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The effect of *Trichoderma harzianum* in combination with organic amendment on soil suppressiveness to *Rhizoctonia solani*

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Summary. The effect of sheep manure amendment and the biocontrol agent *Trichoderma harzianum* Rifai on bean (*Phaseolus vulgaris* L.) damping off and on fungal activity including *Rhizoctonia solani* in the soil was investigated over a 24-month period. A combination of *T. harzianum* and sheep manure at all concentrations tested reduced damping off caused by *R. solani* after manure incubation in the soil for 0–24 months compared to the control at zero time. Disease reduction was 50% after 24 months with the highest concentration of organic amendment (10%). Disease reduction increased with increasing concentration of organic amendment and with the duration of the incubation time. A combination of *T. harzianum* and sheep manure reduced both the total fungal population and the *R. solani* population after 12 and 24 months. However, the population of *T. harzianum* increased with increasing concentration of sheep manure and duration of incubation. The incubation time had a greater effect on the *T. harzianum* population than did the concentration of the sheep manure. Sheep manure alone was less effective in reducing bean damping off and improving bean growth than a combination of both manure and *T. harzianum*. In conclusion, the soil suppressiveness to *R. solani* damping off was enhanced when *T. harzianum* isolate Jn14 was added to organically amended soil, and this degree of enhancement increased over time.

Key words: sheep manure, biological control, damping off, bean.

Introduction

Damping off caused by *Rhizoctonia solani* Kühn is an economically important disease of bean (*Phaseolus vulgaris* L.) in Palestinian agriculture. A variety of fungicides and soil fumigants are currently used to control *R. solani* and other soilborne plant pathogens. However, many of these control products are quite harmful to the environment and to the ground water. Methyl bromide is a good example of an effective soil fumigant that also unfortunately has a great adverse impact on the environment so that it has recently been phased out owing to health concerns. The use of micro-organisms antagonistic to *R. solani* has been investigated as a possible alternative to fungicides. *Trichoderma harzianum* Rifai is well documented as an effective biocontrol agent controlling *R. solani* in the soil (Papavizas, 1985; Coley-Smith *et al*., 1991; Prasun and Kanthadai, 1997; Sivasithamparam and Ghisalberti, 1998).

The feasibility of using organic amendments such as compost, animal manures and organic industrial by-products in order to suppress soilborne plant pathogens has been well documented (Hoitink and Boehm, 1999; Ryckeboer, 2001; Cheuk *et al*., 2005 and Noble and Coventry, 2005). Composts prepared from agricultural waste and used in container media or as soil amendments may have highly suppressive effects against diseases caused by a variety of soilborne plant pathogens such as

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Pythium spp. (Mandelbaum and Hadar, 1990; Pascual *et al*., 2000), *Phytophthora* spp. (Hoitink and Boehm, 1999; Widmer *et al*, 1999), *Rhizoctonia* spp. (Tuitert *et al*., 1998**;** Rivera *et al*., 2004) and *Fusarium* spp. (Cotxarrera *et al*., 2002). The effectiveness of manure amendments against disease depends on the type of manure, the type of soil, and other factors. Mushroom compost and manure decreased damping off of flax caused by *R. solani*, with the compost being more efficient than the manure (Alabouvette *et al*., 2004). The control of damping off (*R. solani*) on cucumber in compost-amended pot media was greatly affected by the stage of maturation of the compost: fresh compost actually stimulated pathogen growth and infection, but long-matured compost consistently suppressed it (Tuitert *et al*., 1998). The severity of bean root rot caused by *R. solani* was reduced by compost (Ferrara *et al*., 1996). Noble and Coventry (2005) reviewed the various combinations of biological control agents (including *T. harzianum*) and organic amendment that were reported to control soilborne plant pathogens. They stated that such combinations could significantly reduce the disease caused by *R. solani*. The aim of the present study was to investigate the effect of a combination of sheep manure organic amendment and a native isolate (Jn14) of the biocontrol agent *T. harzianum* on increasing soil suppressiveness to *R. solani* damping off of bean over time.

Materials and methods

Soil preparation

Soil samples were collected from the Al-Aroub agricultural research station (10 km north of Hebron, Palestinian Authority) and analyzed for physical and chemical properties (clay soil, consisting of sand 19.4%, silt 20.6% and clay 60%; pH 7.29, EC 1.5 ms, organic matter 2.5%, nitrate 45 ppm, ammonium 15.4 ppm, potassium 179.4 ppm, phosphorus 45.4 ppm). Soil samples were passed through a 4-mm mesh sieve to remove plant residues and gravel. Sieved soil samples were amended with air-dried sieved (4 mm) sheep manure at 0% (control), 2%, 6% and 10% (w:w). Manure-amended soils were further treated either with *R. solani* alone, or with *R. solani* plus *T. harzianum* isolate Jn14. The experimental design was completely randomized with three replicates for each treatment. Each replicate was represented by 20 kg soil placed in a 20 l container. The manure-amended soil in some of the containers was inoculated with 0.5 g kg^{-1} soil of *R. solani* inoculum, while that in other containers was inoculated with the same amount of *R. solani* inoculum plus 15 μg mycelium and 10^6 conidia g^{-1} soil of *T. harzianum*. Soil moisture in the containers was maintained at 60–80% of field capacity and the soil was incubated at 22–25°C for 24 months. Different treated soils in the containers were used to determine percent bean damping off, plant fresh weight (g $plant^{-1}$), the *R. solani* population (colony forming units, CFUs), total fungal population (CFUs), the *T. harzianum* population (CFUs), and dehydrogenase activity after 0, 6, 12, and 24 months of incubation.

Preparation of *R. solani* inoculum

The *R. solani* isolate used in the study was isolated from a diseased bean plant and classified as anastomosis group 1 according to Carling (1996). The inoculum was prepared as follows. One hundred grams of barley grains was poured into ten 1 l Erlenmeyer flasks containing 50 ml distilled water each and covered with aluminium foil. The flasks were autoclaved for 1 h daily at 121°C for three consecutive days and allowed to cool down. An antibiotic solution (pH=4) was added (streptomycin sulfate 250 μg g^{-1}, chlorotetracycline 250 μg g^{-1} and oxgall 500 μg g^{-1} [Sigma, Steinheim, Germany]) to each flask, which was then inoculated with a 5-mm mycelium plug cut from a 10-day-old *R. solani* culture growing on potato dextrose agar (PDA; Difco, Detroit, MI, USA). Inoculated flasks were incubated at 25°C for 3 days (12-hour day) in stationary culture to allow the mycelium to grow on the barley grains; flasks were then shaken by hand on a daily basis. After two weeks, the grains were dried under sterilized conditions, ground and sieved down to 3 mm. The manure-amended soils in the containers were inoculated with 0.5 g kg^{-1} soil of this *R. solani* inoculum.

Preparation of *T. harzianum* inoculum

Trichoderma harzianum Jn14 was isolated in 2004 (Barakat *et al*., 2006). This isolate had shown mycoparasitism and antibiosis against *R. solani* in both dual culture and other laboratory bioassays (Barakat *et al*., 2007). The isolate was grown on 90 mm Petri dishes containing PDA at 25°C for 10 days. Autoclaved Erlenmeyer flasks (500 ml) con-

taining 100 ml potato dextrose broth (PDB, Difco) were each inoculated with a 5 mm mycelium plug of the isolate. The flasks were then incubated under shaking (200 rpm) at 25°C in a growth chamber for 12 days. The contents of the flasks were filtered through a sterile glass funnel (pore size: 40–100 μm), and the mycelium mat was homogenized and separated. The filtrate suspension containing spores was centrifuged at 2308 × *g* (RCF) for 15 min. to precipitate the spores and the supernatant was discarded. The pellets containing the spores were then washed using sterile distilled water, vortexed to ensure a homogeneous suspension and centrifuged as previously described (repeated 2 times). After washing, the pellets were recovered in sterile water. The mycelium spore suspension was prepared by mixing homogenized mycelium previously prepared (1.5 g l^{-1}) and 10^8 conidia ml^{-1}. The mycelium spore suspension inoculum of *T. harzianum* was applied to the manure-amended soil at a concentration of 15 μg mycelium and 10^6 conidia g^{-1} of soil.

Evaluation of damping off and bean fresh weights

Five bean seeds per pot were planted in 1 l pots containing *R. solani*-treated soil amended with only manure or with manure plus *T. harzianum*. Damping off (%) caused by *R. solani* and plant fresh weight was evaluated 2–3 weeks after planting. The experimental design was completely randomized with five pots (replicates) for each treatment. The pots were then incubated in a growth chamber at 25ºC. Damping off (%) was evaluated after 0, 6, 12, and 24 months of amended soil incubation.

Fungal population

The total fungal population and the population densities of *R. solani* and *T. harzianum* (CFU g^{-1}) were evaluated in the treated soils after incubation for 0, 6, 12 and 24 months. The total fungal population was evaluated using the dilution plate technique, by seeding 200 μl of 10^{-5} soil dilution on each PDA Petri dish. The *R. solani* CFU was determined using the soil pellet technique (Shidong, 1995; Neate and Schneider, 1996). Samples of treated soil were spread evenly, compacted, and then divided with a small knife into 100 pellets, 5 mm in diameter and with a dry weight of approximately 0.2 g each. The pellets were placed on 90 mm Petri dishes (20 pellets per dish) on a *R. solani*-selective medium (Neate and Schneider, 1996). The basal medium was autoclaved at 121°C for 30 minutes and cooled down to 50–55°C, after which 450 μg Aliette (Bayer Crop Science, Germany), 8 μg pentachloronitrobenzene (PCNB) (Sigma, Steinheim, Germany), 300 μg streptomycin sulfate and 200 μg rose Bengal ml^{-1} (Sigma, Steinheim, Germany) were added. After incubation for 2 days at 25°C, the dishes were inspected and the number of pellets with *R. solani* growth was recorded.

The *T. harzianum* population (CFU g^{-1}) was evaluated using the dilution plate technique on a selective medium (Elad *et al*., 1981). Twenty-five grams of an air-dried soil sample was suspended in 225 ml of 0.1% water agar and shaken on a rotary shaker at 200 rpm for 20–30 minutes. Serial dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} were made for each soil sample, and 0.1 ml of the 10^{-5} soil dilution was plated on 90-mm Petri dishes containing a *Trichoderma*-selective medium (TSM) and spread out using a glass rod. Five Petri dishes, taken to be replicates, were used for each soil sample. Petri dishes were then incubated for 5–7 days at 25°C, and the germinated spores were counted. The experiments were designed as completely randomized; five replicates (plates) were used per treatment (0, 2, 6, and 10% organic amendment, with or without *T. harzianum*).

Dehydrogenase enzyme assay

Dehydrogenase enzymes are involved in the oxidation and reduction within the Krebs tricarboxylic acid cycle. Dehydrogenase activity should therefore indicate the respiration, and hence the activity, of the soil microflora. Soil dehydrogenase activity was determined by measuring the rate at which 2, 3, 5-triphenyltetrazolium chloride (TTC**,** Sigma) was reduced to 2, 3, 5-triphenyltetrazolium formazan (TTF, Sigma) (Smith and Pugh, 1979). Dehydrogenase enzyme activity in the incubated soil was assessed by placing 1.2 g soil in each of three closed test tubes (replicates) for each soil sample. Soil samples were then saturated with 400 μl of a 1% solution of TTC and mixed thoroughly using a vortex shaker (the TTC solution was prepared by dissolving 1 g TTC in 100 ml sterilized distilled water). The sealed tubes were incubated in a covered shaking water bath at 30°C for 24 h. After incubation, 2.4 ml of methanol (Sigma) was added to each tube and the contents vortexed and allowed to stand at room temperature for 10 min.

until the solution became red. The content of the tubes was centrifuged at 2308 × *g* (RCF) for 15 min. The aliquots were removed and the intensity of the red color, produced by the reduction of TTC to TTF, was determined spectrophotometrically at 485 nm. The amount of TTF was calculated with the absorbance of a known standard curve of TTF freshly prepared in methanol. Dehydrogenase activity was assessed in μl hydrogen; the formation of 1 mg of TTF requires 150.35 μl hydrogen. The data are shown after calculation as μl hydrogen g^{-1} dry soil 24 h^{-1} (μl H g^{-1} 24 h^{-1}). The dehydrogenase enzyme assessment was done after soil incubation for 0, 12, and 24 months (Smith and Pugh, 1979).

Statistical analysis

The experimental data were statistically analyzed using one-way repeated analysis of variance (ANOVA), and Fisher's LSD test ($P \leq 0.05$) was used for means separations (Sigma Stat® 2.0 program, SPSS Inc., Chicago, IL, USA)

Results

Damping off and bean fresh weights

Manure amendment significantly (LSD=21.23, $P \leq 0.05$) reduced bean seedling damping off at concentrations of 6% and 10% after 6, 12 and 24 months of incubation compared with the control at zero time. At a manure concentration of 6%, the reduction in damping off was not significant after 6, 12 and 24 months of incubation compared with the control after the same duration. At a manure concentration of 10%, the reduction in damping off was 23, 27, and 31% after these same periods of incubation respectively, compared with the control at zero time; this reduction became significant only after 24 months, when compared with the control at the same time (Fig. 1a). A combination of *T. harzianum* and organic amendment at 2%, 6%, and 10%, significantly (LSD=21.23, $P \leq 0.05$) reduced damping off after soil incubations of 0 to 24 months compared to the control at zero time. The reduction in damping off increased as the organic amendment concentration (r^2=0.83) and the duration of soil incubation (r^2=0.75) increased. Disease reduction was 33% and 50% in soil with *T. harzianum* plus organic amendment at concentrations of 6 and 10% respectively after 24 months of soil incubation (Fig. 1c). A manure concentration of 2% had no effect on damping off severity with any incubation duration in the absence of *T. harzianum*. However, damping off was significantly reduced at this concentration when *T. harzianum* was present in the soil. (Fig. 1c).

As regards bean growth, organic amendment at a concentration of 10% significantly increased bean fresh weight (LSD=2.99, $P \leq 0.05$) after 12 and 24 months of soil incubation in the presence of *T. harzianum* (Fig 1d). However, in the absence of *T. harzianum*, organic amendment at any concentration did not have a significant effect on bean fresh weight (Fig. 1b). A combination of *T. harzianum* and organic amendment at 10% increased bean fresh weight by 75% and 57% after 12 and 24 months of soil incubation respectively compared with the control at the same time (Fig. 1d).

Fungal population and activity

Organic amendment at a concentration of 10% significantly (LSD=3.19, $P \leq 0.05$) increased the total fungal population (CFU) by 47% and 68% after soil incubation for 12 and 24 months respectively (Fig. 2a). However, at lower manure concentrations (2 and 6%), the total fungal population was not affected even after long incubation periods. Organic amendments did not significantly affect the *R. solani* population (Fig. 2b), although they did increase this population slightly at the longer incubation periods, compared with the control at zero time. However, with 10% organic amendment, microbial activity was significantly stimulated after 12 and 24 months of soil incubation (Fig. 2c). At lower concentrations, organic amendments did not affect microbial activity as determined by dehydrogenase activity.

A combination of *T. harzianum* and organic amendment at a concentration of 6% significantly (LSD=3.19, $P \leq 0.05$) reduced the total fungal population by 54% and 79%, and at a concentration of 10% by 57% and 72% after 12 and 24 months respectively (Fig. 3a). Furthermore, a combination of *T. harzianum* and organic amendment significantly (LSD=17.46, $P \leq 0.05$) reduced the pathogen (*R. solani*) population by 29% and 26% at a manure concentration of 6%, and by 22% and 57% at a manure concentration of 10%, after 12 and 24 months respectively, compared with the control (Fig. 3b). The *T. harzianum* population increased significantly (LSD=13.09, $P \leq 0.05$) with increasing organic amendment concentration and increasing duration

of incubation; the population increased 5 and 7-fold with 6% organic amendment, and by 4 and 10-fold with 10% organic amendment after 12 and 24 months of incubation respectively, as compared with the initial population (Fig. 3c). However, with the lower concentrations of organic amendment (0 and 2%) the *T. harzianum* population increased with increasing incubation time. A combination of *T. harzianum* and organic amendment at 2, 6 and 10%, significantly (LSD=0.68, $P \leq 0.05$) increased microbial activity by 53, 66 and 193% respectively after 24 months of soil incubation, compared with the control (Fig. 3d). In addition, a combination of *T. harzianum* and organic amendment at 10% increased microbial activity by 135% after 12 months of soil incubation. In the presence of *T. harzianum,* the duration of manure incubation was more important for microbial activity than the concentration of the manure.

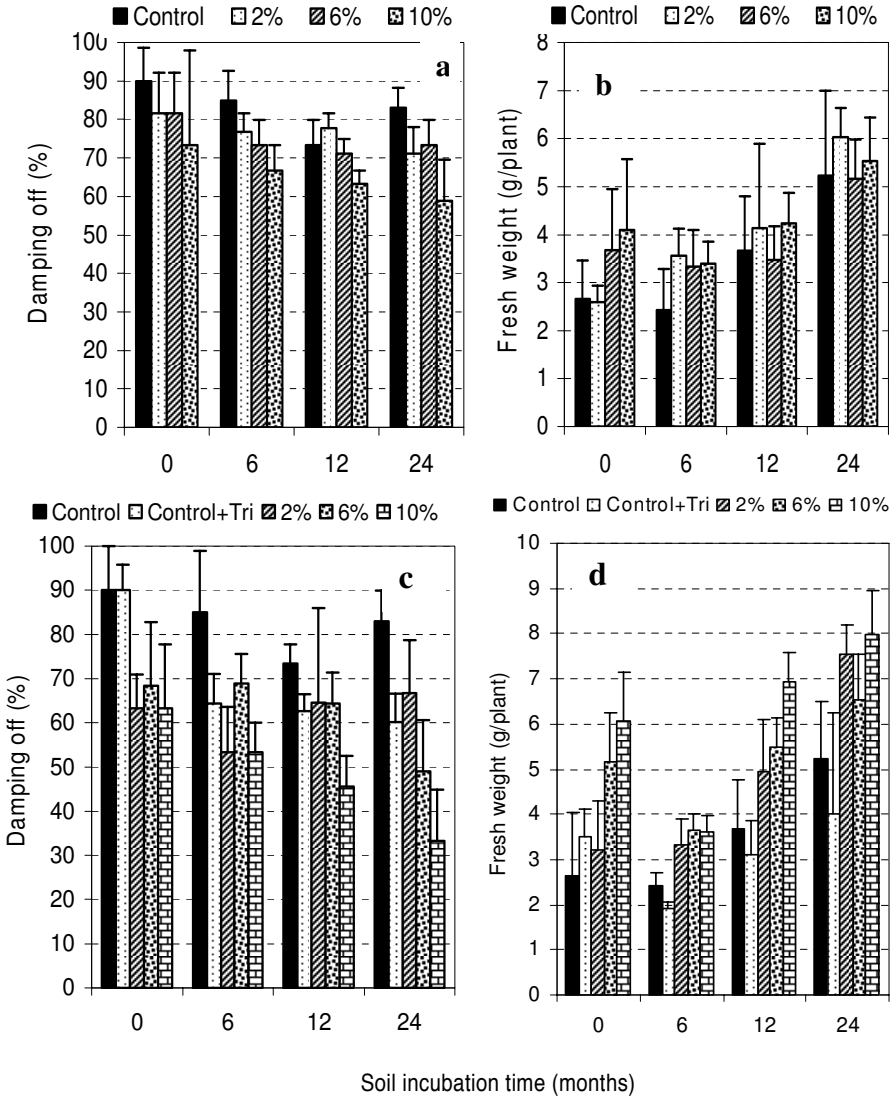

Fig. 1. Effect of incubation of manure amendment in soil on: a, bean damping off (%) caused by *Rhizoctonia solani*; b, bean fresh weight (g plant^{-1}). Effect of a combination of *Trichoderma harzianum* and organic amendment on: c, bean damping off (%), and d, bean fresh weight (g plant^{-1}). Vertical bars represent the standard deviation of replicates from the mean.

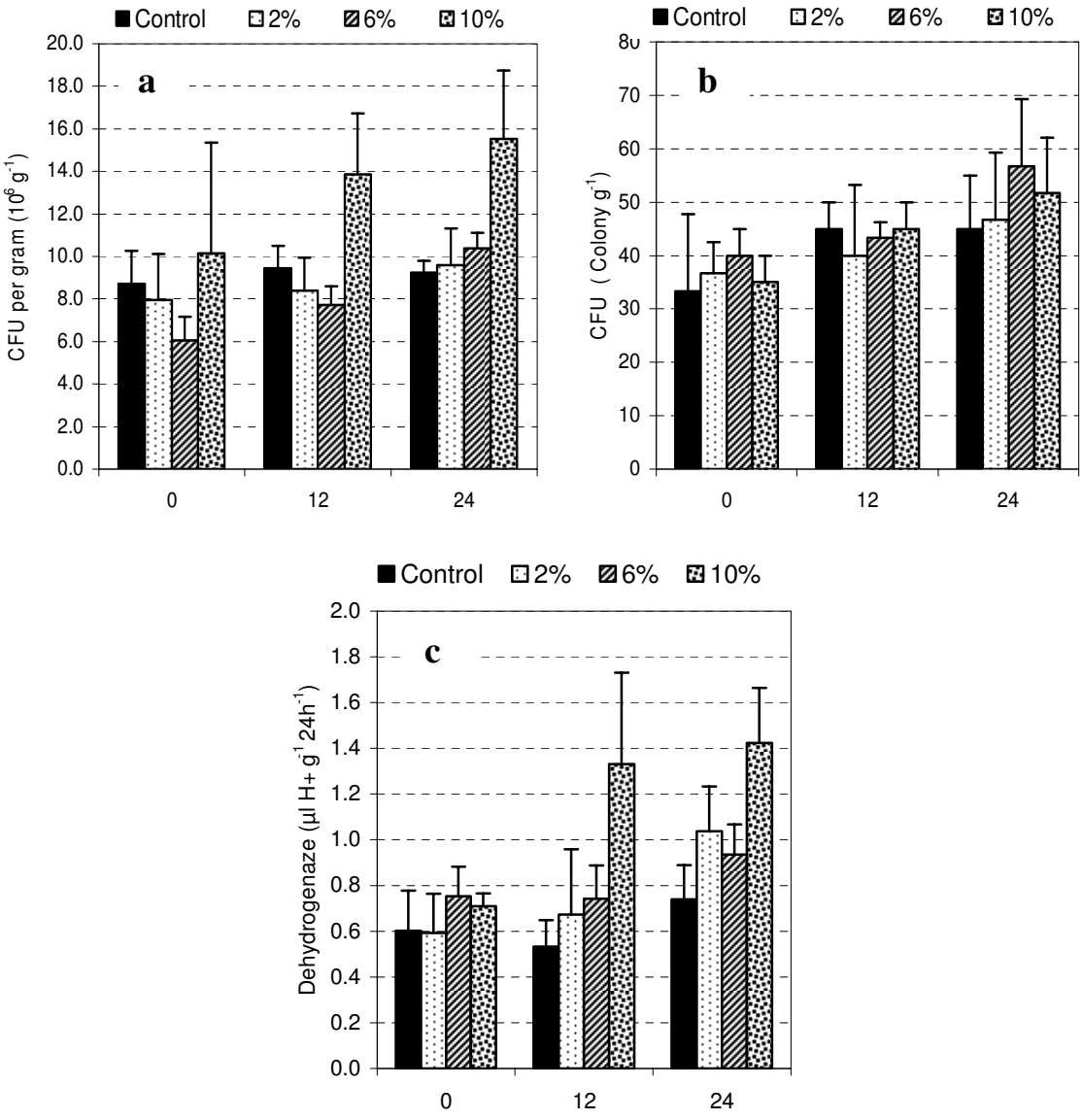

Fig. 2. Effect of incubation of a combination of *Trichoderma harzianum* and organic amendment in the soil on: a, total fungal population (CFU, 10^6 g^{-1}); b, *Rhizoctonia solani* population (CFU, colony g^{-1}); c, *T. harzianum* population (CFU, 10^6 g^{-1}); d, dehydrogenase enzyme activity (hydrogen ions, μl g^{-1} 24 h^{-1}). Vertical bars represent the standard deviation of replicates from the mean.

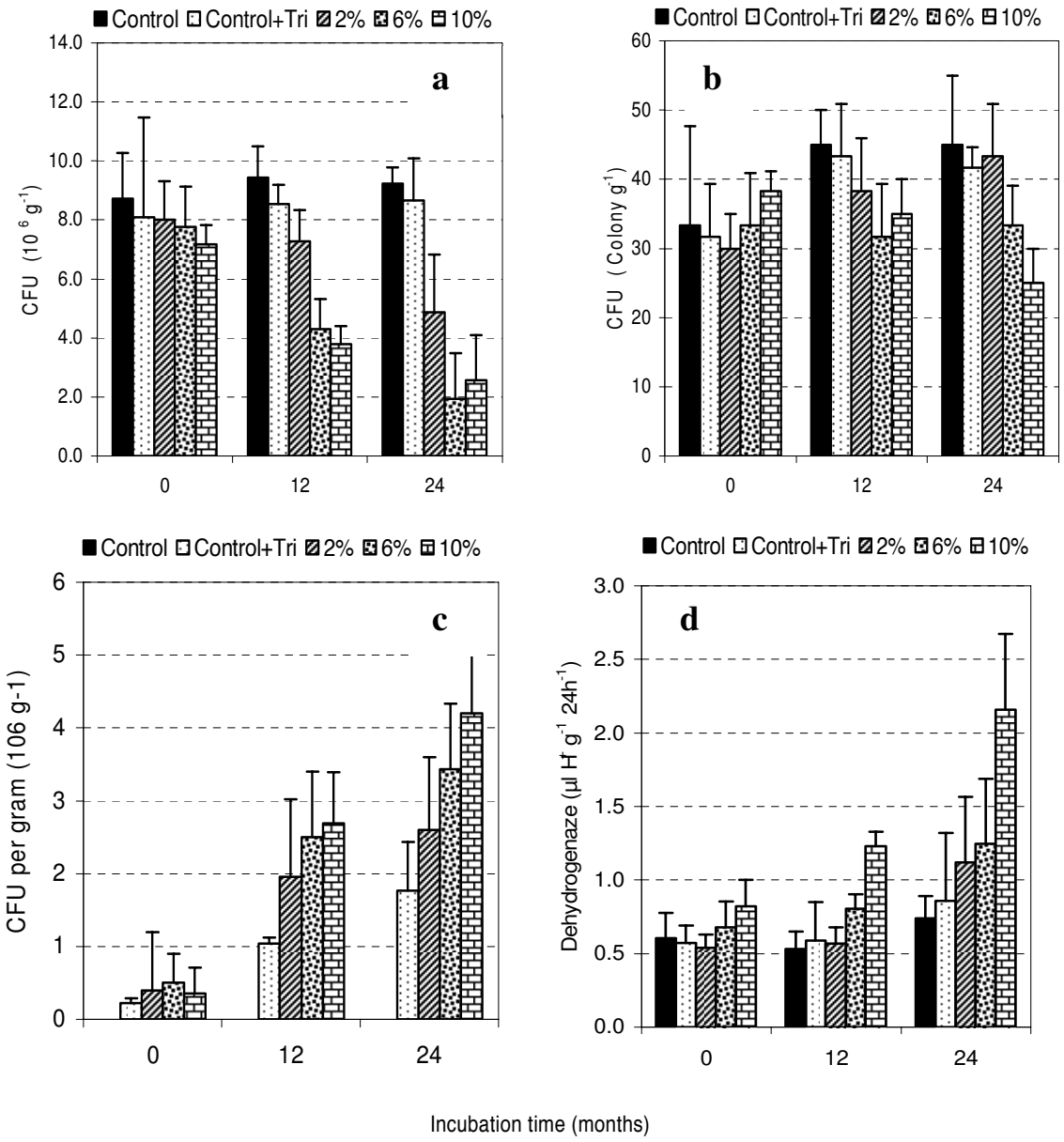

Fig. 3. Effect of incubation of organic amendment in soil on: a, total fungal population (CFU, 106 g-1); b, *Rhizoctonia solani* population (CFU, colony g-1); c, dehydrogenase enzyme activity (hydrogen ions, μl g-1 24 h-1). Vertical bars represent the standard deviation of replicates from the mean.

Discussion

Sheep manure amendment of the soil at the highest concentration (10%) reduced bean seedling damping off significantly after the longest duration of soil incubation (24 months); this reduction was correlated with both the concentration of manure and the duration of manure incubation in the soil. However, with low concentrations of manure, the reduction in damping off was not significant and required longer incubation periods. The reduction in damping off may be due to the mixture of volatile fatty acids, including acetic, propionic, butyric, isobutyric, and valeric acid, which are released during the decomposition of manure; these acids are potential disease suppressors (Tenuta *et al.*, 2002). Several authors studying a variety of crops have found that organic amendment or compost reduces disease caused by *R. solani* (Tuitert *et al*., 1998; Baily and Lazarovit; 2003; Alabouvette *et al*., 2004; Rivera *et al*., 2004; Noble and Coventry, 2005). The effectiveness of manure amendment in reducing disease severity depends on the type of manure, the type of soil and other factors. Mushroom compost was superior to manure in reducing flax damping off caused by *R. solani* (Alabouvette *et al*., 2004). Furthermore, *R. solani* damping off reduction in compost-amended potting soil was affected by the maturation of the compost: fresh compost actually increased pathogen growth and the rate of infection, while only long-matured compost consistently suppressed it (Tuitert *et al*., 1998). Manure amendment at 10% increased bean fresh weight after 12 and 24 months of incubation, probably because the longer maturation time increased the amount of nutrients available to the plants by the decomposition of organic matter. In addition, manure amendment at 10% increased the total fungal population and microbial activity in the soil after 12 and 24 months of incubation. This may explain the lower rates of damping off in amended soil: since the population of *R. solani* was only a small part of the total fungal population in terms of CFUs, there was very likely a fierce competition between the pathogen and the other microbes present in the soil.

A combination of *Trichoderma* and manure amendment at 6 and 10% reduced damping off by 33 and 50% respectively after 24 months of incubation. The population of *Trichoderma* increased with increasing concentration of organic amendment and duration of incubation; the increase was 5 and 7-fold at a concentration of 6%, and 4 and 10-fold at a concentration of 10%, after 12 and 24 months respectively. *Trichoderma*-treated soils without organic amendment did not show any beneficial effect in any of the parameters tested, including % damping off, because the concentration of *Trichoderma* applied was below the minimum inoculum concentration (0.5×10^6 CFU g^{-1} soil) required to control the disease (Barakat *et al*., 2007). With the addition of an organic amendment, however, the *Trichoderma* population was enabled to increase and to reach a critical concentration that enabled it to bring about a reduction in damping off. The organic amendment may have acted as a nutrient source for *Trichoderma*, and thereby increased its population. That *T. harzianum* isolate Jn14 is a mycoparasite on *R. solani* has been reported (Barakat *et al*., 2007). Various hyphal interactions were seen in that study: coiling around the *R. solani* hyphae, penetration of the host hyphae, and subsequent lysis of the infected hyphae, all of which suggested that the interaction between *T. harzianum* and *R. solani* was mycoparasitic. *T. harzianum* isolate Jn14 also reduces mycelial growth of *R. solani* by producing antifungal metabolites at different temperatures, and this suggests antibiosis (Barakat *et al*., 2007). Several authors obtained similar results and reported that the mechanisms that *T. harzianum* employed against *R. solani* included mycoparasitism, induced systemic resistance (ISR) and the production of antifungal metabolites (Papavizas, 1985; Coley-Smith *et al*., 1991; Prasun and Kanthadai, 1997; Sivasithamparam and Ghisalberti, 1998).

In conclusion, a combination of the biocontrol agent *T. harzianum* and sheep manure reduced the damping off of bean caused by *R. solani* and improved bean growth after longer periods of manure incubation. The reduction in damping off increased with increasing concentration of organic amendment and with increasing duration of incubation. Organic amendment alone was less efficient in reducing damping off and improving plant growth than a combination of manure amendment and *T. harzianum*.

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