Biological Control of Grey Mold of Snap Beans by *Trichoderma hamatum*

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ABSTRACT

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Isolates of *Trichoderma* spp. from snap bean foliage were tested for their capacity to suppress grey mold of snap bean pods caused by *Botrytis cinerea*. In a detached blossom-pod assay, an isolate of *Trichoderma hamatum* reduced pod rot by 94% compared with the nontreated control, which was comparable to that obtained with the fungicide vinclozolin. Forty-two colony-forming units (cfu) of *T. hamatum* per blossom reduced pod rot by 77% compared with the nontreated control. Control was 97% when 233 cfu per blossom were applied. Grey mold was reduced only when spores of *T. hamatum* were applied to blossoms before, or simultaneously with, application of conidia of *B. cinerea*. Volatile compounds produced by one isolate of *T. hamatum* reduced mean radial growth of *B. cinerea* to 0.6 mm on potato-dextrose agar, while growth on nontreated plates averaged 23.6 mm. These results suggest the production of inhibitory volatiles as one possible mechanism of biocontrol.

Additional keywords: *Sclerotinia sclerotiorum*

Oregon is the second largest producer of bush snap beans in the United States. Grey mold, caused by *Botrytis cinerea* Pers. ex Fr., can be found on all aerial portions of the snap bean (*Phaseolus vulgarus* L.) plant (4). Economic loss, however, is due primarily to pod rot, which reduces quality and increases processing costs. When conditions are cool and moist, losses from pod rot may be substantial. At the present time, grey mold is managed primarily by application of fungicides. In some cases, however, the application of a fungicide may actually increase the severity of *Botrytis*-caused diseases (3,21). This increase is presumably due to the presence of fungicide-resistant strains of *Botrytis* and suppression of natural antagonists by the fungicide. The development of strains of *B. cinerea* resistant to fungicides (3,6,15,21), coupled with the difficulty of registering new fungicides, has stimulated an interest in biological control.

Botrytis cinerea requires a saprophytic phase before infection of healthy plant tissue (11,16). Wood (20) suggested that control of *B. cinerea* might be achieved by colonization of senescent tissue by competing microorganisms so as to preclude infection by the pathogen. With beans, senescent blossoms provide an important saprophytic base for infection of pods by *B. cinerea* (4). Colonization of blossoms by organisms antagonistic to *B. cinerea* should reduce subsequent infection of the developing pods.

Precolonization of flowers has suppressed *Botrytis*-caused diseases. For example, *Exophiala jeanselmei* applied to cut roses 1 day before inoculation with a conidial suspension of *B. cinerea* provided control of Botrytis blight (18). The inoculation and colonization of dead tomato flowers by *Cladosporium herbarum* prevented the development of grey mold on petals and subsequent fruit infection in glasshouses (14). Grey mold on strawberries was reduced in the field with applications of several species of *Trichoderma*, beginning at early bloom (19). In the field over several seasons, Dubos (8) demonstrated a reduction in grey mold on grapes with applications of *Trichoderma harzianum* to the flowers. Other workers (2) also have inhibited grey mold on grapes in the field by applying conidial suspensions of *Trichoderma* spp., *Cladosporium* sp., and *Aureobasidium* sp. beginning at flowering.

This study examined the potential of *Trichoderma* spp. recovered from snap bean foliage to suppress grey mold of snap beans.

MATERIALS AND METHODS

Collection of potential antagonists. In 1985, blossoms and the terminal leaflet of the first trifoliate leaf were removed from snap bean plants in 15 commercial fields in Oregon's Willamette Valley. Blossoms and leaflets were placed individually in flasks containing 5 or 50 ml of distilled water, respectively, with 0.1 ml of Tween 20 per liter added as a wetting agent. These flasks were placed on a rotary shaker at 150 rpm for 30 min. Afterwards, the wash solution was diluted serially, and 0.2 ml of each dilution was plated on *Trichoderma*-selective medium (TSM) (9). After incubation at room temperature for 7 days, selected isolates were transferred to malt agar and placed in storage at 4 C.

Greenhouse production of bean blossoms and pods. Seeds of cultivar Oregon Trail were seeded in 15-cm-diameter by 18-cm-deep plastic pots containing the Oregon State University greenhouse soil mix (40% pumice, 20% peat, 20% sand, and 20% soil). Before planting, 1.0 kg of calcium nitrate (15.5-0-0, 19% calcium), 1.5 kg of sulfomag (0-0-22-22-19), and 2.0 kg of superphosphate (0-25-0) were incorporated into each cubic meter of soil. Two weeks after planting, seedlings were thinned to three per container, and 6.5 g of Osmocote (14-14-14, 2–3 mo formulation, Sierra Chemical Company, Milpitas, CA) were top-dressed on each container. Blossoms and mature pods (9–12 cm long) were harvested for laboratory trials.

Preparation of spore suspensions. Spores were obtained from sporulating 14-day-old cultures grown on plates of potato-dextrose agar (PDA) at room temperature. To each plate, 10 ml of 0.01 M phosphate-buffered saline solution (with 0.01% Tween 20) (PBST) were added. Spores were removed by gentle agitation with a glass rod. After filtering

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the suspension through two layers of cheesecloth, spore concentrations were determined with a hemacytometer. Concentrations were adjusted by dilution with PBST.

In vivo screening. Fourteen fungal isolates, which gave some reduction in pod rot in preliminary tests, were tested for suppression of grey mold on bean tissue. Blossoms and pods were obtained from greenhouse-grown bean plants. A minimum of 10 blossoms was placed on a wire screen in a covered, clear plastic box (30 × 24 × 10 cm) that contained a thin layer of tap water to maintain high relative humidity. With a chromatography sprayer, 5 ml of a spore suspension containing 10^5 spores per ml of the potential antagonist were sprayed on the blossoms in each box. After incubation in a growth chamber at 17 C for 24 hr (16 hr light, 8 hr dark), the blossoms were sprayed with 5 ml of a conidial suspension of *B. cinerea* containing 10^4 conidia per ml. Inoculated blossoms were placed in contact with apparently healthy bean pods, and the boxes were returned to the growth chamber. After incubation for 4 days, the proportion of blossoms initiating pod rot was determined. For comparison, a treatment with *B. cinerea* alone at 10^4 conidia per ml and a fungicide treatment (vinclozolin, 5 ml at 0.6 g a.i./L) were included. Treatments were arranged in a randomized complete block design with three replications blocked over time. No attempt was made to sterilize flowers or pods before application of the treatments. One isolate of *Trichoderma hamatum* (Bon.) Bainier (Is 117) was selected for subsequent tests.

Spore populations required for disease suppression. The number of spores of *T. hamatum* per bean blossom required to reduce grey mold in vivo was determined. Blossoms were placed on wire screens in plastic boxes as described above. There was a minimum of 15 blossoms per box. Blossoms were sprayed with a 5-ml spore suspension of *T. hamatum* containing either 10^3, 10^4, 10^5, or 10^6 spores per ml. After a 24-hr incubation period at room temperature, the blossoms were sprayed with 5 ml of a condial suspension of *B. cinerea* containing 10^4 conidia per ml. Inoculated blossoms were placed in contact with apparently healthy bean pods. After an additional 4-day incubation period, the proportion of blossoms initiating pod rot was determined. A treatment with *B. cinerea* applied alone was included as a control. There were four replications in a completely randomized design.

Immediately following the application of the antagonist, five blossoms were removed from each box and placed together in 25 ml of distilled water with Tween 20 (0.1 ml/L) and washed on a rotary shaker for 1 hr at 150 rpm. The wash solution was diluted serially, and 0.2 ml of each dilution was plated on each of two plates of TSM. After 4 days incubation at room temperature, colonies characteristic of *T. hamatum* were counted and the number of colony-forming units (cfu) per blossom was calculated.

Inoculation sequence. The effect of the sequence of *T. hamatum* and *B. cinerea* application on incidence of pod rot was tested. Bean blossoms were placed in plastic boxes and inoculated as described above. Treatments consisted of 1) *B. cinerea* applied 24 hr before *T. hamatum*, 2) *T. hamatum* applied 24 hr before *B. cinerea*, 3) *B. cinerea* and *T. hamatum* applied together, and 4) *B. cinerea* applied alone. *Botrytis cinerea* and *T. hamatum* were applied at 10^4 and 10^5 spores per ml, respectively. Inoculated blossoms were placed in contact with symptomless bean pods. After incubation at room temperature for 4 days, the proportion of blossoms initiating pod rot was determined. Treatments were arranged in a randomized complete block design with four replications blocked over time.

Effect of volatiles produced by *T. hamatum*. Inhibition of *B. cinerea* by volatile metabolites produced by *T. hamatum* was tested using a modification of Dennis and Webster's enclosed chamber test (7). A 5-mm-diameter plug was transfered from the margin of a 3-day-old colony of *T. hamatum* grown on PDA to the center of a 100-mm-diameter plastic petri dish containing PDA. One inoculated dish and one containing noninoculated PDA were sealed together in a plastic bag and incubated in the dark at 20 C. After a 48-hr incubation, a 5-mm-diameter plug was transferred from the margin of a 3-day-old *B. cinerea* colony grown on PDA to a 100-mm-diameter glass petri dish containing PDA. With the tops removed, the glass petri dishes were immediately inverted over the plastic dishes containing the 48-hr-old colony of *T. hamatum* or noninoculated PDA. One set of dishes containing *B. cinerea* inverted over *T. hamatum* and one set of dishes containing *B. cinerea* inverted over noninoculated PDA were sealed together in each plastic bag and returned to the 20 C incubator. There were eight replications in a randomized complete block design. After 72 hr, radial growth of the colonies of *B.*

Table 1. Control of grey mold of snap bean pods in vivo by fungal antagonists

Treatment[a]	Proportion of blossoms initiating pod rot[b]	Control[c] (%)
Vinclozolin	0	100
Is 117 (*Trichoderma hamatum*)[d]	0.06	94
Is 114	0.21	78
Is 118	0.21	78
Is 116	0.28	71
Is 147	0.37	61
Is 160	0.39	59
Is 161	0.39	59
Is 143	0.40	58
Is 144	0.44	54
Is 125	0.48	50
Is 164	0.58	39
Is 126	0.65	32
Is 155	0.84	12
Is 115	0.90	5
Botrytis cinerea alone	0.95	...
FPLSD[e] ($P = 0.05$)	0.20	

[a] Spore suspension (10^5 spores/ml) or fungicide (0.6 g a.i./L) applied 1 day before application of a *B. cinerea* spore suspension (10^4/ml).
[b] Inoculated blossoms held at 17C for 4 days.
[c] Percent reduction of pod rot compared with *B. cinerea* applied alone.
[d] Selected for additional testing.
[e] Fisher's protected least significant difference.

Table 2. Effect of population size of *Trichoderma hamatum* on grey mold of snap bean pods

Spores/ml applied[a]	Cfu[b] per blossom	Proportion of blossoms initiating pod rot[c]	Control[d] (%)
0	0	1.00	...
10^3	42	0.23	77
10^4	233	0.03	97
10^5	2,134	0	100
10^6	11,500	0	100
FPLSD[e] ($P = 0.05$)		0.17	

[a] Spore suspensions of *T. hamatum* (5 ml) applied 1 day before application of 5 ml of *Botrytis cinerea* at 10^4 conidia per ml.
[b] Colony-forming units (cfu) determined immediately following application of spores of *T. hamatum*.
[c] Inoculated blossoms held at room temperature for 4 days.
[d] Percent reduction of pod rot compared with *B. cinerea* applied alone.
[e] Fisher's protected least significant difference.

Table 3. Control of grey mold of snap beans with different application sequences of *Trichoderma hamatum* and *Botrytis cinerea*

Treatment[a]	Proportion of blossoms initiating pod rot[b]
B. cinerea first[c]	0.96
B. cinerea alone	0.84
B. cinerea and *T. hamatum* together	0.09
T. hamatum first[d]	0.07
FPLSD[e] ($P = 0.01$)	0.26

[a] Five ml suspensions of *T. hamatum* (10^5 spores/ml) or of *B. cinerea* (10^4 conidia/ml) were applied.
[b] Inoculated blossoms held at room temperature for 4 days.
[c] *Trichoderma hamatum* applied 24 hr later.
[d] *Botrytis cinerea* applied 24 hr later.
[e] Fisher's protected least significant difference.

cinerea was measured.

RESULTS

In vivo screening. Of the 14 isolates tested in the detached blossom-pod assay, 12 significantly reduced ($P = 0.05$) the amount of pod rot compared with *B. cinerea* applied alone (Table 1). *Trichoderma hamatum* (Is 117) reduced the incidence of pod rot by 94%, which was not significantly different ($P = 0.05$) from the fungicide treatment that gave 100% control. This isolate was selected for additional laboratory and field tests.

Spore populations required for disease suppression. As few as 42 *T. hamatum* cfu per blossom significantly reduced ($P = 0.05$) pod rot by 77% (Table 2). With 233 cfu per blossom, control of pod rot was 97%. This was not significantly different from higher populations that gave 100% control of pod rot.

Inoculation sequence. Spores of *T. hamatum* applied before or with conidia of *B. cinerea* significantly reduced ($P = 0.01$) the amount of pod rot compared to *B. cinerea* applied alone (Table 3). When conidia of *B. cinerea* were applied before *T. hamatum*, incidence of pod rot was not significantly reduced.

Effect of volatiles produced by *T. hamatum*. Radial growth of *B. cinerea* was significantly reduced when it was inverted in a closed chamber over a colony of *T. hamatum*. After 72 hr incubation, mean radial growth of *B. cinerea* enclosed with *T. hamatum* was 0.6 mm compared with 23.6 mm when enclosed with noninoculated PDA ($FPLSD_{0.01} = 4.3$ mm).

DISCUSSION

The first step in development of a biological control system is to isolate and identify organisms with potential for disease suppression. Because organisms predictively compete most successfully in the environment in which they have evolved and to which they are adapted, the search for biological control agents should be in the same system in which they will be applied (5).

Approximately 4 weeks elapses between first bloom and harvest of snap beans. During this brief period, natural populations of inhibitors may not increase rapidly enough to achieve the degree of disease suppression required by commercial agriculture. Artificial culture and application of antagonistic organisms to the blossoms might provide the disease control demanded in commercial bean production. Newly opened blossoms presumably are relatively free of microorganisms, resulting in minimal competition for microbes applied as biocontrol agents.

This study suggests that an isolate of *T. hamatum* has potential as a biological control agent against grey mold of snap beans. In the detached blossom-pod assay, control of grey mold was comparable to that obtained with the fungicide vinclozolin. As expected, sequence of application and the number of spores of *T. hamatum* applied to the blossoms were important factors in suppression of grey mold in the detached blossom-pod assay. Grey mold was controlled only when spores of *T. hamatum* were applied to blossoms before or with inoculum of *B. cinerea*.

Some isolates of *Trichoderma* are capable of producing volatile metabolites that, in vitro, inhibit growth of other fungi (7). The isolate of *T. hamatum* used in these trials (Is 117) produced volatile compounds that, in vitro, inhibited the growth of *B. cinerea*. The effect of volatile compounds on bean flowers in the field is not known. The ability of *T. hamatum* to produce volatile compounds that inhibit *B. cinerea* does not preclude other possible mechanisms of inhibition.

Most reports regarding suppression of plant diseases by *T. hamatum* involve soilborne pathogens (10,12). Reports describing control of foliar pathogens by *T. hamatum* are less numerous than are reports of *T. hamatum* suppressing soilborne pathogens. Field studies in Italy demonstrated that four to six applications of conidial suspensions of *T. hamatum* beginning at flowering reduced the incidence of grey mold on grapes (2).

Another important disease on beans in Oregon is white mold, caused by *Sclerotinia sclerotiorum*. The disease cycles of grey mold and white mold are very similar. Like *B. cinerea*, *S. sclerotiorum* requires an exogenous nutrient base for infection of healthy plant tissue (13,17). As with *B. cinerea*, senescing flowers provide an important saprophytic base for invasion of bean pods by *S. sclerotiorum* (1,13). Because fungicides used to control white mold may also affect grey mold and/or the biological control agents used to control grey mold, any biocontrol study of grey mold on beans must also consider white mold. Because of the dependence of *B. cinerea* and *S. sclerotiorum* on senescing flowers for infection of healthy bean pods, it is probable that both pathogens could be suppressed by the same microbe(s). Preliminary field trials indicate that Is 117 of *T. hamatum* is also effective in reducing white mold (*unpublished data*).

It is evident that organisms capable of reducing grey mold exist on snap bean foliage, but at populations too low to be sufficiently effective. If these organisms were cultured and applied to the blossoms, grey mold might be reduced. Field trials under a wide range of conditions are needed to better understand the potential and limitations of *T. hamatum* for biological control of grey mold on beans.

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