Effects of Plant Growth-Promoting Rhizobacteria on Loblolly and Slash Pine Seedlings

S.A. Enebak, G.Wei, and J.W. Kloepper

ABSTRACT. Loblolly and slash pine seed were inoculated at sowing with 1 of 12 different strains of plant growth-promoting rhizobacteria (PGPR) in the greenhouse. Time to germination and seedling densities were determined at 21 days, and seedling biomass was measured at 12 wk after sowing. All bacterial strains significantly increased the speed of seedling emergence over nontreated pine seed. By 12 wk, however, no differences in stand densities were observed between bacteria-treated and nontreated seed for either pine species. Postemergence damping-off was reduced in loblolly pine when seed was treated with 3 of the 12 bacterial strains; however, postemergence damping-off on slash pine seedlings was not affected by rhizobacteria. Treatment with rhizobacteria had a significant positive and negative effect on seedling growth and biomass, which depended on tree species. Loblolly pine shoot and root lengths, as well as the above- and belowground biomass, were significantly reduced when seeds were treated with strains BS1 and BS2. In contrast, loblolly pine seeds treated with strains BS3, PM2, and INR7 significantly increased the below ground biomass of the seedling root systems. Slash pine seedlings had similar interactions with the bacterial strains. Strain BS1 significantly reduced shoot lengths compared with nontreated seeds, while strains 90-166, INR7, and SE49 increased shoot biomass. Slash pine root lengths and biomass were also reduced when treated with strains BS1 and BS2. Unlike loblolly pine, no bacterial strain increased slash pine root length or biomass. This study suggests that the effects of rhizobacteria inoculation on seedling emergence and plant growth are independent and that the effects are species specific. FOR. SCI. 44(1):139-144.
Additional Key Words: Germination, *Pinus taeda* L., *Pinus elliottii* (Engelm.), PGPR, seedling growth.

BAREROOT AND CONTAINERIZED forest tree nurseries in the southern United States currently produce on average 1.1 billion seedlings annually for reforestation programs (Moulton et al. 1995). This intensively managed forest crop is subject to serious foliar and soil-borne pathogens, insects, and weeds that are capable of causing hundreds of thousands of dollars in damage every year (Peterson and Smith 1975, Duryea and Landis 1984). One of the most effective treatments to combat these pests is the use of methyl bromide as a soil sterilant prior to sowing. However, due to concerns of methyl bromide's effect on the ozone layer, Clean Air Act legislation dictates that methyl bromide production be ended by the year 2001, effectively eliminating this compound as a treatment in forest tree nurseries (Anonymous 1993, 1995). There is currently a national effort to identify a replacement for methyl bromide by the year 2001.

One possible alternative to methyl bromide could be the use of specific biological treatments tailored to forest tree nurseries. Plant growth-promoting rhizobacteria (PGPR) are naturally occurring soil bacteria that are able to aggressively colonize plant roots and stimulate plant growth when applied to roots, tubers or seeds (Kloepper et al. 1980, Weller 1988). Some of the other reported benefits of using PGPR include the ability to control soil-borne fungi, enhance plant survival, and induce systemic resistance to foliar pathogens (Liu et al. 1995). While PGPR research on agricultural crops has been ongoing for nearly 15 yr, research on the effects of PGPR on tree species has just begun. There is no *a priori* reason to suggest that PGPR isolates would not be effective against plant pathogens in either forest nurseries or plantations. Indeed, the same pathogenic fungi that have been examined on other agricultural crops are common to both forest nursery and field settings; these include *Fusarium* sp. (Leeman et al. 1995), *Phytophthora* sp. (Lifshitz et al. 1987), Pythium sp. (Howell and Stipanovic 1980), and *Rhizoctonia* sp. (Turner and Backman 1991).

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A number of questions need to be answered before PGPR could be used as an effective tool in forest tree nursery programs. Evidence suggests that there is an important host response specificity in tree species when treated with PGPR (O'Neill et al. 1992, Chanway and Holl 1993) that may be related to local environmental and geographical conditions. Seedling growth response specificity following inoculation with PGPR could be an impediment to the development of effective seed or root inoculates for use in nursery or outplanting programs. More importantly, while a few PGPR strains have been shown to increase seedling growth of a few northern conifers, rhizosphere colonization of southern tree species has yet to be examined. The potential benefits to the forest resource community of using PGPR as either a disease control treatment, to maximize growth promotion, or to increase stand survival are enormous. These greenhouse studies were undertaken to determine if bacterial strains shown to have growth promotion on other agronomic crops would have similar effects on two important conifer species. Specific items examined included how bacterial-treated seeds affected the speed and percent germination, as well as seedling growth promotion, of commercially important pine species in the southern United States.

Materials and Methods

Loblolly *(Pinus taeda L.)* and slash *(Pinus elliottii* Engelm.) pine seed were sown in Ray-Leach® containers (Pine Cell 150 cm^3, 4.0 cm diameter x 20.0 cm deep, Canby, OR) filled with a nonsterile peat-vermiculite potting medium amended with Osmocote® 18-6-9:8-9 month nutrient release at 4 g/L peat (The Scotts Company, Marysville, OH). Loblolly and slash pine families (half-sib) represented second-generation planting material and were collected from seed orchards maintained in Glennville, GA by Rayonier, Inc. Prior to sowing, stratified seed (moistened, then stored at 0°C for 45 days) was surface sterilized with a solution of hydrogen peroxide (10 mL of a 30% solution in 90 mL water) to eliminate any seed-borne organisms. Three days prior to inoculation, 12 rhizobacteria (Table 1) were removed from -80°C storage and streaked onto TSA plates (Tryptic Soy Agar, Difco®, Detroit, MI) and incubated at 21°C for 24 hr. The rhizobacteria used in these studies were from the culture collection of Dr. J.W. Kloepper maintained at the Department of Plant Pathology, Auburn, University, Auburn, AL (Table 1). These 12 bacterial strains were chosen because they had been shown to have either plant growth-promoting properties or disease controlling properties on other agronomic crops (Table 1). From the TSA streak plate, a single colony was-chosen, and another TSA plate (working plates) was streaked with the rhizobacteria and incubated at 21 °C for 24 hr. Suspensions were made by flooding the working plates with 10 mL of sterile water and, using a sterile disposable loop, the bacteria were scraped off the agar surface and poured into 140 mL water. One mL of this bacterial suspension yielded approximately 10^8 cfu/mL. Each cavity received one pine seed and then was immediately inoculated with 1 mL of bacterial suspension. Control containers received the same volume of water without bacteria. Inoculated seeds were covered with washed sand and watered to saturation. After 4 wk seedlings were fertilized with 0.350 g of 20- 10- 10 (Sternes - Miracid®, Port Washington, NY) and 0.020 g $FeSO_4$ per liter once a week.

Data collected included the number of seedlings that had emerged at 3 day intervals (germination speed) until germination was completed, 4 wk after sowing. At this time germination percent was also determined for each half-sib family. At 12 wk, final stand densities and seedling mortality since emergence were recorded. Seedlings were removed from the containers, roots were separated from shoots, and shoot height and root length were measured. Seedling material was then dried for 48 hr (70°C) before root and shoot weights were recorded. The experiment was repeated three times and the data pooled for each species. Data were analyzed using SAS Version 6.10, and differences between treatments used Dunnets' test which

Table 1. Bacterial strains used to inoculate loblolly and slash pine seed, and the beneficial aspect reported on other agronomic crops.'

Strain	Species	Crop	Benefit
90-166	*Serratia marcescens*	Cucumber	Induce plant resistance (Liu et al. 1995)
BS1	*Bacillus subtilis*	Peanut	Control soil-borne pathogens (Turner and Backman 1991)
BS2	*Bacillus subtilis*	Cotton & snap bean	Control soil-borne pathogens (Kloepper 1993)
BS3	*Bacillus subtilis*	Cotton & snap bean	Control soil-borne pathogens (Kloepper 1993)
PM1	*Paenibacillus macerans*	Cucumber	Control soil-borne pathogens (unpublished data-Wei, G.)
PM2	*Paenibacillus macerans*	Cucumber	Control soil-borne pathogens (unpublished data-Wei, G.)
INR7	*Bacillus pumilus*	Cucumber & tomato	Induce desease resistance (Wei et al. 1996)
SE34	*Bacillus pumilus*	Cucumber & tomato	Induce disease resistance (Jetiyanon 1997)
SE49	*Bacillus pumilus*	Cucumber	Induce disease resistance (Jetiyanon 1997)
SE52	*Bacillus pumilus*	Cucumber	Induce disease resistance (Jetiyanon 1997)
SE56	*Bacillus sphaericus*	Cucumber	Induce disease resistance (Jetiyanon 1997)
T4	*Bacillus pumilus*	Cucumber	Induce disease resistance (Jetiyanon 1997)

[1]**Bacterial strains were from the culture collection of J.W. Kloepper maintained at the Department of Plant Pathology, Auburn, University, Auburn, AL.**

Table 2. Germination speed of loblolly and slash pine seedlings from seed inoculated with plant growth-promoting rhizobacteria.

	Species	
Bacterial strain	Loblolly	Slash
90-166	4.25*	4.28*
BS1	4.20*	4.23*
BS2	4.10*	4.33*
BS3	3.99*	3.99*
PM1	4.04*	4.15*
PM2	4.15*	4.15*
INR7	4.25*	4.12*
SE34	4.31*	4.15*
SE49	3.86*	3.96*
SE52	4.02*	3.96*
SE56	4.25*	4.17*
T4	3.86*	3.88*
Control	3.54	3.62

[1]**Germination speed determined as the number of seedlings emerged from potting media as a percentage of total possible sown divided by number of days (21) since sowing. Differences noted with asterisk indicate significant increase in germination speed over nontreated controls (based on Dunnet's test; *n=235).* Differences between bacterial isolates were observed for a number of comparisons; however, data is not presented.**

compared each bacterial treatment to the control (SAS Institute Inc., Cary, NC). Analyses of variance (ANOVA) were conducted for all measurement variables to determine the interactions among bacteria, species, and half-sib families.

Results

The loblolly and slash pine families used in this study contained a high percentage of viable seed. Germination ranged from 90 to 95% and 88 to 93% for loblolly and slash pine seedlots, respectively, when plated onto moistened filter paper in the laboratory. Sowing in potting media and inoculating with the 12 bacterial isolates had no effect on the germination percentage when compared to the moistened filter paper; germination ranged from 86 to 94% and 86 to 92% for loblolly and slash pine, respectively (Table 2). However, the treatment of pine seed with bacteria at the time of sowing significantly increased the speed of emergence over nontreated controls. On average, the bacterial treated seed emerged 1 day sooner than nontreated seed (Table 2). The effects of bacteria on seed and seedling mortality were quantified as pre- and postemergence damping-off, respectively. Pre-emergence damping-off (seed mortality) was unaffected by bacterial treatment of the seed. Nor did the bacteria increase seedling mortality throughout the duration of the study, with postemergence damping-off less than 3.5% for all treatments. However, three bacterial strains (BS1, BS3, and SE56) significantly decreased the amount of postemergence damping-off on loblolly pine when compared to the nontreated control seeds (Table 2).

The effect of rhizobacteria on seedling growth was either stimulatory, benign, or detrimental to the development of pine seedlings and depended on the tree species and bacterial strain used. Overall, treatment of loblolly pine with strains BS1 and BS2 had a negative effect on seedling growth when compared to the nontreated seedling (Table 3). These two isolates consistently produced smaller shoot and root lengths with correspondingly lower dry weights for both parameters. Inoculating loblolly pine seed with strains BS3 and PM2, however, resulted in a significant increase in seedling root biomass over the controls. Isolate INR7 had a stimulatory effect on the number of branches that were formed; however, this did not translate into larger biomass measurements for these treatments (Table 3). No other effects were noted with the remaining bacterial strains on loblolly pine.

Similar growth trends were noted on slash pine. Seed treated with strains BS1 and BS2 at the time of sowing had a negative effect on seedling shoot length and/or root lengths, resulting in smaller seedlings than nontreated seedlings. As observed with the loblolly pine seedlings, the reduction in shoot and root lengths resulted in a reduction in biomass measurements (Table 4). In contrast, treating slash pine seed with strain 90-166 resulted in a significant increase in shoot biomass over nontreated seeds. Bacterial strain PM1 was responsible for increasing the number of branches on the slash pine seedlings, but did not affect seedling shoot and root parameters. No other effects were noted with the remaining bacterial strains on slash pine.

Table 3. Characteristics of loblolly pine seedlings 3 months after treatment with 12 plant growth-promoting rhizobacteria

	Shoot		Root		Branches
Bacterial strain	Length (cm)	Weight (g)	Length (cm)	Weight (g)	#/seedling
90-166	13.6	0.41*	17.4	0.17	0.7
BS1	13.0*	0.43*	15.6*	0.15*	0.5
BS2	13.2*	0.47	15.9*	0.16*	0.6
BS3	14.6	0.51	17.8	0.24**	0.8
PM1	14.6	0.46	17.9	0.19	0.9
PM2	14.8	0.45	17.7	0.23**	0.9
INR7	14.7	0.47	17.5	0.19	1.1**
SE34	14.8	0.47	17.5	0.17	0.9
SE49	14.6	0.45	17.5	0.16	0.6
SE52	13.9	0.44	17.2	0.18	0.8
SE56	14.4	0.45	17.3	0.18	0.8
T4	13.3	0.44	17.4	0.18	1.0
Control	14.9	0.61	17.0	0.20	0.6

***Significant increase or ** significant decrease in seedling parameter measured over nontreated controls (based on Dunnet's test; *n* = 235).**

Discussion

The potential for using PGPR strains in southern nurseries to promote emergence of loblolly and slash pine was demonstrated, as all strains increased the speed of germination. Speed of germination is important in seedling production. Protracted or uneven seedling emergence in container or bareroot nurseries necessitates over-sowing, a procedure that wastes expensive seed collected from tree improvement programs. Also, the more rapidly that seeds emerge from nursery soil, the less chance soil-borne fungi have to colonize the seed and cause pre-emergence, postemergence, or latent infections after outplanting. In addition, a more rapid and uniform germination of seeds has been shown to result in a significant decrease in mortality, as well as an increase in plantable seedlings at the end of the growing season (Boyer et al. 1987).

Growth promotion by most PGPR strains was not evident; in fact, a number of strains reduced seedling growth and biomass of both loblolly and slash pine seedlings. Bacterial strains BS1 and BS2 reduced shoot and root length as well as the amount of biomass produced. Although not a desired trait when it comes to growing conifer seedlings, there have been previous reports of PGPR isolates stunting the growth of wheat and other grasses (Ho11 et al. 1988). The quantity of bacteria in suspension used in these trials (10^8 cfu/mL) was used to produce maximum colonization ability. These quantities have been shown to be effective with other conifer hosts (Ho11 and Chanway 1992, O'Neill et al. 1992). The negative growth impact on both conifer species by strains BS1 and BS2 was unfortunate, as both have excellent biological control properties to soil-borne pathogens commonly found in bareroot nurseries (Schippers et al. 1987, Turner and Backman 1991). The levels of bacteria used on these two tree species may have been detrimental to seedling growth, and a more optimal level of bacteria needs to be identified.

While the dose response may be an important factor in explaining the reduction in plant growth, other direct and indirect mechanisms may be involved in the growth promotion and growth reduction observed on loblolly and slash pine in these studies. Several methods have been suggested to explain the phenomenon of plant growth-promotion when agronomic crops are inoculated with rhizobacteria. These include increases in the nitrogen fixation, the production of auxin, gibberellin, cytokinin, ethylene, the solubilization of phosphorus and oxidation of sulfur, increases in nitrate availability, the extra-cellular production of antibiotics, lytic enzymes, hydrocyanic acid, increases in root permeability, strict competition for the available nutrients and root sites, as well as the induction of plant systemic resistance (discussed in Chanway 1997, Kloepper 1993). For each of the 12 bacterial strains used, one could expect any number of the above mechanisms to occur either alone or in concert on pine seedlings. The complexity of such interactions will require extensive examination of a particular strain and pine species to determine the specific mechanism of growth promotion or growth reduction reported here.

However, these trials used a nonsterile potting medium, and thus were not set up to monitor some of the biological control mechanisms against pathogens of conifer hosts mentioned above. Studies have shown that the positive aspects of these rhizobacteria are not evident unless the plant host is under normal field conditions (Kloepper and Schroth 1981, Kloepper 1993). Even in the absence of growth promotion with many of the bacterial strains used in these trials, a few strains increased the length and/or the biomass of seedlings. Such growth promotion in greenhouse-grown containers is promising, as the potential for seedling growth-promotion increases when these bacterial strains are used in field situations. Bareroot forest tree nurseries are at the mercy of soil-borne fungi and their effects on seedling production and quality (Brown 1985, Juzwik et al. 1988). The ability to produce disease-free, outplantable seedlings is contingent on the ability to control the fungi that feed on the seedling root systems. One of the most favorable factors in using bacteria for growth promotion is their ability to protect against the deleterious fungi and bacteria in the rhizosphere. This can be demonstrated as an increase in growth over nontreated seed. Since these seedlings were grown in a potting medium free of plant pathogens, the ability of the strains to limit the

Table 4. Characteristics of slash pine seedlings 3 months after treatment with 12 plant growth-promoting rhizobacteria.

	Shoot		Root		Branches
Bacterial strain	Length (cm)	Weight(g)	Length (cm)	Weight(g)	#/seedling
90-166	16.3	o.67*	17.5	0.27	0.8
BS1	14.7**	0.51	14.0**	0.19**	0.4**
BS2	15.3	0.57	13.7**	0.18**	0.4**
BS3	16.0	0.58	17.2	0.22	0.9
PM1	16.0	0.66	17.3	0.27	1.1*
PM2	15.8	0.59	17.2	0.23	0.9
INR7	16.1	0.69*	17.6	0.28	0.8
SE34	16.1	0.65	17.2	0.25	0.8
SE49	16.1	0.69*	16.8	0.25	0.9
SE52	15.5	0.63	16.8	0.22	0.9
SE56	16.5	0.64	16.8	0.24	0.8
T4	15.7	0.64	16.9	0.18**	0.9
Control	15.5	0.52	17.1	0.25	0.7

l Significant increase or ** significant decrease in seedling parameter measured over nontreated controls (based on Dunnet's test; *N* = 235).

effects of pathogens was minor. Future trials using these strains must incorporate pathogens commonly found in bareroot nurseries.

Standard nursery practices routinely involve top-pruning of seedlings to maintain a uniform seedling height and root collar diameter for outplanting purposes.Therefore, increases in seedling shoot height by bacterial inoculation may not necessarily be desirable. More important in seedling development and outplanting success is root health and root architecture, which have been shown to greatly influence seedling survival after outplanting (Trappe 1971, Wisniewski et al. 1991). Seedling root biomass was increased in loblolly pine with two PGPR isolates, and was due to an increase in the number as well as the size of first-order lateral roots (data not shown). This positive response occurred in the absence of deleterious root pathogens that are present in many nursery situations. Other bacterial strains used in these trials might have increased root biomass over nontreated controls as well, if root pathogens had been present. Trials that examine co-inoculations of soil-borne pathogens and specific PGPR strains could determine if root growth is increased in the presence of deleterious fungi.

While we identified a number of PGPR strains used in agriculture that could be useful in reforestation programs, a number of bacterial strains have been examined more extensively on conifers in Canada. For example, the use of Bacillus *polymyxa* resulted in a significant increase in the biomass of lodgepole pine *(Pinus contorta* Doug. ex Loud.) and white spruce *(Picea glauca* [Moench] Voss) seedlings 8 wk after sowing (Ho11 and Chanway 1992, O'Neill et al. 1992). Another effect reported on seedlings treated with this *B. polymyxa* was an increase in the rate and percent germination when treated prior to sowing (Chanway et al. 1991). This effect is similar to the data reported for the 12 PGPR strains in this study. Yet another PGPR isolate, *Burkholderia cepacia,* used in containerized nurseries in British Columbia, resulted in a significant decrease in the amount of *Fusarium oxysporium* on Douglas-fir roots *(Pseudotsuga menziesii* [Mirb.] Franco.), increased seedling biomass (Chanway et al. 1991), and increased seedling stand survival for up to two years after outplanting (Dr. M.S. Reddy, pers. comm. Agrium Inc., Saskatoon, Sask.). This study and these examples of other PGPR strains strongly suggest that the beneficial effects of using specific PGPR are not limited to agronomic crops and could be highly beneficial in forestry. Further studies are warranted to characterize the effects of emergence-stimulating bacteria or plant growth-promoting bacteria will have on seedlings before these can be considered for use under operational conditions in forest tree nurseries. Such studies should focus on "fine tuning" specific strains for use on individual tree species for growth promotion, biological control, or induced disease resistance reported for other agronomic crops.

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