roots were characterized as lateral roots that were white or light in color and > 0.5 cm in length.

** Means within a variable followed by the same letter are not significantly different at $P < 0.05$ using the LSD test.

Table 4. Primordia, ectomycorrhizae, and short roots associated with root systems of **12-week-old** shortleaf pine seedlings inoculated or not inoculated with Pisolithus tinctorius and fertilized with 0 (-B) or 25 $\mu\text{g/ml}$ (+B) boric acid.

Variable (units)	Noninoculated		Inoculated	
	-B	+B	-B	+B
----- Root primordia -----				
Number/cm root length	0.15 b	0.20 b	0.48 a	0.15 b
Ectomycorrhizae + Short Roots (uncolonized)				
Number/seedling	442 a *	467 a	395 a	460 a
Number/cm root length	1.8 a	2.0 a	1.4 b	1.4 b

* Means within a variable followed by the same letter are not significantly different at $P < 0.05$ using the LSD test.

Assessment of ectomycorrhizal colonization indicated that boric acid fertilization treatment significantly increased the spread of P. tinctorius colonization (Table 5). The percentage of primary lateral roots colonized by P. tinctorius was 22 percent greater in response to boric acid fertilization. However, the intensity of ectomycorrhizal colonization on seedling root systems was unaffected by boric acid fertilization.

Table 5. Ectomycorrhizal colonization of 12-week-old shortleaf pine seedlings inoculated or not inoculated with Pisolithus tinctorius and fertilized with 0 (-B) or 25 $\mu\text{g/ml}$ (+B) boric acid.

Variable	Noninoculated		Inoculated	
	-B	+B	-B	+B
Percent primary lateral root infection *	6.2 bC**	0.3 bC	77.8 aB	94.8 aA
Infection points/primary lateral root	2.8 b	0.6 b	14.1 a	14.2 a

* Percent primary lateral root infection = (Number of colonized primary lateral roots/total number of primary lateral roots) x 100.

** Means within a variable followed by the same lower- or uppercase letter are not significantly different at $P < 0.05$ or $P < 0.10$, respectively, using the LSD test.

Discussion

Past research has suggested that boric acid fertilization is beneficial for synthesis of mycorrhizal symbioses. However, the effect of boric acid fertilization on mycorrhizal colonization appears to be dependent upon host and fungus species. Lambert et al. (1980) found that boric acid fertilization of alfalfa (Medicago sativa L.) seedlings inoculated with Glomus fasciculatus [(Thaxt. sensu Gerd.) Gerd. & Trappe] resulted in an 88 percent increase in root infection. However, they also found that boric acid fertilization of alfalfa seedlings inoculated with Glomus mossae [(Nicol. & Gerd.) Gerd. & Trappe], and red clover (Trifolium pratense L.) seedlings inoculated with Gigaspora gigantea [(Nicol. & Gerd.) Gerd. and Trappe] resulted in only 10 and 16 percent increases in root infection, respectively.

In the present experiment, boric acid fertilization had no effect on the number of mycorrhizae per infected primary lateral root of 12-week-old shortleaf pine seedlings inoculated with P. tinctorius. However, fertilization with boric acid did result in a slight enhancement in the spread of colonization by P. tinctorius throughout the root system. The 22-percent increase in primary lateral root infection is similar in some ways to results obtained by Mitchell et al. (1987). Using similar host and fungus

species, Mitchell et al. (1987) found 111 and 53 percent increases in the number of mycorrhizae per infected primary lateral root and the percentage of primary lateral roots infected by *P. tinctorius*, respectively, in response to boric acid fertilization following a 16-week cultural period. Similarities in the trend but differences in the intensity of the ectomycorrhizal response to boric acid fertilization treatment suggests that, in addition to host and fungus species, seed and fungal isolate sources as well as cultural environment may dictate the effect of boric acid fertilization on mycorrhizal colonization. Variation in colonization rates between the present experiment and that of Mitchell et al. (1987) illustrates the sensitivity of the shortleaf pine-*P. tinctorius* ectomycorrhizal association to boric acid fertilization and the need for better definition of conditions under which optimum ectomycorrhizal colonization is obtained.

Root morphological changes often accompany enhanced mycorrhizal colonization which occurs in response to boric acid fertilization. Lambert et al. (1980) found that the root system length of 15- and 30-day-old endomycorrhizal alfalfa seedlings increased 100 and 92 percent, respectively, due to boric acid fertilization. Mitchell et al. (1987) found a reduction in the number of primary lateral roots of 16-week-old shortleaf pine seedlings inoculated with *P. tinctorius* due to, boric acid fertilization. However, although not significant, Mitchell et al. (1987) also found 38 and 17 percent increases in root dry weights of 16-week-old shortleaf pine seedlings inoculated with *P. tinctorius* and foliar- and soil-fertilized with boric acid, respectively. An increase in root dry weight but a reduction in number of primary lateral roots of shortleaf pine seedlings inoculated with *P. tinctorius* in response to boric acid fertilization suggests that lateral root branching and/or short root production may have been altered.

Following a 12-week cultural period, we found that root dry weights of shortleaf pine seedlings inoculated with *P. tinctorius* were unaffected by boric acid fertilization. However, the root system length was significantly greater than that of seedlings inoculated with *P. tinctorius* but not fertilized with boric acid. Perhaps specific fungus-host interactions in the presence of boric acid resulted in a physiological environment which was conducive to root system development.

Past research has suggested that *P. tinctorius* is capable of producing indole-3-acetic acid (IAA) (Ek et al., 1983; Mitchell et al., 1986). Mitchell et al. (1986) determined the IAA content of uninoculated shortleaf pine seedling root tissue as approximately 120,000 ng/mg dry weight. Ek et al. (1983) found that one isolate of *P. tinctorius*, grown *in vitro* for 5 weeks, produced 19,760 ng/mg dry weight; whereas, a second isolate of *P. tinctorius* only produced 840 ng/mg dry weight when grown *in vitro* for the same period of time. Ectomycorrhizal fungus-produced IAA may supplement endogenous compounds. As a result, inoculation with an isolate of *P. tinctorius*, known to produce large quantities of IAA, may result in elevated root tissue IAA content. This was demonstrated by Mitchell et al. (1986) who found a 2.25-fold increase in the IAA concentration of shortleaf pine seedling root tissue due to inoculation with *P. tinctorius*. Physiological functions, such as those involved in root development, which are regulated by IAA, may be modified by ectomycorrhizal fungus-induced elevated root IAA content.

Boric acid fertilization may further modify the physiological environment in which root development occurs. Furthermore, boron nutrition has been associated with the metabolism of IAA in plants. Boron may regulate transport of IAA from the site of synthesis by altering membrane function (Pollard et al., 1977; Goldbach and Amberger 1986; Schon et al., 1990). As a result, boron deficiency may be associated with elevated IAA concentrations (Smirnov et al., 1977). In addition, past research has indicated that catalytic oxidation of IAA may be modified by boron nutrition (Smirnov et al., 1977; Bohnsack and Albert 1977; Mitchell et al., 1986).

Interaction between fungus-induced elevated IAA and boron may result in alteration of root morphology and subsequently, mycorrhizal colonization. Root developmental processes which may be influenced by interaction between ectomycorrhizal fungus-induced, elevated IAA and boric acid include the rate of root primordia formation, the quantity of root primordia which elongate and root elongation rate (Torrey 1956; Pecket 1957; Whittington 1959; Blakely 1972; Cohen and Lepper 1977; Jarvis et al., 1984; Ali and Jarvis 1988; MacIsaac et al., 1989). In our experiment, the number of primordia in root systems of shortleaf pine seedlings inoculated with *P. tinctorius* but not fertilized with boric acid compared to those of seedlings in the remaining three treatments, suggests that inoculation with *P. tinctorius* may have resulted in higher concentrations of root IAA which-stimulated induction or initiation phases of root primordia formation.

Following enhanced development of organized root primordia under conditions of elevated IAA, root primordia elongation in shortleaf pine seedlings inoculated with *P. tinctorius* may have been inhibited. Although elevated root IAA content may be beneficial for development of root primordia, these conditions may be inhibitory toward their elongation (Thimann 1936; Blakely et al., 1972; Jarvis et al., 1984). Other studies have suggested that root primordia elongation may be regulated by both auxin and cytokinin (Blakely et al., 1972; Wightman and Thimann 1980; MacIsaac et al., 1989).

Application of boron to primary roots of seedlings or the hypocotyl of cuttings promotes root primordia elongation (Whittington 1959; Albert and Wilson 1961; Cohen and Leppers 1977; Jarvis et al., 1984; Ali and Jarvis 1988). Cohen and Leppers (1977) determined that boron was necessary for cytokinesis in root meristems. Other studies have indicated that boron may regulate cytokinesis by modification of ribonucleic acid (RNA) metabolism (Dave and Kannan 1980, Ali and Jarvis 1988).

In our experiment, after 12 semiweekly applications of 20 ml of 25 µg/ml boric acid, lateral root branching of seedlings inoculated with *P. tinctorius* was enhanced. Perhaps inoculation with *P. tinctorius* resulted elevated root IAA content such that the rate of root primordia production increased. Initiation of boric acid fertilization treatments 6 weeks following 90 percent germination may have led to either reduced IAA content or a more favorable IAA:cytokinin ratio such that the number of primordia elongating into lateral or short roots was increased. As a result, root primordia may have been "released" for elongation leading to an increase in secondary and tertiary lateral roots. This sequence of events may explain

both reduction in the number of root primordia and increase in the number of secondary and tertiary lateral roots of inoculated shortleaf pine seedlings following boric acid fertilization.

We found that new lateral root growth was stimulated by boric acid fertilization. The percentage of root system which was new lateral root growth increased 56 percent in noninoculated seedlings, but only 15 percent in seedlings inoculated with *P. tinctorius*. These results suggest that the action of boric acid may not have been as strong in inoculated as in non-inoculated seedlings. However, lateral root branching analyses do not support this possibility. More likely, the availability of boric acid for interaction with IAA and subsequent modification of root morphology may have become increasingly limiting in seedlings inoculated with *P. tinctorius*. As the cultural period progressed, development of a dense fungal network, both internal and external to the root system, may have restricted boric acid movement into the root and, as a result, reduced the effect of boron on root development of ectomycorrhizal seedlings when compared to that of noninoculated seedlings.

In our study, boric acid fertilization was initiated early during development of the ectomycorrhizal association. At this time, boric acid may have easily diffused through the growth medium and into root tissue. As a result, lateral and short root formation may have been enhanced by interaction between *P. tinctorius*-produced IAA and boric acid. However, further development of the ectomycorrhizal association, which was accompanied by proliferation of the fungus mantle, extramatrical hyphae and rhizomorph-like structures, may have presented an increase in potential sites for fixation of boric acid. As a result, less boric acid may have diffused to locations of primordia development within the host.

In conclusion, our findings indicate that boric acid fertilization of shortleaf pine seedlings inoculated with *P. tinctorius* resulted in an increase in root system length which was attributed to an increase in lateral root branching and elongation. This response may be valuable for improvement of conifer seedling root system morphology and synthesis of ectomycorrhizal associations. However, results of this study also suggest that further definition of fungus, host and cultural environment characteristics as well as the optimum timetable for boric acid fertilization is necessary for maximization of the root morphological response to boric acid fertilization.

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