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Biological Control of the Artillery Fungus, *Sphaerobolus stellatus*, with *Trichoderma harzianum* and *Bacillus subtilis*[1]

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Abstract

Three strains of the fungus *Trichoderma harzianum* Rifai and two strains of the bacterium *Bacillus subtilis* (Ehrenberg) Cohn were evaluated for their ability to suppress colonization and sporulation of the artillery fungus (*Sphaerobolus stellatus* Tode:Pers.) on oatmeal agar. All five biological control agents inhibited growth of *S. stellatus*, but efficacy depended on time of application. Simultaneous inoculation of agar with *S. stellatus* and the biocontrol agents, as well as inoculation of biocontrol agents 14 days prior to *S. stellatus*, resulted in complete inhibition of *S. stellatus*. Inoculation of agar with biocontrol agents 14 days after inoculation with *S. stellatus* reduced, but did not completely suppress *S. stellatus* colonization and sporulation. In this experiment, gleba (spore masses) treated with all strains of *T. harzianum* and strain GBO3 of *B. subtilis* did not germinate, but 13% of gleba treated with strain MBI 600 of *B. subtilis* did germinate. *Trichoderma harzianum* was more effective than *B. subtilis* as a biocontrol agent.

Index words: artillery fungus, *Trichoderma harzianum*, *Bacillus subtilis*.

Significance to the Nursery Industry

The artillery fungus grows in landscape wood and bark mulch, producing masses of spores that adhere to plant tissue, automobiles, and house siding. Strains of the fungus *Trichoderma harzianum* and the bacterium *Bacillus subtilis* were found to suppress growth of the artillery fungus on agar, when their inoculation occurred simultaneously, or prior to, inoculation with the artillery fungus. Based on these results, biological fungicides for use as suppression agents for the artillery fungus should be evaluated in field trials.

Introduction

The artillery fungus (*Sphaerobolus stellatus*) is a ubiquitous, white-rotting, wood-decay fungus commonly found in moist, decomposing landscape mulch. *Sphaerobolus* produces sticky, pinhead-size (1 mm) masses of spores called gleba that are forcibly ejected from the fruiting bodies of the fungus and adhere tightly to surfaces with which they come in contact. This fungus derives its common name, 'artillery fungus,' from this ballistic spore discharge mechanism. Artillery fungi are phototropic, discharging their spore masses towards the light, or towards light-colored reflective surfaces such as the sides of houses (1, 9, 19), and are often intercepted by plant tissues (3, 4, 25).

Mulch producers, landscape contractors, and insurance companies have all been subject to complaints or claims from homeowners requesting cleaning, painting, or replacement of their gleba-speckled house siding. Therefore, an immediate need exists to determine ways to inhibit growth and sporulation of the artillery fungus in landscape mulch. One such possibility is control of *S. stellatus* through establishment of competing biocontrol agents within the mulch. Species of the fungus *Trichoderma* and the bacterium *Bacillus* were chosen for this study based on their potential occurrence in landscape mulches and use as biocontrol agents.

Species of *Trichoderma* are common colonizers of soils, freshly cut wood, as well as wood chips used for landscape mulch (14). *Trichoderma* spp. extract soluble sugars from wood, preventing successional decay fungi from colonizing (15). Species of *Trichoderma* also produce volatile and non-volatile antibiotics that inhibit specific wood decay fungi (6, 10), and may be mycoparasitic against wood decay fungi (8, 20). In our preliminary studies, *Trichoderma* spp. were observed to be common contaminants on many different mulches. *Bacillus* species also have been studied for their potential use as biological control agents (18) including wood decay fungi (12, 21). Bernier et al. (2) found that *B. subtilis* inhibited growth of three sapwood-inhabiting fungi, and Krebs et al. (18) reported the primary antifungal substances in *B. subtilis* were cyclic lipopeptides. However, *Bacillus* may be fungistatic rather than fungitoxic (23). Romaine (22) stated that *Bacillus* spp. were common inhabitants of organic substrates such as spent mushroom substrate.

Rootshield™ is produced by BioWorks, Inc. (Geneva, NY) and contains *T. harzianum* strain T-22 (KRL-AG2) as its active ingredient. It is used as a contact fungicide to control species of *Fusarium*, *Pythium*, *Rhizoctonia*, *Sclerotinia* and other fungi in turf and ornamentals. Epic® and Kodiak® are biological control agents produced by Gustafson, Inc. (Plano, TX) with *B. subtilis* as the active ingredient (strains MBI 600 and GBO3, respectively). They are used for suppressing species of *Alternaria*, *Aspergillus*, *Fusarium*, *Rhizoctonia* and other fungi on developing root systems of field crops, such as wheat, soybeans, and corn. The objective of this study was to evaluate the efficacy of three strains of *T. harzianum* and two strains of *B. subtilis* in preventing growth and sporulation of *S. stellatus* on oatmeal agar.

Materials and Methods

S. stellatus cultures. Initial *S. stellatus* isolates were collected on the Pennsylvania State University campus (University Park, PA), grown in pure culture, and maintained on oatmeal agar (11). Following sporulation in culture, individual

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gleba were collected from the lids of petri plates and surface-sterilized using a 1.05% sodium hypochlorite solution (20% Clorox®) followed by a distilled water wash and a 70% ethanol spray. Gleba were placed on oatmeal agar plates on which they germinated and produced new cultures that sporulated after 21–25 days at room temperature. Gleba collected from the new cultures served as the inoculum source for each treatment.

Biological control agents. Five biocontrol agents were evaluated, three of which are commercially available; RootShield™, Epic®, and Kodiak®. *Trichoderma harzianum* strains 299 and 382 were provided as pure, numbered cultures by Dr. H. Hoitink, Ohio State University, and subcultured on potato dextrose agar. The following suspensions of each biocontrol agent were used: 1.2 g RootShield™/100 ml of distilled water, 0.1 g *T. harzianum* 299 or 382/20–25 ml distilled water, and 0.1 g of Epic® or Kodiak® in 20–25 ml of distilled water. Approximately 2 ml of each suspension were dispensed onto each 100 × 15 mm petri plate containing 10 ml oatmeal agar.

Treatments. The ability of the five biological control agents to inhibit *S. stellatus* was evaluated using three treatments:

- Simultaneous inoculation of agar with *S. stellatus* gleba and biocontrol agents.
- Inoculation of agar with *S. stellatus* gleba, followed by biocontrol agents 14 days later.
- Inoculation of agar with biocontrol agents, followed by *S. stellatus* gleba 14 days later.

Cultures were maintained on laboratory benches at room temperature (*ca.* 21–23C) with natural daylight. Ceiling fluorescent lights were operating in the laboratory for approximately 10 hours per day from 800–1800 hr. Treatments were replicated five times for each biocontrol agent.

Growth and sporulation were evaluated after 42 days, the time required for *S. stellatus* to sporulate (1, 9). Growth was evaluated as either present or absent. Sporulation was evaluated by counting the number of gleba adhering to each petri plate lid. After counting, the original gleba were removed, surface-sterilized, and subcultured on oatmeal agar plates. Viability and subsequent growth were evaluated 14 days later. Controls (gleba with no biocontrol agent and biocontrol agents with no gleba) were included with each treatment. The experiment was replicated 3 times. Duncan's multiple range test (P = 0.05) was used to determine if significant differences occurred among treatments.

Results and Discussion

Inoculating agar with any of the five biocontrol agents prior to adding *S. stellatus* completely inhibited growth of *S. stellatus* (data not shown). Simultaneous inoculation of agar with *S. stellatus* and any of the biocontrol agents also resulted in total inhibition of growth and sporulation (data not shown). These findings imply death of the gleba *in situ*, death of germ tubes, or inhibition of germ tube growth without death of the gleba.

Bruce et al. (7, 8) reported that the ability of *Trichoderma* spp. to control wood decay fungi was dependent on the nutrient status of the growth medium. Species that performed well as control agents on malt agar were reduced in growth on nutrient-limited media. However, suppression of glebal germination by the biocontrol agents in our study is probably due to chemical inhibition. It is unlikely that competition for available nutrients was the reason for suppression, because oatmeal agar is an extremely rich medium with a high level of available nutrients. When *S. stellatus* was allowed to colonize the agar prior to inoculation with the biocontrol agents, sporulation was reduced, but not eliminated (Table 1).

Table 1. Glebal production by *Sphaerobolus stellatus* (n = 15) after 42 days. Plates were inoculated with biocontrol agents 14 days after inoculation with *S. stellatus* gleba.

Trade name (Biocontrol agent; strain)	Average no. gleba[z]	% germination[y]
S. stellatus control	12.85a	100
Epic® (*Bacillus subtilis*; MBI 600)	7.00b	13.3
Kodiak® (*B. subtilis*; GBO3)	6.30b	0
Rootshield™ (*Trichoderma harzianum*; T-22)	0.53c	0
(*T. harzianum*; 299)	0.47c	0
(*T. harzianum*; 382)	0.40c	0

[z]Means with the same letter are not significantly different according to Duncan's multiple range test (P = 0.05).

[y]Percentage of gleba (n = 15) that germinated (when placed on fresh media) following treatment with biocontrol agent.

Gleba removed from *T. harzianum* treatments, in which *S. stellatus* did not grow in the presence of the biocontrol agent, at the end of the experiment did not germinate when surface-sterilized and placed onto fresh media (Table 1). Likewise, 13 gleba that were cultured with Epic® did not germinate after sterilization; however, 2 gleba from this treatment did germinate and grow. The average number of gleba produced from these plates after 42 days was 11. None of the gleba originally treated with Kodiak® germinated.

Trichoderma harzianum. Although all biocontrol agents used in this study had some antibiotic effect, the fungus *T. harzianum* reduced sporulation to a greater extent than did the bacterium *B. subtilis*. There were no significant differences among the three *Trichoderma* strains in their ability to suppress sporulation, nor between the two *Bacillus* strains.

Our findings imply that *Trichoderma* has a fungitoxic effect on *Sphaerobolus*. Grondona et al. (13) found differences among isolates of *T. harzianum* in ability to suppress growth of various fungi. *Trichoderma harzianum* isolates had greater inhibition of growth on fungi with cellulose cell walls as compared to fungi with chitin and glucan cell walls. Hüttermann and Cwielong (16) tested a lytic enzyme system from *T. harzianum* as a biological control for annosus root rot in spruce. The fungal cell wall was hydrolyzed by this system, leaving the host tree unaffected. The authors found an inexpensive way to mass produce the enzyme system using a *Penicillium* species as the substrate. Such a system should be considered for application to wood and bark mulch to inhibit *S. stellatus*.

When *S. stellatus* was allowed to grow for 14 days before application of the control agents, the most recent hyphal growth of *S. stellatus* was affected by the biocontrol agents, but *Trichoderma* and *Bacillus* were unable to completely suppress growth and sporulation. This is consistent with the findings of Dennis and Webster (10) who evaluated the reaction of different species and strains of *Trichoderma* (including *T. harzianum*) against root rot fungi. In cultures where *Trichoderma* suppressed growth of the test fungi, the re-

searchers were able to subculture the test fungi onto fresh agar where they grew normally. However, in cultures where growth of the test fungi was completely inhibited initially, no subsequent growth occurred after subculturing. This implies a difference in efficacy among species and strains of *Trichoderma*, and suggests that some strains are fungistatic whereas others are fungicidal. The results of re-isolating gleba after the biocontrol contact could imply fungitoxic metabolites in both of the *T. harzianum* strains and the GBO3 strain of *B. subtilis* and fungistatic metabolites in the *B. subtilis* MBI 600 strain.

Bacillus subtilis. Complete inhibition of *S. stellatus* growth occurred when *B. subtilis* strains were applied prior to or simultaneously with *S. stellatus* on oatmeal agar. Kreber and Morrell (17) reported that an isolate of *B. subtilis* completely inhibited growth of fungal competitors on ponderosa pine sapwood for the first 4 weeks after inoculation, but lost much of its inhibitory ability after 4 additional weeks. Siefert et al. (23) postulated this weakening of antagonism was due to the inability of *Bacillus* to fully colonize the substrate. *Bacillus subtilis* may not be able to prevent subsequent growth of wood decay fungi, but rather just delay it through fungistatic means. However, lysis of *Phytophthora* spp. mycelium by species of *Bacillus* on agar was reported by Broadbent et al. (5). The evaluation of *B. subtilis* as a control agent for a *Phytophthora* species demonstrated the significant differences in effectiveness between strains on cornmeal agar (24). Utkhede (24) also reported that strains of *B. subtilis* produced antibiotics which inhibited mycelial growth of *Phytophthora*.

Although the strains of *B. subtilis* used in this *S. stellatus* experiment completely suppressed growth in two of the three treatments (prior to and simultaneous inoculation with gleba), results of these *in vitro* experiments may not be fully applicable to the landscape mulch situation. Differences in the nutrient status of oatmeal agar versus wood and bark mulch may influence the efficacy of the control agent.

The experiment reported herein demonstrates the suppressive ability of strains of *Trichoderma harzianum* and *Bacillus subtilis* as biological control agents for *S. stellatus in vitro*. When *S. stellatus* was allowed to colonize the substrate initially, *Trichoderma* was more effective at suppressing sporulation than was *Bacillus*. However, it is not known how or when *S. stellatus* enters landscape mulch *in vivo*; therefore, it is not known when to apply a potential biocontrol agent to the mulch. The artillery fungus may be present on the site, be on wood prior to the chipping process, or may infest landscape materials at mulch-producing facilities following chipping. Or, it may enter mulch after application in the landscape, arriving on infected plant material or from surrounding areas such as other mulch beds, decaying organic matter, and forested areas, or some combination of these scenarios. Also, the suppressive potential shown in this study on oatmeal agar may not be as great on wood and bark mulch substrates. However, if additional research supports these findings, it may be feasible to treat landscape mulches at various points of production, application or post-application with suspensions of these biological agents as protection against *S. stellatus* colonization.

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