# Evaluation of Trichoderma harzianum-Impregnated Clay Granules as a Biocontrol for Phytophthora cinnamomi Causing Damping-Off of Pine Seedlings

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ABSTRACT

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A molasses-amended, *Trichoderma harzianum*-infested preparation of attapulgus clay granules was tested in the greenhouse as a biocontrol for *Phytophthora cinnamomi*-induced damping-off of shortleaf pine seedlings. Tests were conducted in flats containing either autoclaved or nonautoclaved soil; flats were watered twice weekly or kept near saturation under a mist system. When incorporated into the soil simultaneously with the *P. cinnamomi* inoculum, *T. harzianum* was most effective in reducing disease incidence in flats that received water twice weekly; an additional application of Trichoderma-granules to the soil surface after 2 weeks resulted in a reduced seedling stand, indicating that leaching possibly provided a nutrient source for *P. cinnamomi*. Seedling survival was poorest in flats in which soil moisture was maintained at near saturation, indicating that *T. harzianum* could not compete and that the residual nutrients contained in the granules were utilized by *P. cinnamomi*. Examination of seedlings along demarcation lines between treatments in multi-treatment flats revealed that neither *T. harzianum* nor *P. cinnamomi* affected seedling survival in adjacent treatments; thus, growth and/or mobility of each organism beyond its respective inoculated area apparently was restricted. For nonautoclaved soil, evaluation of *T. harzianum* for control of *P. cinnamomi* was hindered owing to interactions with other damping-off pathogens. Results indicate that such a preparation of *T. harzianum* cannot be considered an effective biocontrol agent for *P. cinnamomi*.

*Additional key words: Pinus echinata,* blackstrap molasses.

*Phytophthora cinnamomi* Rands is an effective root pathogen on a wide variety of hosts (12, 13), and also is a damping-off pathogen of pine seedlings (4, 15). As a damping-off pathogen it can be expected to function within the top 15 cm of soil, the area most affected by cultural practices; chemical or biological agents can be delivered to this zone with relative ease. *Trichoderma* spp. also are most active within this same area of the soil and have been shown to be effective in reducing disease incidence in other crops (2, 14).

Little information is available concerning biological control of *P. cinnamomi*. Marx (8, 9) has shown that the mantles of ectomycorrhizal roots of pine serve as a barrier to penetration by *P. cinnamomi*, and Anderson (1) has reported that fumigation of soil with chloropicrin increased the population of *Trichoderma viride* and controlled pineapple root rot caused by *P. cinnamomi*. In laboratory tests, 11 of 30 *Trichoderma* isolates were antagonistic to *P. cinnamomi* and other *Phytophthora* spp. (H. D. Wells, *personal communication*). Laboratory tests also have shown that *T. harzianum* Rifai reduces enzymatic activities of *P. cinnamomi* in mixed culture in sterilized soil (6); in a similar study, chlamydospore production by the pathogen also was reduced (7). Thus, *T. harzianum* was selected as the test antagonist in this study. No published information is available concerning direct application to soil of antagonistic fungi to control *P. cinnamomi;* however, use of nutrient-enriched clay granules for growing such fungi and as a carrier to facilitate dispersal in the soil has been tested recently against other soil-borne pathogens (2). Such a system provides a mass of inoculum with a minimum of bulk that can be distributed and incorporated with standard agricultural equipment.

This study was conducted to evaluate *T. harzianum*-infested clay granules for control of *P. cinnamomi*-induced damping-off of shortleaf pine (*Pinus echinata* Mill.) seedlings under two soil moisture regimes in the greenhouse.

## MATERIALS AND METHODS

Sources of *P. cinnamomi* 1-281 and *T. harzianum* T-386 used in this study were previously reported (7). Soil used was a Dothan loamy sand from the Auburn Forest Nursery, Lee County, Alabama (Alabama Forestry Commission). Soil acidity was pH 5.4.

**Preparation of soil flats.**—Soil was screened through a 9.5-mm wire mesh sieve to remove rocks and debris, and 9.09 kg (20 lb) of soil (moist weight) was placed in each greenhouse flat (galvanized metal, 30 × 50 × 9 cm). Flats in the autoclaved soil portions of the experiments described below were autoclaved for 1 hour at 121 C and

one atmosphere (15 psi) pressure prior to infestation and planting; other flats in the nonautoclaved portions were infested and planted without further treatment.

The number of flats used and the treatments included in each of four experiments were as follows. The first experiment consisted of five treatments replicated three times. Treatments in autoclaved soil were (i) control, (ii) *T. harzianum*-granules, (iii) *P. cinnamomi,* and (iv) combination of *T. harzianum*-granules and *P. cinnamomi.* The fifth treatment (v) was a nonautoclaved soil control.

The second experiment was like the first except that 2 weeks after initiation of the experiment a second application of *T. harzianum*-granules was applied to the soil surface in all flats originally receiving this treatment.

The third experiment consisted of three flats containing autoclaved soil and three containing nonautoclaved soil. Soil in each flat was divided into quadrants of equal size and each quadrant received a different treatment: (i) control, (ii) *T. harzianum*-granules, (iii) combination of *T. harzianum*-granules and *P. cinnamomi,* and (iv) *P. cinnamomi.*

The fourth experiment was like the third except that after infesting the soil and planting was completed the flats were placed under a mist system calibrated to maintain the soil moisture level at saturation.

**Preparation of Trichoderma harzianum-granules.**—Attapulgus clay granules [700- to 246-$\mu$m (30/60 mesh), Englehard Corp., Minerals and Chemicals Division, Attapulgus, Georgia 31715] were mixed with a blackstrap molasses solution at a ratio of one part molasses to two parts clay granules (v/v). Blackstrap molasses solution consisted of 100 ml blackstrap molasses, 6 g $KNO_3$, and water to 1,000 ml. Clay granules were spread in aluminum foil-lined trays to a depth of about 12 mm and the molasses solution was evenly distributed over the granules. Trays were then covered with aluminum foil, autoclaved for 30 minutes (121 C), cooled to room temperature, and *T. harzianum* T-386 was added to the granules. Mycelium and agar from one-half of a standard-size petri dish containing a 6- to 10-day-old culture of *T. harzianum* on malt extract agar (5) was blended for 30 seconds in 80 ml of sterile water in a semi-microblender. The resulting suspension was decanted into a flask containing 160 ml of sterile water, the flask was swirled, and 40 ml of suspension was distributed uniformly over the surface of each 1,000 ml of clay granules. The granules then were held for 9 days at 26 C before being air-dried and stored at 5 C until used. The stored granules were used within 2 weeks; however, viability tests on water agar showed that *T. harzianum* remains viable under such conditions for up to 4 months.

In experiments 1 and 2, 200 g of the *T. harzianum*-granules was used in each flat. In experiments 3 and 4, appropriate quadrants of a flat received 50 g of the granules.

**Preparation of Phytophthora cinnamomi inoculum.**—Mycelial mats of 14-day-old cultures of *P. cinnamomi* growing on oatmeal-V-8 agar (10) were stripped from the medium and prepared as inoculum. Inoculum (500 ml) for each flat was individually prepared. For experiments 1 and 2, mycelial mats from five dishes were blended for 30 seconds in 400 ml of sterile water in a Waring Blendor. The resulting suspension was decanted into a sterile 500-ml screw-cap flask, the blender reservoir was rinsed and drained into the flask, and water was added to a total volume of 500 ml. Inoculum used in experiments 3 and 4 were prepared in a similar manner,

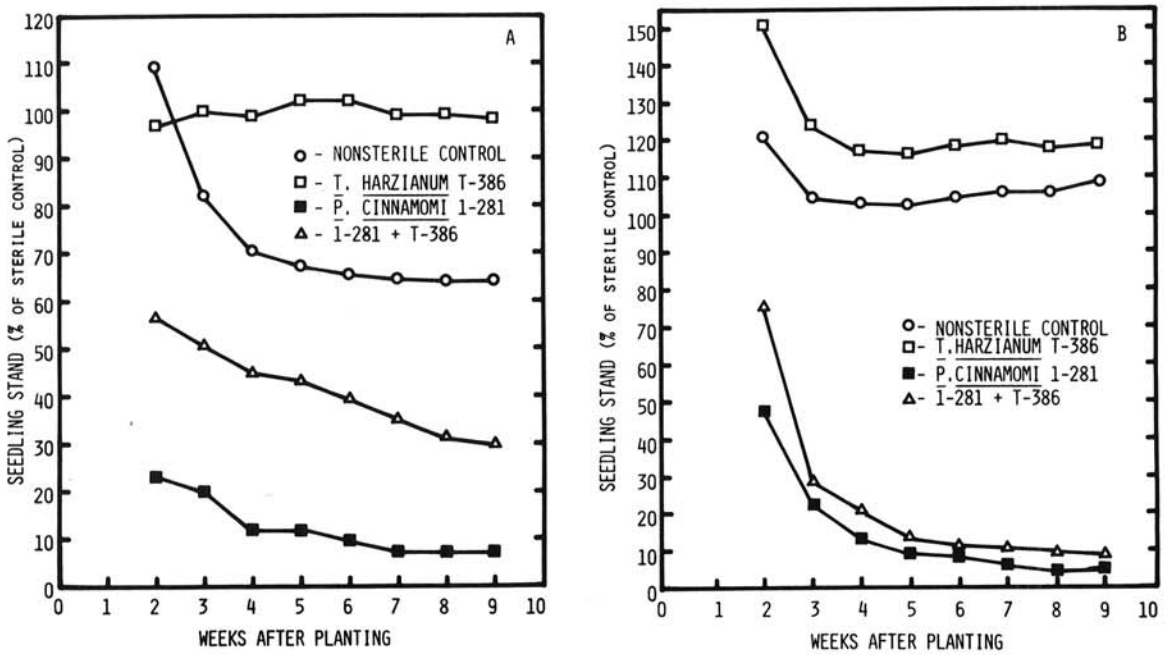


**Fig. 1-(A, B).** Effect of *Trichoderma harzianum* on incidence of damping-off caused by *Phytophthora cinnamomi* in soil flats watered twice weekly. Each flat contained one treatment. **A)** *T. harzianum* incorporated prior to planting. **B)** *T. harzianum* incorporated prior to planting, followed by a second application to the soil surface 2 weeks later.

except that mycelial mats from 1.25 dishes were blended in a semi-microblender containing 100 ml of water, and the total volume of the suspension was 125 ml.

**Incorporation of inoculum into soil.**—Soil in individual flats was emptied onto polyethylene sheets, thoroughly mixed with inoculum, and then replaced in the flat. For experiments 3 and 4, a cardboard divider was used to separate each quadrant until the soil had been replaced in each quadrant. The dividers were then removed and the flat was bumped against a greenhouse bench to remove airpockets between the quadrants. In all experiments, the inoculum density in the soil after mixing was approximately 32 *P. cinnamomi* chlamydospores per gram of soil (11).

**Planting flats with pine seeds.**—A template with 10 rows of 20 holes was used to space the seed. Shortleaf pine seeds, previously soaked for 24 hours in 3% $H_2O_2$ to promote germination, were pressed into firm contact with the soil and lightly covered with a layer of vermiculite. Seeds were from a mixed seed lot collected in Texas in 1967 and were provided by the Eastern Tree Seed Laboratory, U.S. Forest Service, Macon, Georgia. Flats were placed randomly on a greenhouse bench and, except for those under a mist system (experiment 4), were watered twice weekly.

**Seedling counts.**—Beginning 2 weeks after planting, and at weekly intervals thereafter for 9 weeks, all surviving seedlings were counted and recorded for each treatment. In addition, seedlings proximal to demarcation lines between treatments in experiments 3 and 4 were examined to determine whether either fungus had affected seedlings survival in adjacent treatments.

All experiments were repeated at least once and data presented represent overall averages. For each experiment, data from each treatment are plotted as the percentage of the seedling stand in the autoclaved control for that sampling date. Data were subjected to analysis of variance, and means were compared for significance by Duncan's multiple range test. Differences referred to in this paper were significant $P = 0.01$.

## RESULTS

In experiment 1, seedling survival in *T. harzianum*-treated flats approximated that in the autoclaved control flats and was significantly higher than that in other treatments (Fig. 1-A). Survival in the combination treatment (*P. cinnamomi* + *T. harzianum*) was intermediate between that of the nonautoclaved control and the *P. cinnamomi* treatment, being significantly lower than the former and higher than the latter. Seedling survival in the combination treatment from week 3 through 9 averaged 38.7% of the autoclaved control. In the *P. cinnamomi* treatment seedling survival averaged 10.2% of the autoclaved control; thus the presence of *T. harzianum* in the combination treatment accounted for a 28.5% increase in seedling stand.

For experiment 2, in which *T. harzianum* was added a second time 2 weeks after the study was initiated, no significant difference in seedling survival was observed between the *P. cinnamomi* and the combination treatments (Fig. 1-B). However, seedling survival in both was significantly less than that in the other treatments. Average seedling survival in the *P. cinnamomi* and the combination treatments from week 3 through week 9 was 10.9% and 16.4%, respectively, of that in the autoclaved

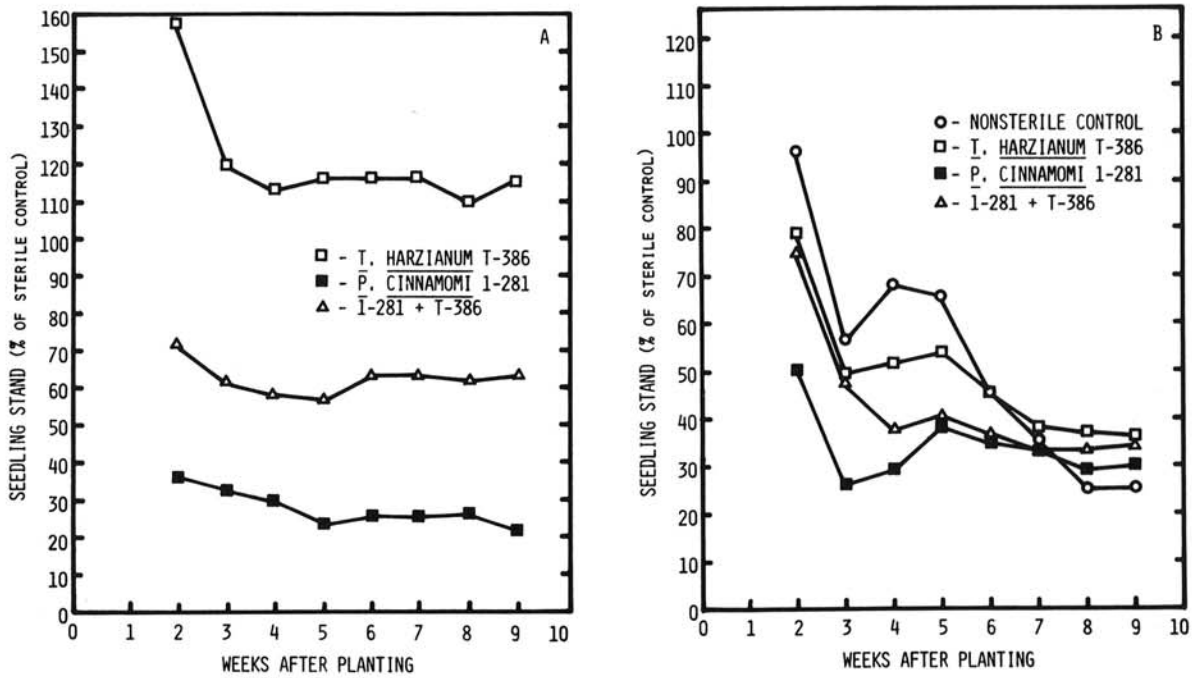


**Fig. 2-(A, B).** Effect of *Trichoderma harzianum* on incidence of damping-off caused by *Phytophthora cinnamomi* in soil flats watered twice weekly. Soil in each flat was divided into quadrants of equal size, and each quadrant received a different treatment. **A)** Autoclaved soil. **B)** Nonautoclaved soil.

control. Thus, *T. harzianum* in the combination treatment accounted for only a 5.5% increase in seedling stand.

Results for treatments involving autoclaved soil in experiment 3 (Fig. 2-A) were similar to those in experiment 1. Seedling survival in the combination and the *P. cinnamomi* treatments was significantly less than that in other treatments; survival in the *P. cinnamomi* treatment was significantly less than that in the combination treatment. Average seedling survival in the combination and the *P. cinnamomi* treatments from week 3 through week 9 was 61.3% and 25.9%, respectively, of the seedling survival recorded for the autoclaved control. Examination of seedlings proximal to demarcation lines between treatments indicated that neither *P. cinnamomi* nor *T. harzianum* affected seedling survival in adjacent treatments.

Seedling survival was poor in all treatments involving nonsterile soil in experiment 3 (Fig. 2-B); effects among treatments were masked by other factors.

Under the high soil moisture condition of experiment 4 (Fig. 3-A), seedling survival in quadrants containing the *P. cinnamomi* and the combination treatments in autoclaved soil was quite different from that in the other experiments. As before, seedling survival in the quadrant treated with *T. harzianum* approximated that in the autoclaved control. However, seedling survival in the combination treatment was significantly below that of all other treatments. Seedling survival in the *P. cinnamomi* quadrant was significantly less than that in the autoclaved control only at weeks 6 through 9. During this period, average seedling survival in the *P. cinnamomi* quadrant was 72.5% of that in the autoclaved control; average seedling survival in the combination treatment was only 10% of that in the autoclaved control.

As in experiment 3, there was no indication that either *P. cinnamomi* or *T. harzianum* affected seedlings in adjacent treatments.

Seedling survival was poor for all treatments involving nonautoclaved soil in experiment 4 (Fig. 3-B); these results were similar to those obtained for treatments involving nonautoclaved soil in experiment 3 (Fig. 2-B). As in experiment 3, treatment effects were masked by other facts.

## DISCUSSION

Baker and Cook (3) define biological control as ". . . the reduction of inoculum density or disease-producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host, or antagonist, or by mass introduction of one or more antagonists." In portions of this study biological control of *P. cinnamomi* was attained by mass introduction of *T. harzianum* (Fig. 1-A, 2-A); however, the reduction in disease incidence was relatively small. Results indicate that *T. harzianum* is a more effective antagonist in moist soil than in wet soil; similar results were reported for *T. viride* by Anderson (1). Backman and Rodriguez-Kabana (2) controlled *Sclerotium rolfsii* Sacc. in peanut fields by applying *T. harzianum*-granules to the soil surface, the area where *S. rolfsii* is most active. Their results show that *T. harzianum* can effectively utilize the residual nutrients contained in the granules and antagonize *S. rolfsii* at the soil surface. Results of the present study indicate that (i) *T. harzianum* is not an effective antagonist beneath the soil surface, (ii)

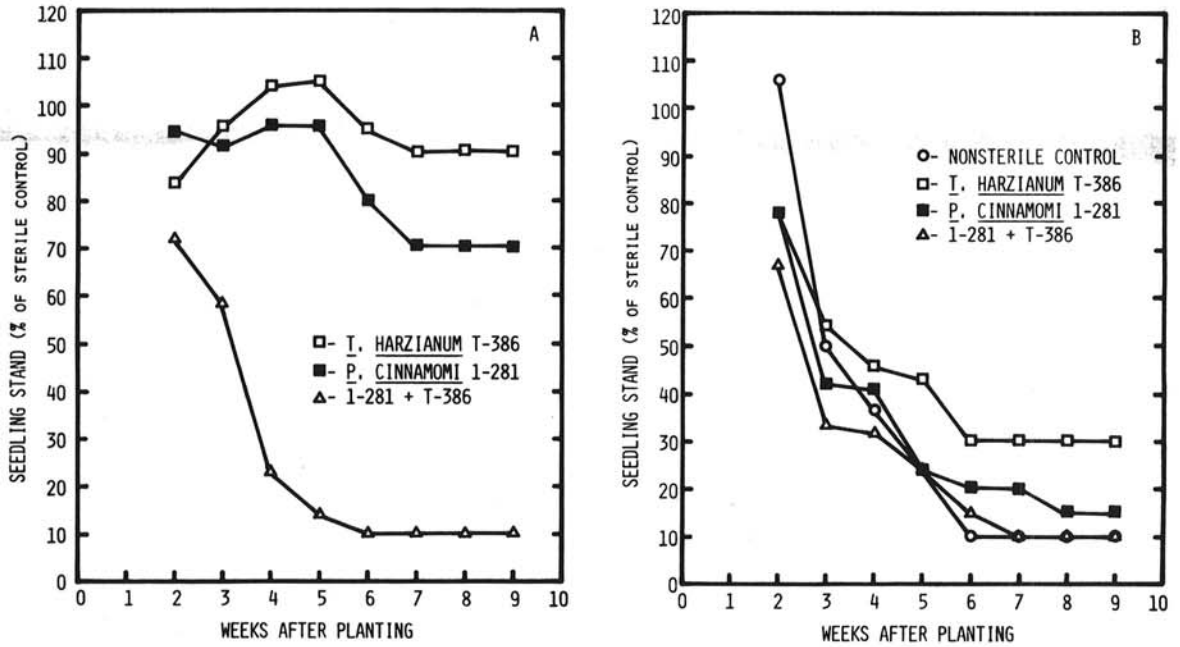


**Fig. 3-(A, B).** Effect of *Trichoderma harzianum* on incidence of damping-off caused by *Phytophthora cinnamomi* in soil flats with the moisture level maintained at saturation. Soil in each flat was divided into quadrants of equal size and each quadrant received a different treatment. **A)** Autoclaved soil. **B)** Nonautoclaved soil.

*P. cinnamomi* is more resistant to antagonistic action by *T. harzianum* than is *S. rolfsii,* or (iii) both of the above. *P. cinnamomi* is more resistant to antagonistic action by *T. harzianum* than is *S. rolfsii,* or (iii) both of the above.

The residual nutrients contained in the clay granules used in this study were intended to serve as a nutrient source for *T. harzianum,* thus increasing its competitive advantage and antagonistic qualities. However, when applied to the soil surface (Fig. 1-B) or incorporated in soil maintained at high moisture levels (Fig. 3-A), these residual nutrients apparently benefited *P. cinnamomi,* resulting in greater incidence of disease. Under such conditions *P. cinnamomi* had a competitive advantage over *T. harzianum.* Seedling survival in the autoclaved soil quadrant containing *P. cinnamomi* alone in experiment 3 was much higher than expected (Fig. 3-A). The high soil moisture condition should have approached the optimum for *P. cinnamomi,* resulting in a high incidence of disease. The fact that disease incidence was significantly higher in the combination treatment, where residual nutrients in the granules were available to *P. cinnamomi,* suggests the possibility that the autoclaved soil in the *P. cinnamomi*-alone quadrant lacked a sufficient food base for *P. cinnamomi* to establish infection.

Soil used in these studies necessarily was collected at several locations within the Auburn Forest Nursery at various times of the year over a 3-year period. Thus, considerable variation in the effects of indigenous damping-off pathogens was incurred in nonautoclaved soil in this study. For the most part, high rates of damping-off masked any effect of *T. harzianum* on *P. cinnamomi* (Fig. 2-B, 3-B). Concurrently, their presence provided an opportunity to evaluate the potential of *T. harzianum* as a biocontrol agent for unidentified damping-off pathogens in their natural habitat; the results indicate that it is ineffective. Such results lend credence to the statement above that *T. harzianum* is not an effective antagonist beneath the soil surface.

Results of this study indicate that (i) under high soil moisture conditions residual nutrients in the *T. harzianum*-granules may be used as an energy source by *P. cinnamomi,* resulting in an increase in disease; (ii) growth and/or mobility of both *P. cinnamomi* and *T. harzianum* was restricted to that area of the soil originally inoculated; (iii) *T. harzianum* was ineffective in reducing incidence of damping-off in nonautoclaved soil containing indigenous, unidentified damping-off pathogens; (iv) under the conditions of this study, the *T. harzianum* isolate tested generally was ineffective as a biocontrol for *P. cinnamomi,* thus corroborating conclusions reached earlier (6, 7); and (v) similar preparations of potential antagonists more suited to conditions favorable to *P. cinnamomi* should be tested.

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