Evidence for Induced Systemic Protection to Fusiform Rust in Loblolly Pine by Plant Growth-Promoting Rhizobacteria

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ABSTRACT

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Trials conducted in 1997 and 1998 tested eight strains of plant growth-promoting rhizobacteria (PGPR) for their capacity to induce systemic protection in loblolly pine to the causal agent of fusiform rust. Pine seeds were treated with bacteria at time of sowing, and seedlings were artificially inoculated with basidiospores of *Cronartium quercuum* f. sp. *fusiforme* 1 month later. Six months after basidiospore inoculation, seedlings were evaluated for the fusoid swelling or galls characteristic of rust infection. Compared with seedlings from seed not treated with bacteria, two bacterial isolates, *Bacillus pumilus* (SE34) and *Serratia marcescens* (90-166), significantly ($P = 0.05$) reduced the number of galls in 1997 and 1998. Combined data from 1997 and 1998 resulted in two additional isolates, *B. pumilus* (INR7) and *B. pumilus* (SE52), significantly ($P = 0.05$) reducing the number of galls. Averaged over both years, 31% of control seedlings were infected with fusiform rust, while those seedlings treated with bacterial strains SE34, 90-166, INR7, and SE52 had 13, 14, 15, and 16% infection, respectively. These four PGPR strains appear to have induced systemic resistance to fusiform rust in loblolly pine, resulting in less infection over nontreated control seedlings.

Additional keywords: ISR, *Pinus taeda*

Fusiform rust of southern pines, caused by *Cronartium quercuum* f. sp. *fusiforme*, is the most important disease in the southern forest industry. Losses to fusiform rust on loblolly (*Pinus taeda*) and slash (*P. elliotii* var. *elliottii*) pines in the southern United States are \$35 million annually (17). Considered an oddity in 1930 (3), this disease has kept many forest pathologists and geneticists occupied over the past 50 years attempting to limit its effects on pine regeneration and productivity. The disease is most prevalent in vigorously growing 1- to 8-year-old plantations. One hundred percent infection by age five is common in high rust hazard areas (17). Main-stem infections limit the use of the tree for either solid wood, particle board, or pulp wood products and increase the risk of main-stem breakage. While the amount (hectares) and severity (incidence) of forest land affected varies within the region, reports indicate that disease incidence is increasing at 3% per year (19). This is attributed to increased planting of susceptible pine hosts and the reduced use of fire to control the abundance of *Quercus* species.

In forest-tree nurseries, fusiform rust is controlled using the systemic fungicide Bayleton (triadimefon; Bayer, Kansas City, MO) as a seed treatment and again as a foliar spray until the threat of basidiospore infection has passed, which is about 3 months postsowing. After outplanting, however, the only practical strategy to reduce infection is the deployment of pine families with limited genetic resistance, as the widespread use of triadimefon in the forest is neither economical nor legal. A potential alternative to the use of fungicides to control fusiform rust is the use of plant growth-promoting rhizobacteria (PGPR) (2,8,9,23). Plant growth-promoting rhizobacteria are naturally occurring soil bacteria that aggressively colonize plant roots (27).

Reported benefits of PGPR include reduced damage by soil-borne fungi, enhanced plant survival, increased plant growth, and induced systemic resistance (ISR) to several plant pathogens (11,15). Induced systemic resistance is a process of active resistance dependent on the host plant's physical or chemical barriers activated by biotic or abiotic agents (14). The ISR stimuli were shown to be salicylic acid (4), avirulent pathogens (13), and non-pathogens such as rhizobacteria (26). Rhizobacteria have been used as seed treatments to induce resistance to many foliar and root pathogens on cucumber (*Cucumis* sp.) (26), bean (*Phaseolus* sp.) (4), tobacco (*Nicotiana* sp.) (22), tomato (*Lycopersicon*

sp.) (5), and radish (*Raphanus* sp.) (14). In these examples, ISR involves the elicitation of latent resistance mechanisms present in the host plant (24). Because phenotypic selection, artificial inoculations, and long-term progeny trials have identified quantitative differences in levels of rust resistance among pine genotypes (1,20), resistance mechanisms that could be stimulated by PGPR may be present in loblolly and slash pine.

A few of the PGPR strains used in the present study have promoted seedling growth and enhanced seed germination of both loblolly and slash pine seedlings (7). The ability of these bacteria to induce resistance to fusiform was untested. Inducing seedling field resistance to fusiform rust and reducing infection by as little as 1% could have substantial benefits to the forest product industry (19). We conducted experiments to determine if PGPR were capable of inducing resistance in loblolly pine seedlings to *C. quercuum* f. sp. *fusiforme*. Results of preliminary experiments have been reported (6).

MATERIALS AND METHODS

Seedling inoculation with PGPR.

Seeds from an open-pollinated, half-sib family (6008) of loblolly pine were collected from a second generation seed orchard in Glennville, Georgia, maintained by Rayonier, Inc. Prior to sowing, stratified seed (moistened, then stored at 3°C for 45 days) were surface-sterilized with a solution of hydrogen peroxide (10 ml of a 30% solution in 90 ml of water) to eliminate seed-infesting organisms. Three days prior to inoculation, eight rhizobacteria strains were removed from -80°C storage and streaked onto TSA plates (Tryptic Soy Agar; Difco, Detroit, MI) and incubated at 21°C for 24 h. Strains were from the culture collection of J. W. Kloepper maintained at the Department of Entomology and Plant Pathology, Auburn University, Alabama. These bacterial strains were chosen because they had previously demonstrated either disease reduction or induced systemic resistance to pathogens on agronomic crops. They included four *Bacillus pumilus* strains (INR7, SE34, SE49, SE52), *B. subtilis* (BS1), *B. sphaericus* (SE56), *Serratia marcescens* (90-166), and *Paenibacillus macerans* (PM2). From the TSA streak plate, a single colony was chosen, and another sterile TSA plate was streaked with the rhizobacteria and simi-

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larly incubated. After 24 h, bacterial suspensions were made by flooding the plate with 10 ml of sterile water and, using a sterile, disposable loop, the bacteria were dislodged from the agar surface and poured into 140 ml of water. Serial dilutions and plating onto TSA determined that this bacterial suspension yielded approximately 10^8 CFU/ml. Stratified loblolly pine seeds were sown in March 1997 and March 1998 in Ray-Leach containers (Pine Cell 150 cm³, 4 cm diameter $\times$ 20 cm deep, Canby, OR) filled with a nonsterile peat-vermiculite potting medium amended with Osmocote 18-6-9 (The Scotts Company, Marysville, OH). Each container cell received one pine seed and was inoculated with 1 ml of the bacterial suspension, which was pipetted onto the seed and potting medium. The control seed received 1 ml of sterile water. Inoculated seeds were covered with washed sand, and containers were watered to saturation. One month after germination, the systemic fungicide Bayleton DF (45 g a.i./ha) was used to treat five blocks of 20 seedlings each. These served as a positive control.

Basidiospore inoculations. Water oak (*Quercus nigra*) leaves, bearing the telia stage of *C. quercuum* f. sp. *fusiforme*, were gathered on the Auburn University campus in May 1997 and May 1998 and placed in moist chambers over acidified distilled water (16). Cast basidiospores were collected on 0.10- μ m filters. The filters were rinsed with distilled water and, using a hemacytometer, the spore suspension was adjusted to 90,000 basidiospores per ml. Ten container blocks, each consisting of 20 4-week-old seedlings representing one replication of each treatment (8 bacteria, 1 triadimefon treated, and 1 untreated) were then sprayed with 25 ml of the basidiospore suspension. Inoculated seedlings were immediately placed in moist chambers at 100% RH and 20°C for 24 h. This inoculation procedure was performed five times. After incubation, the seedling blocks were randomly placed on greenhouse benches, where they were maintained throughout the summer at approximately 27°C/20°C (day/night) using no artificial lights.

Seedling assessment and data analysis. The experiment consisted of five blocks of 20 seedlings for each of the 10 treatments in a randomized complete block design. Six months after inoculation, seedlings were examined for main-stem swellings or galls symptomatic of fusiform rust infection. Mortality and the number of galled stems were recorded for each treatment block of 20 seedlings. Seedlings were harvested and then separated into root and shoot portions, dried at 70°C for 48 h, and weighed. Dunnett's test ($P = 0.05$) was used to compare percent infection and seedling biomass differences between PGPR and Bayleton treatments and the nontreated (check) blocks (SAS Institute Inc., Cary, NC).

RESULTS

For both 1997 and 1998, bacterial seed treatments had no effect on either germination, seedling mortality, or seedling biomass. The range of infection as measured by gall formation ranged from 5 to 42% in 1997 and 3 to 25% in 1998 (Fig. 1A and B). Nontreated control seedlings had significantly more fusiform galls than triadimefon-treated seedlings, 30% versus 5%, respectively. Some bacterial treatments also significantly reduced basidiospore infection and subsequent gall formation in both 1997 and 1998. In 1997, bacterial strains 90-166, SE56, and SE34 had 11, 15, and 19%, fusiform rust infection, respectively, compared with 31% for the nontreated control seedlings (Fig. 1A). In 1998, bacterial strains SE34 and 90-166 had 5 and 2.5% fusiform rust infection,

respectively, compared with 25% for the nontreated control seedlings (Fig. 1B). When experiments were pooled over both years, 31% of nonbacteria treated seedlings were infected by *C. quercuum* f. sp. *fusiforme*, and those treated with SE34, 90-166, INR7, and SE52 had 13, 14, 15, and 16% infection, respectively (Fig. 1C).

DISCUSSION

Seed treatments with rhizobacteria strains *Bacillus pumilus* (INR7, SE34, SE52) and *Serratia marcescens* (90-166) significantly reduced the percentage of infected loblolly pine seedlings after artificial inoculation over nontreated controls. Rust infection in these four treatments ranged from 15 to 18% less than in the nontreated loblolly pine seedlings, which had 30% (Fig. 1C). These four strains have

Fig. 1. Percent loblolly pine seedlings with fusiform rust symptoms 6 months after sowing seed treated with plant growth-promoting rhizobacteria (PGPR) in (A) 1997, (B) 1998, and (C) pooled 1997 and 1998 data. Letters above a column indicate a significant decrease in infection measured over nontreated controls based on Dunnett's t test ($n = 5$, $P = 0.05$). BS1 = *Bacillus subtilis*, PM2 = *Paenibacillus macerans*, INR7 = *B. pumilus*, SE34 = *B. pumilus*, SE49 = *B. pumilus*, SE52 = *B. pumilus*, SE56 = *B. sphaericus*, and 90-166 = *Serratia marcescens*.

also induced resistance and protection in other crops against other pathogens (15,26). However, this is the first time PGPR have been shown to reduce infection of an obligate pathogen on a pine host and indicate that PGPR-mediated systemic protection occurs for fusiform rust. To confirm that this protection is truly ISR (12), studies on the physical and chemical barriers need to be completed.

The short-lived basidiospores of *C. quercuum* f. sp. *fusiforme* are produced typically in April and May, depending on the temperature and relative humidity (17). Rust management in the nursery uses timely applications of Bayleton to ensure rust-free seedlings for outplanting at the end of the growing season (10). However, after outplanting, infection of pine varies with the genetic makeup of the seedlings, the quantity of alternate hosts, and the local environment (17). Induced resistance in pine could significantly reduce the development of this important pine disease.

The duration of the disease protection observed in our studies is unknown. In pathosystems of agronomic crops, induced resistance has been reported to last for the length of the growing season with little or no apparent reduction in efficacy (26). In field-grown cucumber, PGPR decreased the amount of two different foliar diseases, anthracnose caused by *Colletotrichum orbiculare* and angular leaf spot caused by *Pseudomonas syringae* pv. *lachrymans*, over the growing season until late fall for 2 consecutive years. To be an effective treatment for fusiform rust in loblolly pine, a PGPR-mediated ISR needs to last for the initial 1- to 5-year period after outplanting when rust infection is most damaging.

Most of the \$35 million annual loss attributed to fusiform rust results from rust-associated mortality that is strongly correlated with infections before age 3 to 5 (18). For example, 71% of loblolly pine seedlings infected before age 3 died by age 15, compared with only 3% of those infected after age 3 (21). Only main-stem galls contribute significantly to mortality, and galls more than 38 cm from the main stem tend to be self pruning (25). Based on seedling size, infection at the nursery and during the first 3 years after outplanting will almost invariably be close enough to reach the main stem, resulting in mortality. It therefore seems reasonable to predict that a small percentage reduction in infection during the first year after outplanting would produce proportionally larger impacts on final plantation volume and value at harvest.

Thus, protecting seedlings for 2 years in the field can result in significant reduction in tree mortality over the life of the plantation.

Our experiment tested one basidiospore inoculation using a higher inoculum density than would occur naturally. In field trials of annual crops, resistance was expressed through numerous, naturally occurring multiple inoculation events (26). Multiple inoculation events of loblolly pine occur in forest tree nurseries and field plantings in the southeastern United States, since seedlings are exposed to basidiospores for about 2 months each spring. Future tests using several inoculations over time should be carried out to mimic more normal densities and infection periods.

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