Effects of Temperature, Soil Moisture, and Wheat Bran on Growth of *Trichoderma harzianum* from Alginate Pellets

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ABSTRACT

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Radial growth rates and hyphal densities were quantified for hyphae originating from alginate pellets containing hyphae of the biocontrol fungus *Trichoderma harzianum*. Pellets containing 1.8 mg of hyphal biomass (mean dry weight) and either 0 or 2.5 mg of wheat bran were buried beneath sheets of nylon mesh in a steamed silt loam soil, then incubated at 15, 20, or 25 C and −0.03, −0.1, or −0.5 MPa soil matric potential. Extent of hyphal growth was visually mapped after 0, 1, 2, 3, 5, 7, and 14 days, and mean radii were calculated from digitized maps. A visual assessment key for hyphal density was developed with a computer graphics program. With the key, hyphal density at 5, 10, 20, 30, and 40 mm from pellets was quantified after 7 and 14 days. For radial extension of hyphae, a logistic growth model was fit, with the assumption of a 24-hr lag ("germination") period (mean R^2 for all treatments = 0.71). Multiple regression of environmental variables against the slope of each growth curve showed that temperature had a significant positive effect on radial growth rate but that matric potential and bran effects were not significant. Hyphal density declined exponentially with distance from pellets. Hyphal density was significantly higher with bran, in drier soil, and after 14 versus 7 days. Temperature did not significantly affect hyphal density at 7 or 14 days. Methods and results presented may be useful for comparison of nutritional additives or other formulations for pelletized biocontrol fungi or as a basis to develop predictive models for performance of pelletized biocontrol fungi at different application rates and under varying environmental conditions.

Trichoderma harzianum Rifai can act as an antagonist of various soil fungi via mycoparasitism of hyphae and sclerotia or other mechanisms (for example, antibiosis). Thus, *T. harzianum* has potential as a biological control (biocontrol) agent against soilborne pathogens (1,3–8,10,14,15,18,19). To demonstrate this capacity, *Trichoderma* spp. have been applied to soil or seed as conidial suspensions or as hyphae in colonized organic matter (3,5,11,15,18). A recent development is incorporation of hyphal biomass into alginate pellets, with or without an additional nutrient source (9,12,13,16). For mycoparasitism to occur in soil, hyphae of the fungal antagonist must grow from the pellets, contact target propagules (for example, sclerotia), and parasitize them. Thus, the distance over which hyphae grow from pellets to infect target propagules may be analogous to the distance over which some soilborne pathogens grow to infect plant roots.

Most investigators testing survival and/or proliferation of biocontrol fungi in soil have used the technique of counting colonies on soil dilution plates. Papavizas et al (16) studied the effect of formulation procedures, including incorporation of wheat bran, on proliferation of alginate-encapsulated propagules (conidia, ascospores, or hyphae) of *Talaromyces flavus* (Klöcker) Stolk & Samson in soil. They found that ascospores and especially conidia in alginate-bran pellets resulted in rapid proliferation (measured as colony-forming units per gram in soil dilutions), whereas hyphal preparations did not. In contrast, Lewis and Papavizas (12) found that, for the faster growing species *Trichoderma* and *Gliocladium*, hyphal biomass resulted in significant proliferation when added with traces of food base. However, in these and other studies (2,9,11) where dilution plating was used to quantify populations, it is difficult to distinguish between different aspects of proliferation, that is, between hyphal growth and sporulation.

Numerous biotic and abiotic factors may affect the extent and patterns of growth of biocontrol fungi from pellets. Identification and quantification of these variables may make biocontrol more predictable and effective. The objectives of this study were to develop and use methods to quantify influences of temperature, matric potential, and a nutrient source (wheat bran) on the hyphal growth potential of pelletized *T. harzianum*.

MATERIALS AND METHODS

Formulation of *T. harzianum*. Isolate ThzID1 of *T. harzianum* was obtained from Palouse silt loam soil on the University of Idaho Plant Science Farm in Moscow, ID. ThzID1 was mycoparasitic on sclerotia of *Sclerotinia sclerotiorum* (Lib.) de Bary on agar plates and in sterile soil (G. R. Knudsen and D. J. Eschen, *unpublished*). To produce alginate pellets with THzID1, we varied the methods of Fravel et al (9) as follows. Pieces of 4 cm^2 were cut from a culture sporulating on potato-dextrose agar (PDA) (Difco Laboratories, Detroit, MI) plates, and each piece was placed in 500 ml of potato-dextrose broth in a 1-L flask. Flasks were plugged with cotton and incubated at room temperature (approximately 22 C) on a rotary platform shaker (120 rpm) for 1 wk. The mycelial biomass then was strained, rinsed with sterile water, added to 100 ml of 1% aqueous sodium alginate solution, and stirred gently until evenly dispersed.

Wet weight of hyphae averaged 37 g/flask. Ten subsamples were taken randomly from flasks, oven dried (100 C overnight), and weighed. Pellets were made by adding the hyphae alginate mixture dropwise to 0.25 M aqueous $CaCl_2$ (9). In one treatment, 2 g of wheat bran was added to the hyphae alginate mixture before pelletizing. Pellets formed in the $CaCl_2$ solution were removed within 10 min, rinsed with distilled water, allowed to air dry on waxed paper, and stored in glass beakers at room temperature and ambient humidity. An average of about 750 pellets per flask was obtained. Mean oven-dried hyphal weight per pellet was 1.8 mg. Mean weight of bran in pellets containing bran was 2.5 mg/pellet. *T. harzianum* grew from all pellets plated on PDA, even after several months of storage. Pellets used in these experiments were 2–8 wk old.

Soil conditions. Experiments were conducted in a Palouse silt loam soil obtained near Moscow, ID. Soil (2.5-kg volumes) was steamed in an autoclave by adding steam until a temperature of 100–110 C was reached and then holding the temperature at 90–110 C for 1 hr. This method nearly eliminated resident fungi

(for example, *Rhizopus*, *Penicillium*) and was necessary to help distinguish hyphae of *Trichoderma* in soil. Similarly, soil bacterial populations were reduced but not totally eliminated. Soil was allowed to air dry under a transfer hood. For experiments, soil moisture was adjusted gravimetrically according to a previously derived soil moisture release curve. Soil pH was approximately 6.0.

Mapping hyphal growth in soil. Treatments were designed to determine radial growth and density of hyphae under different conditions of temperature (15, 20, 25 C), soil matric potential (−0.03, −0.1, −0.5 MPa), and incorporated bran (0 or 2.5 mg of bran per pellet). For each treatment, moisture content of a batch of soil was adjusted to one of the experimental levels as described above, and then soil was added to either 9-cm-diameter glass petri dishes, 15-cm-diameter petri dishes, or 30 × 20 × 5 cm glass trays. Because treatments were to be sampled destructively over time, larger containers were used for longer incubation periods when more extensive hyphal growth was anticipated.

Containers were filled about halfway with soil, and then one or more pellets were placed on the soil surface. Pellets and the soil surface were overlaid with two layers of 1-mm^2 pore-size nylon mesh, which then were covered with soil to fill the containers. Soil was compressed to a bulk density of 1.2 g/cm^3. Containers were placed in plastic bags and incubated at one of the above temperatures for 1, 2, 3, 5, 7, or 14 days. In preliminary experiments (*unpublished*), we weighed, oven dried (48 hr, 100 C), and reweighed soil samples from treatment jars and observed that with this system soil moisture loss was less than 1% over 14 days.

Radial extension of hyphae was measured after first removing the upper layer of nylon mesh and the soil above it. Hyphal growth in the soil pore space underneath the lower mesh layer was observed with a binocular microscope. Hyphae generally did not adhere to the nylon mesh, and only those hyphae distinctly beneath the mesh were measured. Hyphal maps were constructed by noting the presence or absence of hyphae beneath the 1-mm^2 squares of the nylon mesh and marking corresponding squares on graph paper.

In preliminary experiments, containers were oriented in different ways (for example, flat or on edge), and we observed that the extent of radial growth was independent of orientation. Thus, we assumed that hyphal extension in the measured plane was representative of radial growth in a roughly spherical pattern. We determined the area encompassed by the perimeter of hyphal extension marked on each map by digitizing the maps, using a digitizer board and software (Jandel Scientific, Corte Madera, CA). Based on our observations of maps, we made the assumption that growth patterns were generally circular in the plane of observation and, thus, calculated the radius of each colony as a function of its area (that is, radius = [area/3.1415]$^{0.5}$). The experiment was performed once with three replicates per treatment

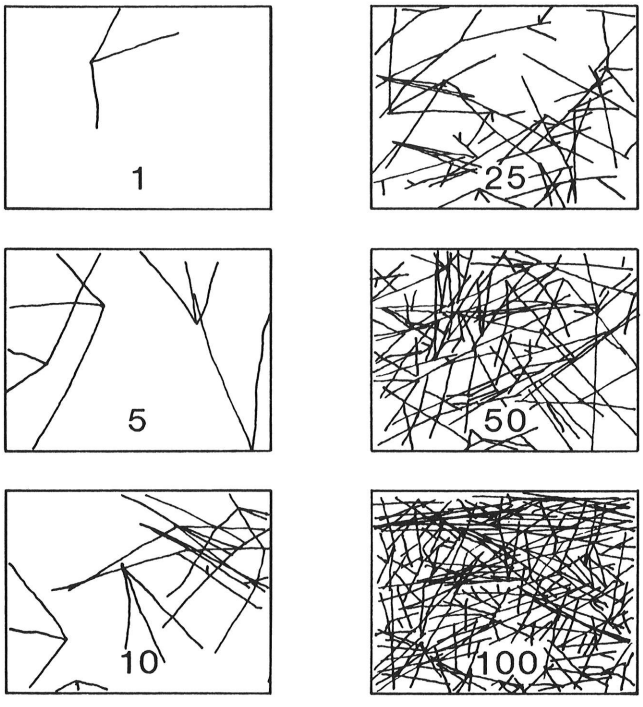

Fig. 1. Visual assessment key for estimating hyphal density in soil. A computer graphics program was written to generate enlarged simulated images of 4-mm^2 sample grids containing 1, 5, 10, 25, 50, or 100 linear mm of hyphae/mm^3.

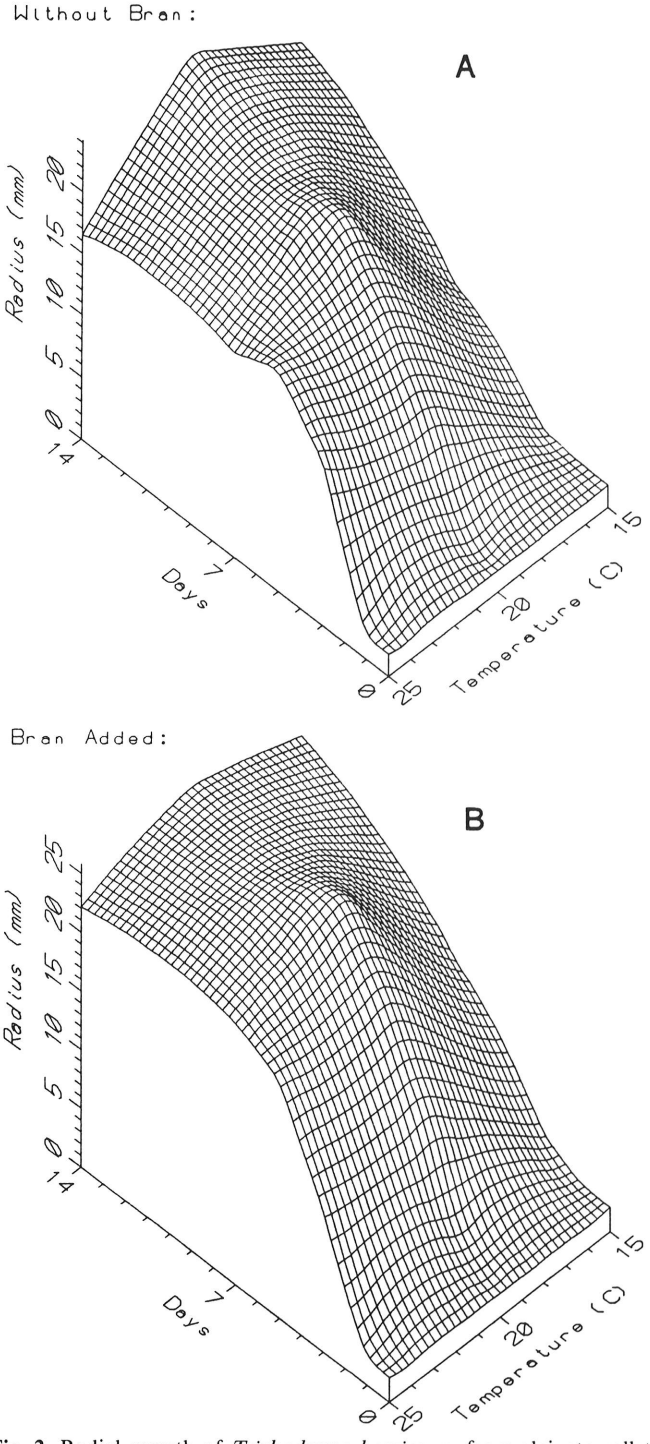

Fig. 2. Radial growth of *Trichoderma harzianum* from alginate pellets over time, at different temperatures, and **A,** without bran, or **B,** with wheat bran added. Matric potential did not significantly affect radial growth rate; therefore, data shown are averaged over all matric potential levels. Plots are shown after kriging and cubic spline smoothing.

per sample time and, subsequently, repeated with two replicates. Analysis of variance indicated no significant differences between repetitions of the experiment ($P < 0.05$); therefore, the experiment was analyzed as a single data set with five replicates and six sample times for each of the 18 treatments (three temperatures, three matric potentials, two bran levels), for a total of 540 hyphal maps. Colony radius values for time = 0 days were arbitrarily set at the mean measured radii of pellets: 1.8 mm for pellets without bran, and 2.07 mm for pellets with bran.

After 7 and 14 days of incubation, estimates of hyphal density were made with a visual reference key (Fig. 1). The key was developed by writing a computer graphics program to generate enlarged simulated images of 4-mm^2 sample grids containing 1, 5, 10, 25, 50, or 100 linear millimeters of hyphae per cubic millimeter. For each of four replicate pellets per treatment, we made four separate visual assessments at each of five distances from the pellet (5, 10, 20, 30, and 40 mm) in four directions, using a transparent plastic template. For each assessment, the visible hyphal pattern was compared with the key, and hyphal density thus was estimated. For each pellet, densities at each radial distance were averaged over the four directions.

Data analysis. A geostatistical package (Surfer, Golden Software, Inc., Golden, CO) was used to interpolate and plot radial growth data by a kriging routine (17) and cubic-spline smoothing. Radial growth was plotted as a function of temperature and matric potential for each level of bran over time. A general pattern was observed of no growth for 1–2 days, then an increase in radius with a gradual leveling off after several days. Radius values greater than 30 mm were rare (four of 540 observations), and we estimated this level as the asymptote of growth curves. Using the SAS statistical package (SAS Institute, Inc., Cary, NC), we tested a logistic growth model for each treatment. We assumed a lag period of 24 hr. The logistic model was fit by transforming all observations made after 24 hr with the following logit transformation:

$$\text{logit}(x) = \log_e (x/[1 - x/K]) \quad (1)$$

where x = colony radius in millimeters and K = 30 mm, and then regressing logit(x) against time. Radius values greater than 30 mm (three out of 540) were arbitrarily set at 29 mm. For regression of transformed data, the intercept was forced through the transformed value for the mean radius of ungerminated pellets. Analysis of variance then was performed, with the slope of each growth curve as the dependent variable and bran, temperature, and matric potential as independent variables.

Hyphal densities observed in the different treatments after 7 or 14 days were plotted. Based on observation of the plotted data, linear regression of log(density) versus distance from the pellet center was performed. Analysis of variance was performed to determine effects of bran, temperature, and matric potential on hyphal density at different distances from pellets after 7 or 14 days.

RESULTS

Radial growth. Plots of radial growth over time within the experimental range, after kriging and smoothing, are shown in Figure 2. For radial extension of hyphae, the logistic growth model was generally a good fit (mean R^2 for all treatments = 0.71, median = 0.74, range = 0.38–0.90). Analysis of variance indicated that temperature had a highly significant positive effect on radial growth rate ($P < 0.01$), but matric potential and bran effects on radial growth rate were not significant ($P > 0.10$). There were no significant interactive effects.

Hyphal density. Mean hyphal densities after 7 and 14 days are shown in Figure 3. The exponential model was a generally good fit to the data (mean R^2 = 0.75, median = 0.75, range = 0.41–0.93). Analysis of variance showed that hyphal density was significantly higher with bran ($P < 0.01$), in drier soil ($P < 0.01$), and after 14 versus 7 days ($P < 0.01$). Temperature did not significantly affect density ($P > 0.05$). There were no significant interactive effects. In Figure 3, the differential effect of bran on hyphal density is especially apparent.

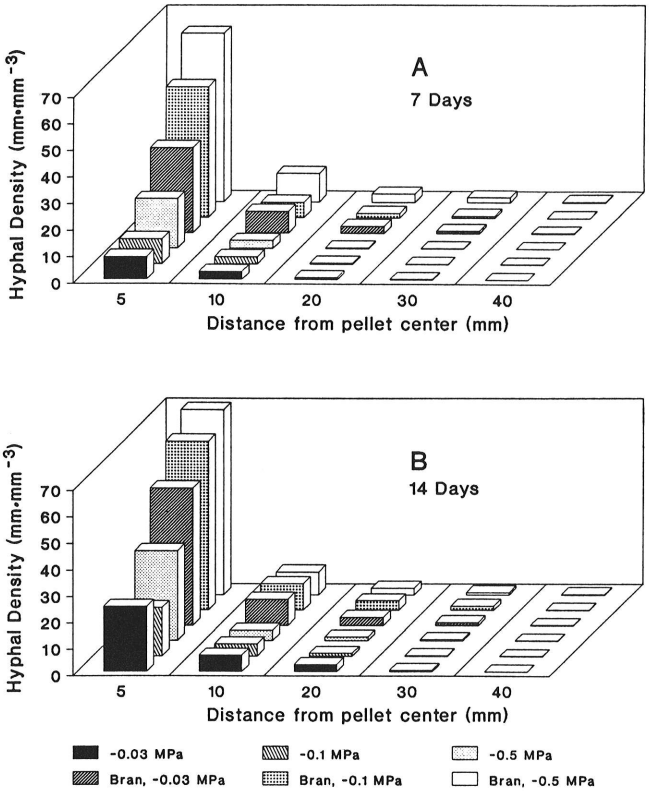

Fig. 3. Effects of bran and soil matric potential on hyphal density of *Trichoderma harzianum* originating from alginate pellets, **A**, after 7 days, and **B**, after 14 days. Temperature did not significantly affect density levels; therefore, data shown are averaged over all temperature levels.

DISCUSSION

For control of sclerotia-forming pathogens in soil, the ability of the biocontrol agent to effectively "explore" the three-dimensional soil space via hyphal growth may be more important than proliferation via spatially localized sporulation. The extent of mycoparasitism probably depends on the density and spatial arrangement of sclerotia and hyphae of the biocontrol agent.

Soil temperature affected radial growth rates of hyphae from alginate pellets, although radial extension at all temperatures tested appeared to reach a maximum of 30 mm or less within 7–14 days. Presumably, because growth in all treatments had more or less reached a limit by 7–14 days, there was no observed effect of temperature on hyphal density measured at these times. Soil moisture between −0.03 and −0.5 Mpa did not significantly affect radial growth rates; however, there was a significant effect of soil matric potential on hyphal density at these times. We speculate that reduced availability of oxygen in the wetter soil may have adversely affected hyphal proliferation.

Addition of a food base (bran) did not significantly increase radial growth rates; however, bran did have a highly significant effect on hyphal density within the limits of radial growth. Thus, if proliferation is defined as an increase in biomass rather than an expanding zone of hyphal extension, bran had a positive effect on proliferation of the biocontrol agent, and, thus, we agree with the observations of Lewis and Papavizas (12). Although sporulation was not quantified in these experiments, we consistently noted that hyphae from pellets with bran sporulated sooner and much more profusely than those from pellets without bran.

The methods presented here should be useful for comparison of nutritional additives or other formulations for pelletized biocontrol fungi and as a basis to develop predictive models for performance of such formulations at different application rates

and under varied environmental conditions. Two potential sources of experimental error should be noted. First, because the visual reference key for hyphal density has density classes that differ by roughly exponential levels of magnitude, it is possible that observations made with the key will have some bias toward an exponential pattern. Second, it was necessary to steam the soil used to confidently identify hyphae as originating from a particular alginate pellet. Thus, it is possible that interactions with other soil microbes will be underestimated with these methods. We currently are investigating how restoration of specific components of the resident bacterial population affects hyphal growth patterns.

A logical next step will be to extend this model system to quantify growth in raw soil and under fluctuating environmental conditions. Although visualization of hyphal growth from a single source in nonsterile soil may be technically unfeasible, indirect methods (for example, baiting for hyphae) should allow validation of predictions made based on the methods presented here.

LITERATURE CITED

1. Abd-El Moity, T. H., Papavizas, G. C., and Shatla, M. N. 1982. Induction of new isolates of *Trichoderma harzianum* tolerant to fungicides and their experimental use for control of white rot of onion. Phytopathology 72:396-400.
2. Ahmad, J. S., and Baker, R. 1987. Rhizosphere competence of *Trichoderma harzianum*. Phytopathology 77:182-189.
3. Backman, P. A., and Rodriguez-Kabana, R. 1975. A system for the growth and delivery of biocontrol agents to the soil. Phytopathology 65:819-821.
4. Beagle-Ristaino, J. E., and Papavizas, G. C. 1985. Survival and proliferation of propagules of *Trichoderma* spp. and *Gliocladium virens* in soil and in plant rhizospheres. Phytopathology 75:729-732.
5. Chet, I., Hadar, Y., Elad, Y., Katan, J., and Henis, Y. 1979. Biological control of soil-borne plant pathogens by *Trichoderma harzianum*. Pages 585-591 in: Soil-Borne Plant Pathogens. B. Schippers and W. Gams, eds. Academic Press, New York.
6. Elad, Y., Chet, I., and Henis, Y. 1981. Biological control of *Rhizoctonia solani* in strawberry fields by *Trichoderma harzianum*. Plant Soil 60:245-254.
7. Elad, Y., Chet, I., and Katan, J. 1980. *Trichoderma harzianum*: A biocontrol agent effective against *Sclerotium rolfsii* and *Rhizoctonia solani*. Phytopathology 70:119-121.
8. Elad, Y., Hadar, Y., Chet, I., and Henis, Y. 1982. Prevention, with *Trichoderma harzianum* Rifai aggr., of infestation by *Sclerotium rolfsii* Sacc. and *Rhizoctonia solani* Kuhn, of soil fumigated with methyl bromide, and improvement of disease control in tomatoes and peanuts. Crop Prot. 1:199-211.
9. Fravel, D. R., Marois, J. J., Lumsden, R. D., and Connick, W. J., Jr. 1985. Encapsulation of potential biocontrol agents in an alginate-clay matrix. Phytopathology 75:774-777.
10. Hadar, Y., Chet, I., and Henis, Y. 1979. Biological control of *Rhizoctonia solani* damping-off with wheat bran culture of *Trichoderma harzianum*. Phytopathology 69:64-68.
11. Lewis, J. A., and Papavizas, G. C. 1984. A new approach to stimulate population proliferation of *Trichoderma* species and other potential biocontrol fungi introduced into natural soils. Phytopathology 74:1240-1244.
12. Lewis, J. A., and Papavizas, G. C. 1985. Characteristics of alginate pellets formulated with *Trichoderma* and *Gliocladium* and their effect on the proliferation of the fungi in soil. Plant Pathol. 34:571-577.
13. Lewis, J. A., and Papavizas, G. C. 1987. Application of *Trichoderma* and *Gliocladium* in alginate pellets for control of *Rhizoctonia* damping-off. Plant Pathol. 36:438-446.
14. Papavizas, G. C. 1981. Survival of *Trichoderma harzianum* in soil and in pea and bean rhizosphere. Phytopathology 71:121-125.
15. Papavizas, G. C. 1985. *Trichoderma* and *Gliocladium*: Biology, ecology, and potential for biocontrol. Annu. Rev. Phytopathol. 23:23-54.
16. Papavizas, G. C., Fravel, D. R., and Lewis, J. A. 1987. Proliferation of *Talaromyces flavus* in soil and survival in alginate pellets. Phytopathology 77:131-136.
17. Ripley, B. D. 1981. Spatial Statistics. Wiley-Interscience, Inc. 252 pp.
18. Ruppel, E. G., Baker, R., Harman, G. E., Hubbard, J. P., Hecker, R. J., and Chet, I. 1983. Field tests of *Trichoderma harzianum* as a biocontrol agent of seedling disease in several crops and Rhizoctonia root rot of sugar beet. Crop Prot. 2:399-408.
19. Wells, H. D., Bell, D. K., and Jaworski, C. A. 1972. Efficacy of *Trichoderma harzianum* as a biocontrol for *Sclerotium rolfsii*. Phytopathology 62:442-447.