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Influence of Soil Microbial Biomass on Growth and Biocontrol Efficacy of *Trichoderma harzianum*

Yeoung-Seuk Bae[1], Guy R. Knudsen* and Louise-Marie C. Dandurand

**Plant Pathology Division, National Institute of Agricultural Science and Technology, RDA, Suwon, 441-707, Korea*

[1]Department of Plant, Soil, and Entomological Sciences, College of Agriculture, University of Idaho, Moscow, Idaho 83844-2339, USA

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The hyphal growth and biocontrol efficacy of *Trichoderma harzianum* in soil may depend on its interactions with biotic components of the soil environment. The effect of soil microbial biomass on growth and biocontrol efficacy of *T. harzianum* isolate ThzID1-M3 (green fluorescent protein transformant) was investigated using artificially prepared different levels of soil microbial biomass (153, 328, or 517 µg biomass carbon per g of dry soil; BC). The hyphal growth of *T. harzianum* was significantly inhibited in the soil with 328 or 517 µg BC compared with 153 µg BC. When ThzID1-M3 was added to the soils as an alginate pellet formulation, the recoverable population of ThzID1-M3 varied, but the highest population occurred in 517 µg BC. Addition of alginate pellets of ThzID1-M3 to the soils (10 per 50 g) resulted in increased indigenous microbial populations (total fungi, bacteria, fluorescent *Pseudomonas* spp., and actinomycetes). Furthermore, colonizing ability of ThzID1-M3 on sclerotia of *Sclerotinia sclerotiorum* was significantly reduced in the soil with high level of BC. These results suggest that increased soil microbial biomass contributes to increased interactions between introduced *T. harzianum* and soil microorganisms, consequently reducing the biocontrol efficacy of *T. harzianum*.

Keywords **:** biological control, *Sclerotinia sclerotiorum*, soil biomass, soil microorganisms, *Trichoderma harzianum*.

Isolates of *Trichoderma* spp. have been known as potential biocontrol agents for many soilborne fungal pathogens of plants (Chet, 1987; Papavizas, 1985). For effective biocontrol of soilborne plant pathogens, hyphal growth of *Trichoderma* through soil is one of the important factors to extend towards and colonize target propagules after introduced into soil (Knudsen et al., 1990, 1991). However, numerous biotic and abiotic factors may reduce growth and establishment of biocontrol fungi in soil ecosystems (Bae and Knudsen, 2001; Dandurand and Knudsen, 1993; Eastburn and Butler, 1988a, b; Hubbard et al., 1983; Knudsen and Bin, 1990; Papavizas, 1985). Interactions between introduced biocontrol agents and other microorganisms in soil ecosystems are important, but frequently have been assessed the effect to a single microorganism against biocontrol agent *Trichoderma* sp. rather than by regarding whole ecological communities (Bin et al., 1991; Dandurand and Knudsen, 1993). For example, fluorescent *Pseudomonas* species inhibited and lysed germlings of *Trichoderma* sp. under conditions of iron deprivation, resulting in failure of biological control of Pythium seed rots (Hubbard et al., 1983). In a previous study in our laboratory, Bin et al. (1991) observed that *P. fluorescens* 2-79RN10 inhibited radial growth and hyphal density of *T. harzianum* on agar and in sterile bulk soil.

Microbial biomass is an active component of soil ecosystem, primarily composed of bacteria and fungal mycelia and spores (Lumsden, 1990). This component is usually influenced by seasonal moisture and temperature fluctuations, addition of organic matters, and tillage management (Dalal et al., 1991; Lemanceau et al., 1995; Lundquist et al., 1999). Microbial biomass generally increases at or near the soil surface (Lundquist et al., 1999), which may be important for biocontrol since hyphal growth of *Trichoderma* spp. may also occur at or near the soil surface (Eastburn and Butler, 1988b). There is little information available on the role of total soil microbial biomass on the growth and biocontrol efficacy of introduced *T. harzianum* into the soil.

The objectives of this study were: first, to determine whether increased soil microbial biomass influences hyphal growth, population, and biocontrol efficacy of introduced *T. harzianum,* and, second, to determine whether the introduction of *T. harzianum* as alginate pellets into soil changes the population of indigenous soil microorganisms.

*Corresponding author.
Phone) +82-31-290-0562, FAX) +82-31-290-0453
E-mail) ysbae@rda.go.kr

Materials and Methods

Isolates and media. *T. harzianum* isolate ThzID1-M3 containing three foreign genes (hygromycin B resistance, β-glucuronidase, and green fluorescent protein genes, Bae and Knudsen, 2000) was used in this experiment. Alginate pellets of ThzID1-M3 that contain hyphal biomass and spores with wheat bran plus polyethylene glycol 8,000 (PEG) were formulated as described previously (Bae and Knudsen, 2000). Pellets were air-dried on waxed paper and were stored at 4°C in a covered glass beaker. Pellets used in this experiment were 2-4 wk old.

An isolate of *S. sclerotiorum* (Knudsen and Bin, 1990) maintained on potato dextrose agar (PDA) was transferred to and cultured on PDA at 25°C for 7 days. Mycelial disks were transferred to sterilized sliced carrots contained in 2-L Erlenmeyer flasks. After 6-8 weeks of incubation at room temperature, sclerotia were harvested, rinsed with sterile distilled water, and air-dried for 2-3 days. The sclerotia were attached to plastic toothpicks with cyanoacrylate glue and allowed to dry overnight. All sclerotia were surface-sterilized with a sterile solution (1 min, 10% ethanol, 10% bleach in sterile distilled water) and stored at 4°C before use.

The media used were half strength of tryptic soy agar (1/2 TSA, Difco), King's B medium (KB), and Actinomycete Isolation Agar (Difco) for counting total bacteria, fluorescent *Pseudomonas* spp., and actinomycetes, respectively. Total fungi, *Trichoderma* spp., and ThzID1-M3 were counted on peptone-rose bengal agar (Dhingra and Sinclair, 1985), *Trichoderma* selective medium (TSM, Elad et al., 1981), and TSM amended with 50 μg per ml of hygromycin B, respectively.

Soil preparation and determination of microbial biomass carbon. Palouse silt loam soil was obtained from the University of Idaho Parker Farm near Moscow. Soil analysis (University of Idaho Analytical Services Laboratory) indicated that the soil contained 20% sand, 20% clay, and 60% silt by weight, with 82.2 μg per g of plant available iron. Soil pH in soil to water (2:1) solution was approximately 5.9. Soil was sieved through a 2-mm mesh and kept at room temperature prior to use.

To generate soils containing different levels of soil biomass, one hundred gram of the soil was inoculated into a 1-liter flask containing 500 ml of autoclaved nutrient solution (5 g of glucose and 1 g of yeast extract in 500 ml of distilled water). The flask was incubated for 5 days with shaking at approx. 200 rpm at room temperature (hereafter named soil culture). Then, a 2.5-kg amount of air-dried soil was amended with 100 ml or 500 ml of the soil culture, and another 2.5 kg of air-dired soil was used as non-amended soil. Therefore, treatments consisted of non-amended soil and amended soils with 100 ml or 500 ml of the soil culture. All soils were adjusted the soil moisture content to -50 kPa. Then, the soils were separately placed in plastic containers and incubated at 25°C for 2 days to stabilize microorganisms. The soils were sieved through a 2-mm mesh and vigorously mixed again just before use.

Soil microbial biomass carbon (BC) was determined by the chloroform fumigation procedure of Jenkinson and Powlson (1976) with minor modifications. Two 20-g soil samples of each soil were placed in 100-ml glass beakers with three replicates. One sample was used as a control, while the other was fumigated with 50 ml of ethanol-free chloroform (Fisher Scientific, Cat. No. C607-4) in a vacuum dessicator lined with moist paper. Vacuum was applied until the chloroform boiled vigorously for 2 min. The non-fumigated and fumigated soils were incubated in room temperature for 24 hr in the dark. After removing the moist paper, the residual chloroform vapor was removed from the soil samples by repeating evacuation with a vacuum. Then, both soil samples (non-fumigated and fumigated) were adjusted to 30% soil moisture by weight. Instead of water, a soil suspension prepared from 10 g of non-fumigated soil in 100 ml of distilled water was used as a microbial inoculum for the fumigated soils. Each sample including a blank (without soil) was placed in a mason jar with a vial containing 5 ml of 1 M NaOH as a CO_2 trap. Twenty ml of sterile water was added in the bottom of the jar. The jars were tightly closed with lids and incubated at 25°C for 10 days in the dark. In order to determine the evolved CO_2 in the NaOH solution and BC, the method described by Jenkinson and Powlson (1976) was used. CO_2-C evolved was calculated by the equation as follows:

$$CO_2\text{-C } (\mu g\ g^{-1} \text{ dry soil}) = (B-S) \times M \times E \times A / DW$$

where B is the amount of acid needed to titrate the NaOH solution in the bottle containing the soil sample from pH 8.3 to 3.7 in microliter, S is the amount of acid needed to titrate the NaOH solution in the blank from pH 8.3 to 3.7 in microlitter, M is the molarity of the HCl, E = 6 (equivalent weight to express the data as carbon), A is the ratio of the total volume of the NaOH to the titrated volume of the NaOH, and DW is the dry weight of the soil in grams. BC was calculated by

$$BC\ (\mu g\ g^{-1} \text{ dry soil}) = (F-NF) / k$$

where F is CO_2-C evolved from the fumigated sample, NF is CO_2-C evolved from the non-fumigated sample, and $k = 0.45$, a constant representing the estimate of the proportion of microbial biomass C that is mineralized to CO_2 over a 10-day incubation period.

Effect of soil microbial biomass on radial growth of ThzID1-M3. Radial growth of ThzID1-M3 was measured by a previously described method (Bae and Knudsen, 2000). Glass petri dishes (15-cm diameter) were half filled with the amended soil described above and a glass slide pre-coated with 1.8% water agar was placed on the soil surface. A single pellet sized at the average of 3-mm in diameter was glued in the middle of the glass slide with cyanoacrylate glue. The glass slide and pellet were covered with the soil to fill the plate, then the plates were placed in a covered plastic container lined with moist paper towels and incubated at 25°C for 3, 5, 7, and 14 days. At each sampling time, glass slides were removed and examined using epifluorescence microscopy. Radial growth was quantified by measuring colony diameters at magnifications of ×250 or ×400. There were five replicates of slides per treatment.

Assessment of ThzID1-M3 and microbial populations. To measure ThzID1-M3 and microbial populations, a 50-g sample of each amended soil was placed in a 250-ml glass container. Ten alginate pellets of ThzID1-M3 were added and thoroughly mixed

in. The container was covered by aluminum foil to obtain a relatively constant soil moisture and incubated at 25°C for 3, 5, 7, and 14 days in the dark. At each sampling time, populations of introduced ThzID1-M3, total *Trichoderma* spp. (indigenous *Trichoderma* spp plus ThzID1-M3), total fungi, total bacteria, fluorescent *Pseudomonas* spp., or actinomycetes were determined as follows. The soil in a 250-ml flask was thoroughly mixed by voltexing at maximum speed, then a 1-g sample was randomly taken and suspended in 9 ml sterile distilled water. Serial dilutions were made, and aliquots (100 μl) were spread on each plate of media for counting microbes described above. Plates were incubated at 25°C, and colonies developed on each medium were counted after 3 days for bacteria and fluorescent *Pseudomonas* spp., 5-10 days for fungi including ThzID1-M3, 10-14 days for actinomycetes. Other soils were used to measure soil moisture content. There were three replicates per treatment.

Effect of soil microbial biomass on colonization of sclerotia by ThzID1-M3. A 150-g amount of each soil, as described above, was mixed with or without 20 pellets of ThzID1-M3 in a plastic bag, and placed in a 250-ml glass jar. Then, ten sclerotia glued on toothpicks were randomly distributed in the soil at the depth of 2 cm. Jars were covered to maintain a relatively constant moisture. Treatments were non-amended soil and amended soils (100 or 500 ml of soil culture) added with or without ThzID1-M3 alginate pellets. After 14 days of incubation at 25°C, the sclerotia from each jar were recovered, surface-sterilized, and placed on PDA containing 0.5 ml of tergitol (Sigma) and 0.15 g of tetracycline per liter to reduce colony growth (Fravel et al., 1985). Plates were incubated at 25°C for 10 days, and sclerotia were observed to determine whether *S. sclerotiorum*, or *Trichoderma* spp. were growing from them. For ThzID1-M3, mycelia grown from sclerotia were examined for green fluorescent protein activities using epifluorescence microscopy. Proportions of sclerotia colonized by *Trichoderma* spp. or ThzID1-M3 were recorded. There were four replicates.

Statistical analysis. All data except BC data were subjected to factorial analysis of variance (ANOVA) using Statistical Analysis System software (SAS Institute, Inc., Cary, NC). BC data were subjected to analysis of variance. Statistical analyses and inferences were based on log transformations for all data and arcsine transformation for percentage data. Means and the standard error of the mean were calculated for all data. The experiments were repeated once with similar results and results from one experiment are presented here.

Results

Soil microbial biomass carbon. BC in non-amended soil was 153 μg per g of dry soil, whereas it was 328 or 517 μg per g of dry soil in soils amended with 100 or 500 ml of the soil culture to 2.5 kg of the dried soil (data not shown).

Effect of soil microbial biomass on radial growth and population of ThzID1-M3. Increased soil microbial biomass resulted in inhibitory affects on the radial growth of *T. harzianum* ThzID1-M3 over time (Fig. 1). However,

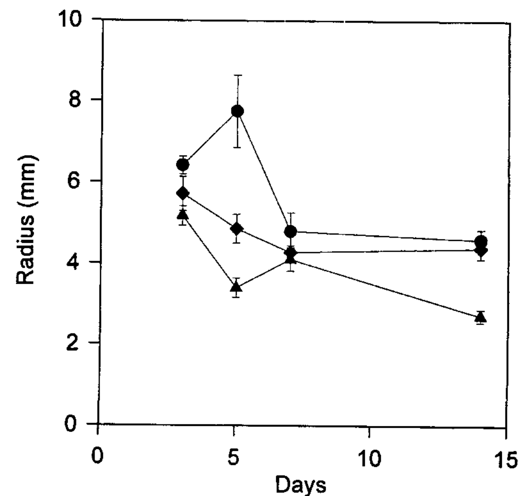

Fig. 1. Effect of soil microbial biomass on hyphal radial growth of *T. harzianum* isolate ThzID1-M3 originating from an alginate pellet in soil containing 153 (●), 328 (◆), or 517 (▲) μg biomass carbon per g of dry soil at 3, 5, 7, 14 days. Vertical bars represent ±1 SEM.

analysis of variance indicated significant ($P < 0.05$) main effect (soil microbial biomass and sample time) and two-factor interaction (soil microbial biomass x sample time).

Mean recoverable number of ThzID1-M3 is shown in Fig. 2. Main effects (soil microbial biomass and sample time) were significant ($P < 0.05$), but there was no significant ($P > 0.05$) two-factor interaction (soil microbial biomass x sample time). Mean recoverable numbers of ThzID1-M3 in all treatments gradually increased until 7 days and decreased by day 14, with the highest measured

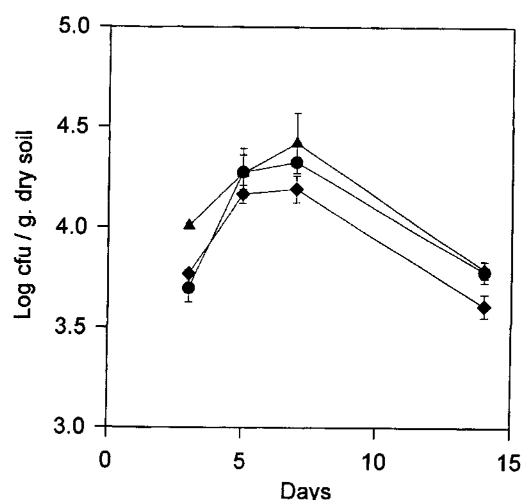

Fig. 2. Effect of soil microbial biomass on population density of *T. harzianum* isolate ThzID1-M3 originating from alginate pellets in soil containing 153 (●), 328 (◆), or 517 (▲) μg biomass carbon per g of dry soil at 3, 5, 7, 14 days. Vertical bars represent ±1 SEM.

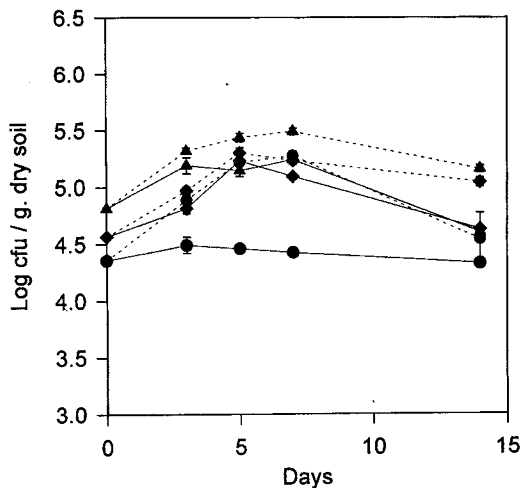

Fig. 3. Effect of alginate pellet addition of *T. harzianum* isolate ThzID1-M3 on population density of total *Trichoderma* spp. in soil containing 153 (●), 328 (◆), or 517 (▲) μg biomass carbon per g of dry soil at 3, 5, 7, 14 days. The soils were mixed with (dotted line) or without (solid line) alginate pellets. Vertical bars represent ±1 SEM.

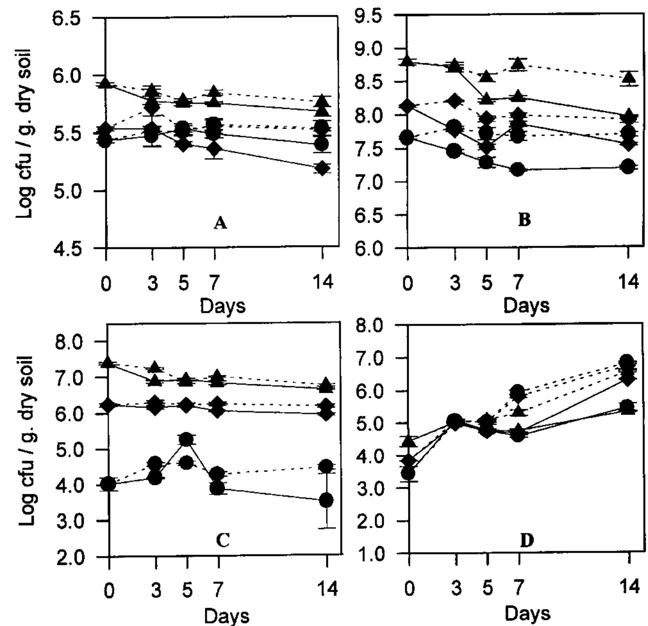

Fig. 4. Effect of alginate pellet addition of *T. harzianum* isolate ThzID1-M3 on population densities of total fungi (A), bacteria (B), fluorescent *Pseudomonas* spp. (C), and actinomycetes (D) in soil containing 153 (●), 328 (◆), or 517 (▲) μg biomass carbon per g of dry soil at 3, 5, 7, 14 days. The soils were mixed with (dotted line) or without (solid line) alginate pellets. Vertical bars represent ±1 SEM.

population densities at day 7. At day 3, the mean number of ThzID1-M3 was significantly higher in the soil containing the high BC (517 μg per g of dry soil). After day 5, there was no significant difference between the soils of 153 and 517 μg BC. The soil of 328 μg BC showed lower population densities at day 7 and 14 than those in the other soils.

Populations of *Trichoderma* spp. and total fungi. The amendment of soil culture to the dried soil increased the initial mean population densities of recoverable *Trichoderma* spp. in the soils (Fig. 3). Addition of *T. harzianum* ThzID1-M3 pellets to the soils resulted in significantly higher population densities of *Trichoderma* spp. over those of non-amended soils over time, showing the highest population densities in the soil of 517 μg BC during the experimental period. Analysis of variance indicated significant ($P < 0.05$) main effect (soil microbial biomass, ThzID1-M3 addition, and sample time), two-factor interaction (soil microbial biomass x ThzID1-M3 addition and soil microbial biomass x sample time), and three-factor interaction (soil microbial biomass x ThzID1-M3 addition x sample time).

Analysis of variance for the mean culturable numbers of total fungi showed significant ($P < 0.05$) main effect and two-factor interaction, but three-factor interaction was not significant ($P > 0.05$, Fig. 4A).

Populations of total bacteria, fluorescent *Pseudomonas*, and actinomycetes. Mean culturable number of total bacteria decreased below initial densities in all soils during the experimental period (Fig. 4B). There were significant ($P < 0.05$) main effect (soil microbial biomass, ThzID1-M3 addition, and sample time), two-factor interaction (soil microbial biomass x sample time), and three-factor interaction. But there was no significant ($P > 0.05$) interaction between soil microbial biomass and ThzID1-M3 addition.

Analysis of variance for the mean population density of culturable fluorescent *Pseudomonas* species indicated significant ($P < 0.05$) main effect and two-factor interaction of soil microbial biomass x sample time (Fig. 4C). However, interaction of soil microbial biomass x ThzID1-M3 addition and three-factor interaction (soil microbial biomass x ThzID1-M3 addition x sample time) were not significant ($P > 0.05$).

The mean population density of actinomycetes increased over initial densities in all soils during the experimental period (Fig. 4D). Analysis of variance showed significant ($P < 0.05$) main effect, two-factor interaction, and three-factor interaction.

Effect of soil biomass on colonization of sclerotia of *S. sclerotiorum* by ThzID1-M3. The mean percentage of sclerotia colonized by ThzID1-M3 or *Trichoderma* spp. is shown in Fig. 5. Analysis of variance for the mean percentage of sclerotia colonized by ThzID1-M3 showed significant ($P < 0.10$) main effect of soil biomass or ThzID1-M3 and two-factor interaction (soil microbial biomass x ThzID1-M3 addition).

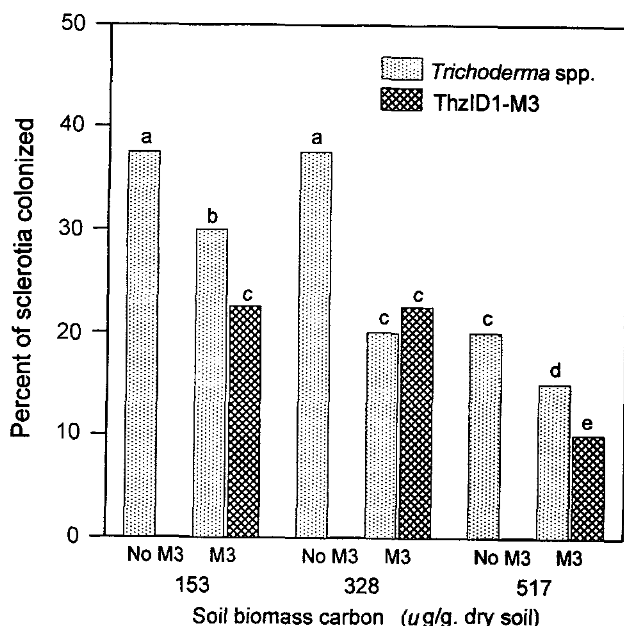

Fig. 5. Effect of soil microbial biomass on colonization of sclerotia of *S. sclerotiorum* by *T. harzianum* isolate ThzID1-M3 at different levels of soil microbial biomass at 14 days. Soil used contained 153, 328, or 517 μg microbial biomass carbon per g of dry soil and each soil was amended with 0 (no M3) or 20 pellets (M3) of ThzID1-M3 per 150 g of soil. Ten sclerotia were randomly distributed in 150 g of soil. Means followed by the same letter are not significantly different at $P < 0.05$.

There was significant ($P < 0.05$) main effect of soil microbial biomass or ThzID1-M3 addition on the mean percentage of sclerotia colonized by *Trichoderma* spp., but no significant interaction between soil microbial biomass and ThzID1-M3 addition ($P > 0.05$).

Discussion

When *T. harzianum* is introduced as alginate pellet formulation into soil ecosystems, the biocontrol efficacy may depend on its ability to explore through soil to the target propagules of soilborne pathogens during a short period of favorable environmental conditions (Dandurand et al., 1993; Knusden et al., 1990, 1991). However, natural soils are often unfavorable to introduced biocontrol agents due to abiotic or biotic factors (Bae and Knudsen, 2001; Knudsen and Bin, 1990; Hadar et al., 1984; Hubbard et al., 1983; Papavizas, 1985). We studied how soil microbial biomass, as a total biotic factor, affects the growth and biocontrol efficacy of introduced *T. harzianum* in soil.

The amount of soil microbial biomass in natural soil ranges between 90 and 2300 mg per g of dry soil (Jenkinson and Oades, 1979; Oades and Jenkinson, 1979; Van De Werf and Verstraete, 1987). However, active microbial biomass ranges between 75 and 272 mg per g of dry soil in agricultural soils regardless of texture, crop or fertilization level (Van de Werf and Verstraete, 1987), indicating that much of soil microbial biomass may be present in a dormant state. In this experiment, we used the soils containing soil biomass carbon of between 153 and 517 mg per g of dry soil. In the present study, increased soil biomass in the soil significantly reduced hyphal growth and biocontrol efficacy (measured as colonization of sclerotia) of *T. harzianum* during 14 days of incubation. This result may be caused by nutrient or niche competition (Eastburn and Butler, 1988ab; Hadar et al., 1984; Hubbard et al., 1983; Stack et al., 1987), antibiotic production (Fravel, 1988), or volatile organic compound production by other microbes (Mackie and Wheatley, 1999) or any combination of these. Athough recoverable numbers of ThzID1-M3 varied in the soils, the highest density of ThzID1-M3 occurred in the highest level of BC. This result suggests that hyphal growth of *T. harzianum* may not always accord with an increased soil population of *T. harzianum*. Taken all together, our results confirmed that biocontrol effectiveness mainly depends on the initial hyphal growth originating from pellets (Bin et al., 1991; Knudsen et al., 1991).

Eastburn and Butler (1988b) reported that an increase of *T. harzianum* in natural soil microsites resulted in an increase of total bacterial populations. In the present study, the addition of *T. harzianum* as alginate pellets into soil resulted in an increased population density of both total bacteria including fluorescent *Pseudomonas* species and actinomyctes over non-treated controls during the experimental period in all soils used. This result indicates that *T. harzianum* pellets may become nutrient source for other soil microorganisms, being degradated hyphae of *T. harzianum* or wheat bran in alginate pellets, or alginate itself. Many researchers (Hadar et al., 1984; Hubbard et al., 1983; Papavizas, 1985) emphasized that biotic components affect biocontrol efficacy of *T. harzianum* when newly introduced into soil ecosystems. Our results suggest that increased soil microbial biomass contributes to increased interaction between introduced *T. harzianum* and soil microorganisms. This appears to reduce the biocontrol efficacy of *T. harzianum*.

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