Induction of Suppressiveness to *Rhizoctonia solani* in Soil

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ABSTRACT

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When soil was infested with large (>589 μm) propagules of *Rhizoctonia solani*, soil suppressiveness to *R. solani* was generated only in acidified or naturally acidic soils after replanting radish, alfalfa, or sugar beet at weekly intervals. As suppressiveness increased, the propagule density of *Trichoderma harzianum* also increased. Acidic pH levels enhanced in vitro growth of *T. harzianum* more than that of *R. solani* and stimulated its conidiophore formation and spore germination. The mechanism of antagonism was found to be parasitism followed by lysis rather than antibiosis; the fungus released active β-(1-3) glucanase and chitinase when grown on *R. solani* cell walls and lysed both living mycelium and cell walls of that fungus. In raw soil, *T. harzianum* also controlled damping-off of radish.

Additional key words: lytic enzymes.

Soil suppressive to *Rhizoctonia solani* Kühn was induced in Fort Collins clay loam infested with the pathogen after four or five successive weekly plantings of radishes (10). Recently, Liu and Baker (16) found that progagule density of *Trichoderma harzianum* Rifai increased in soil as suppressiveness increased and that inoculum density of *R. solani* was inversely proportional to propagule density of the antagonistic fungus. Suppressiveness also was found after similar successive plantings of cucumber, but not after sugar beet, alfalfa, or wheat monocultures. Moreover, such suppressiveness could not be generated when large propagules (>589 μm) of *R. solani* were used for soil infestation (16,23).

The antagonistic activities of *T. harzianum* against several pathogenic fungi has been reported previously by several groups working on biological control (1,3,4,8,14,22). Some isolates effectively controlled disease induced by *R. solani* in the greenhouse (6).

An objective of this study was to explore methods of inducing soil suppressiveness to *R. solani* by monoculturing plants (alfalfa and sugar beet) which so far have been ineffective (16). Since large propagules of the pathogen may be important in survival, experiments also were done to induce suppressiveness when these were present in soil. An obvious strategy for enhancing activity and thallus development of *Trichoderma* is by providing favorable (acidic) soil hydrogen ion concentrations. Finally, the antagonistic relationship of *Trichoderma* to *R. solani* was studied.

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MATERIALS AND METHODS

Fort Collins clay loam was sieved through a 2-mm screen. Before each experiment, soil moisture was adjusted and then maintained at 15% of moisture holding capacity (about −0.7 bars water potential). In all experiments with successive plantings, plastic pots (80 mm deep, 110 mm top diameter) were filled to depth of 2–3 cm with 100 g of soil. A pellet of isolate R-3 of *R. solani* grown in a chopped potato-soil (CPS) mixture prepared according to Ko and Hora (15) was introduced in the center of each pot. Each pot was seeded with 32 radish (*Raphanus sativus* L. 'Early Scarlet Globe'), alfalfa (*Medicago sativa* L. 'Ranger') or sugar beet (*Beta vulgaris* L. 'MonoHy D_2') seeds having 94–97% germinability. All pots were covered with transparent Mylar® (E. I. DuPont de Nemours Co., Wilmington, DE 19898) sheets to reduce evaporation. All treatments were randomly arranged on benches at 25 ± 1 C under continuous fluorescent illumination (approximately 5,000 lx). After 7 days, the conducive index (CI) was determined. Relative suppressiveness of soil was assessed by CI. The CI value of a completely suppressive soil is 0; a completely conducive soil has a value of 1 (10). In subsequent replants the soil in the five replications of each of the various treatments was bulked, mixed, and redistributed into five portions. Each of these portions was considered to be a replication. Seeds were replanted in this soil which contained randomly distributed inoculum. After 1 wk, disease incidence (DI) was determined (10) and the process was repeated each week. After six or seven replants, the CI was determined again for each treatment.

Inoculum density of *R. solani* was determined on the selective medium of Ko and Hora (15) by using the multiple pellet soil

sampler (11). *Trichoderma* density in soil was determined by the dilution plate method with a selective synthetic medium supplemented with chloramphenicol rosebengal, Dexon and pentachloronitrobenzene (PCNB) prepared according to Elad, Henis, and Chet (*unpublished*). Conidia of *T. harzianum,* Fort Collins isolate (16) were produced on potato dextrose agar (PDA). Conidia were collected and suspended in distilled water. Suspension density was determined with the aid of Helber Chamber Counter (Hausser & Sons, Philadelphia, PA 19100).

Enzymatic and antibiotic activity of *T. harzianum*. Hyphal cell walls of *R. solani* were prepared according to the method of Chet and Henis (2). One tenth ml of a conidium suspension (10^7conidia per milliliter) of *T. harzianum* was added to Erlenmeyer flasks each containing 100 ml of salt medium with *R. solani* cell walls as the sole carbon source (19). Cultures were shaken on a rotary shaker for 7 days at 25 ± 1 C and then centrifuged aseptically at 20,000 *g* for 15 min at 4 C. The supernatant was used for determining activity of extracellular enzymes.

The activity of β-(1-3) glucanase (E.C. 3.2.1.39) was determined by following the release of free glucose with Glucostat reagent (Worthington Biochemical Corp., Freehold, NJ 00728). Specific activity was expressed as μmole glucose per milligram protein per hour. The reaction mixture contained 2 ml of 0.1 M citrate buffer (pH 4.5), 2 mg cell walls of *R. solani*, and 5 ml of the cell-free supernatant of the *Trichoderma* culture. The reaction was carried out for 2 hr at 45 C.

Enzymatic activity of chitinase (E.C. 3.2.1.14) was determined by following the release of *N*-acetylglucosamine (20). Specific activity was expressed as μmole *N*-acetylglucosamine per milligram protein per hour. The reaction was maintained at 37 C for 2 hr.

Antibiotic activity of *T. harzianum* was tested by the cellophane method of Mughogho (18) and the sprayed conidia method of Hsu and Lockwood (12).

Lytic activity of *T. harzianum* was tested as follows: Cultures of *Rhizoctonia* were grown for 14 days in shaking flasks containing potato dextrose broth (PDB) and transferred to salt medium. Two-tenths ml of conidial suspension (10^7/ml) of *T. harzianum* was introduced into these flasks. The cultures were shaken on a rotary shaker at 25 ± 1 C. Aliquots of the two-membered culture were taken weekly, observed under the microscope and transferred to *Rhizoctonia* selective medium to test the viability of the pathogen.

RESULTS

The effect of soil pH on the development of soil suppressiveness to *R. solani*. Samples of Fort Collins clay loam (pH 8.1) were acidified to pH 5.7 and pH 6.5, which was maintained by irrigation with buffered solution (21). Radish, alfalfa, and sugar beet were seeded in 100-g aliquots of these samples and repeatedly replanted at weekly intervals. Large propagules of *R. solani* were used as inoculum. With all three species, soil suppressiveness to the pathogen was generated only in the acidified soil, but not in untreated soil (Fig. 1a–c). At pH 6.5 the CI decreased from its initial level (approximately 0.7) to 0.16 and 0.09 for alfalfa and radish, respectively, whereas with sugar beet it reached a level of 0.4. No significant difference in suppression was observed between pH 6.5 and pH 5.7.

Similar results were obtained with acidic Rocky Mountains clay loam (pH 6.1) obtained from the Poudre Valley 40 km west of Fort Collins, CO. The density of *T. harzianum* in Fort Collins soil increased from 1×10^2 propagules per gram in the beginning of the experiment to 2×10^5 propagules per gram in the soil of treatment groups in which disease incidence decreased after 7 wk of monoculturing.

The effect of pH on *T. harzianum* and *R. solani* in culture. The pH of PDA was adjusted with Good's buffers (5) in 1.0-pH-unit increments from 3.5 to 8.5. The pH of the medium did not change during the experiment. Agar disks covered with mycelium of *R. solani* or *T. harzianum* were transferred from the edge of 3-day-old colonies to the center of petri plates containing these media. Linear growth of mycelium of *Trichoderma* was greatest at pH 5.5 and grew well at pH 3.5 (Fig. 2). Optimum linear growth of *R. solani* occurred at pH 6.5–7.5. Similarly, when conidia of *T. harzianum* germinated on water agar different pH levels, 95% germination occurred after 25 hr at pH 4–6.2. However, at pH levels above 7.8 only 36% or less germinated after 40 hr. Fig. 3 which shows the percentage of germinating conidia after 15 hr, indicates a pronounced decrease above pH 6.5. The maximal number of conidiophores was formed on media at pH 4–6, whereas only 35% and 23% of this maximum were formed at pH 8 and pH 9, respectively (Fig. 4).

Antagonistic activity of *T. harzianum*. Conidia of *T. harzianum* were germinated in a liquid salt medium (pH 6) containing living

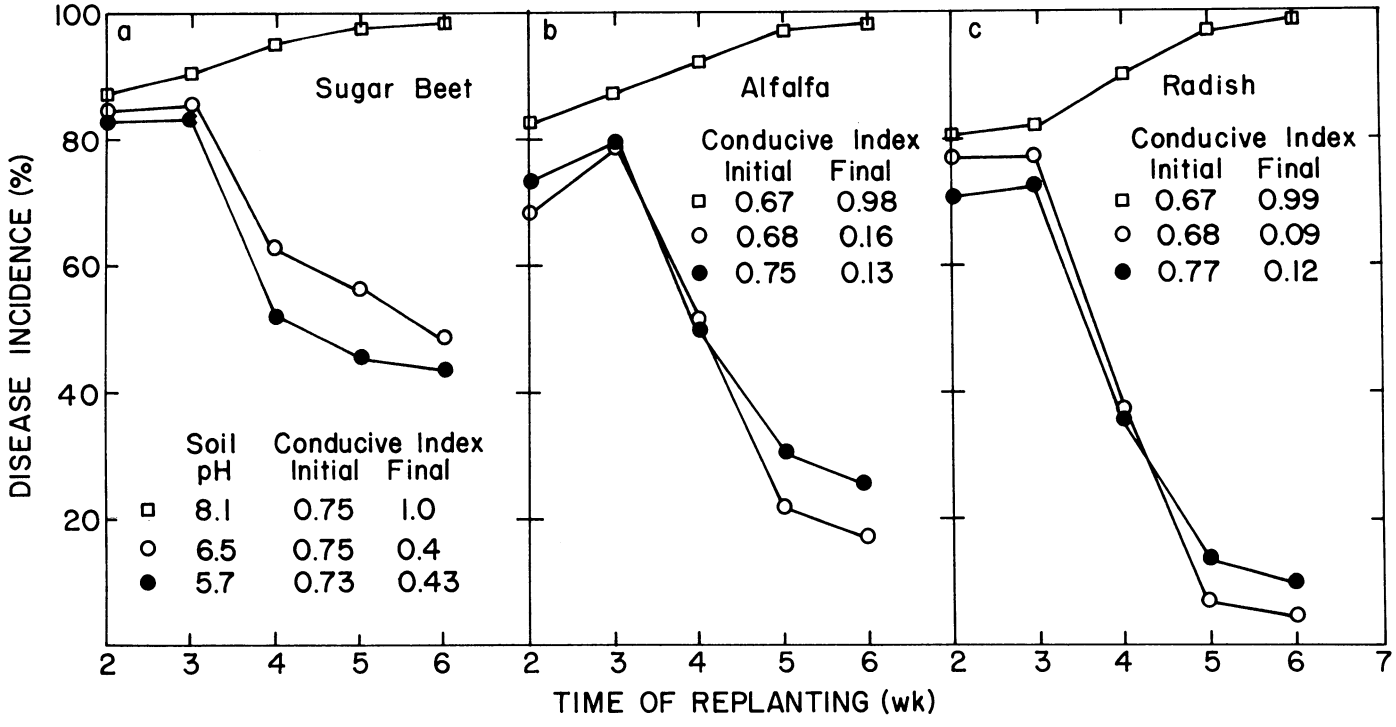

Fig. 1. Effect of soil pH on the development of soil suppressiveness with successive crops of **a,** sugar beet; **b,** alfalfa; and **c,** radish replanted in the soil at 7-day intervals. Soil was infested with large (>589 μm) propagules of *Rhizoctonia solani*.

mycelium of *R. solani*. The cultures were shaken together and samples were taken every 7 days. After 7 days of incubation, coiling of *Trichoderma* on the hyphae of the pathogen could be seen as reported by Liu and Baker (16). Within 5–6 wk *R. solani* mycelium was completely lysed. No growth from this lysed mycelium could be detected on the *Rhizoctonia*-selective medium.

The possibility of antibiosis was tested by two methods (12,18) but no antibiotic substances from *T. harzianum* which were active against *R. solani* could be detected.

T. harzianum released extracellular β-(1-3) gluconase and chitinase which degraded β-(1-3) glucose and chitin from cell walls when grown on media with cell walls of *Rhizoctonia* as the sole carbon source. The optimal pH for *Trichoderma* β-(1-3) glucanase and chitinase activity was 4.5 and 5.3, respectively. Operating at optimal pH levels, β-(1-3) glucanase released 17.2 μmole glucose per milligram protein per hour from the cell walls, whereas, chitinase released 1.2 μmole *N*-acetylglucosamine per milligram protein per hour.

The antagonistic activity of *T. harzianum* in naturally infested soil. Soil was collected from an area that had been used as a *Rhizoctonia* disease nursery with sugar beets. This soil had an inoculum density of two propagules of *Rhizoctonia* per gram of soil. Eighty percent of these propagules were large (10). Some of this soil was mixed with 1×10^6 conidia of *T. harzianum* per gram of soil, whereas soil in control pots was not treated. The results of growing radishes weekly in monoculture in this experiment are shown in Fig. 5. Without added *T. harzianum*, the disease incidence increased in the second replanting and suppressiveness developed slowly after the 5th wk by replanting. Propagule density of *Trichoderma* increased during 8 wk from 3×10^2 propagules per gram of soil to 4×10^4 propagules per gram of soil. Conversely, when *Trichoderma* propagules were mixed with the soil at the beginning of the experiment, a rapid decrease in disease incidence was observed from the first planting. Population density of *T. hamatum* did not change during the experiment.

The influence on damping-off of suppressive soil transferred with the seedlings was studied. Conidia of *T. harzianum* (10^6 conidia per gram of soil) were added to soil containing germinating radish seeds. After 4 days, seedlings were transplanted, either with a

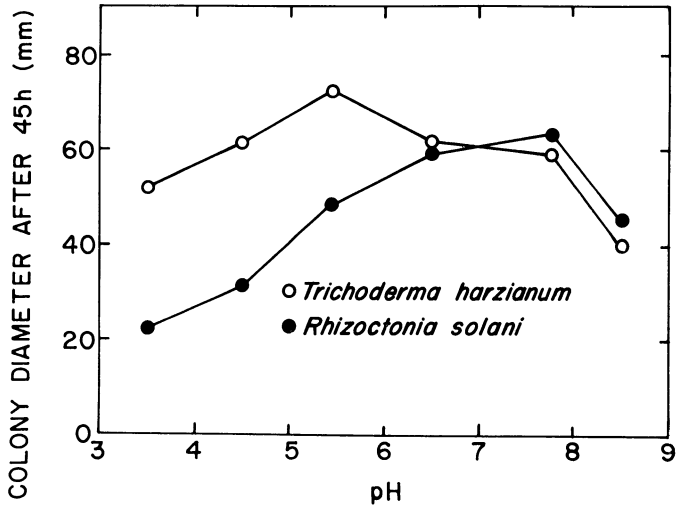

Fig. 2. The effect of pH, adjusted with Good's buffers on linear growth of *Rhizoctonia solani* and *Trichoderma harzianum* in potato dextrose agar.

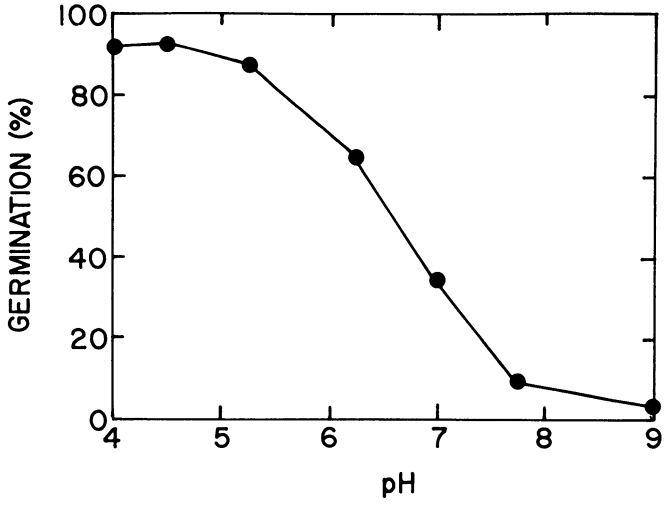

Fig. 3. The effect of pH adjusted with Good's buffers on conidium germination of *T. harzianum* in 2% water agar, after 15 hr.

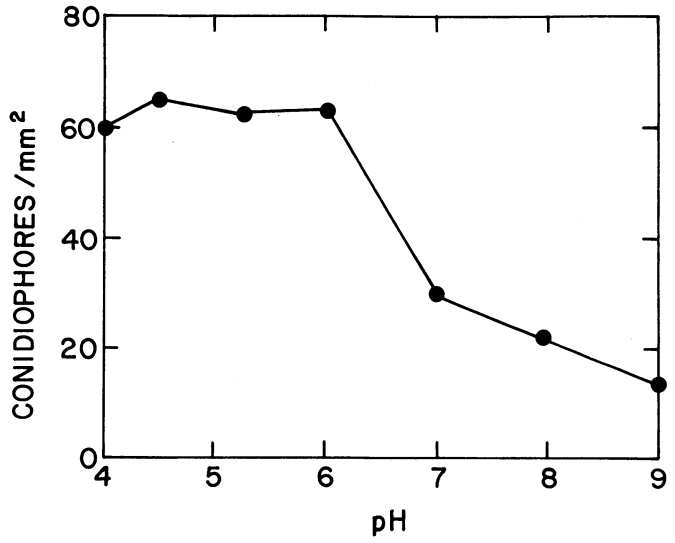

Fig. 4. The effect of pH adjusted with Good's buffers on conidiophore formation of *Trichoderma harzianum* on water agar.

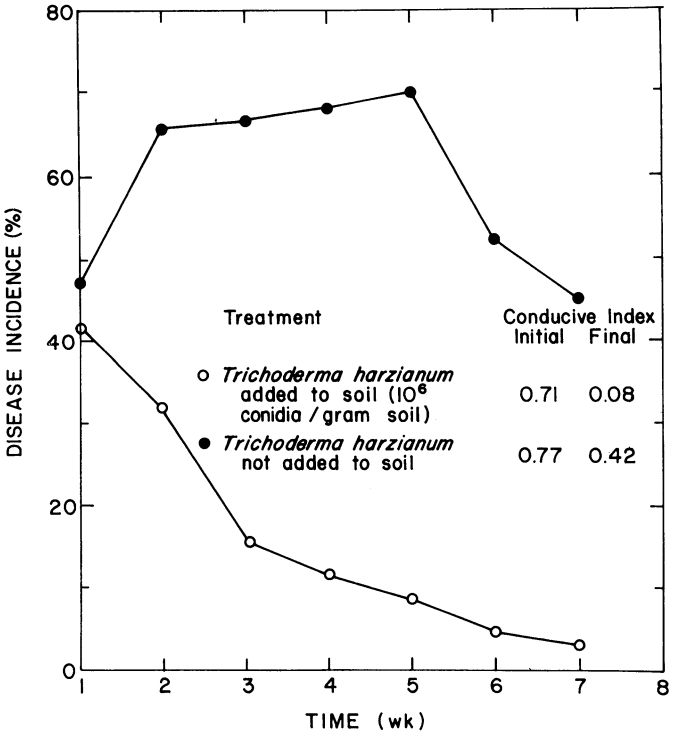

Fig. 5. The development of soil suppressiveness in naturally infested soil during successive replantings of radish compared with damping-off observed during monoculture in the same soil initially infested with 10^6 conidia of *Trichoderma harzianum* per gram of soil.

soil mass corresponding in size to root extension or with roots washed to remove the soil, to conducive soil naturally infested with *R. solani*.

The percentage of damping off in the two treatments is shown in Fig. 6. Symptoms appeared later in seedlings transferred with the mass of soil than in those transferred without soil. After 14 days disease incidence reached 40% and 90%, respectively.

DISCUSSION

Previous research reported that soil became suppressive to *R. solani* after growth of radishes in monoculture in soil infested with *R. solani* (10). However, when large propagules of the pathogen were incorporated (23), or plants other than radishes and cucumbers were tested (16), the soil remained relatively nonsuppressive. These results were obtained in Fort Collins clay loam which has a relatively high pH (8.1). When hosts other than radish or cucumbers were used in monoculture and/or when large propagules were incorporated into acidified Fort Collins clay loam, however, suppressiveness developed during monoculture (Fig. 1). Further, development of suppressiveness was accompanied by an increase in the population density of *T. harzianum,* from approximately 10^2 to 10^5 propagules per gram of soil. These findings, together with the report that large propagules induce more severe symptoms on hosts than do small propagules (7), suggest that the antagonism of *Trichoderma* was more readily enhanced in acidic than in alkaline soils.

The hypothesis that low pH is favorable to the activity of *Trichoderma* was substantiated by tests of the effect of pH on growth of the pathogen and the antagonist in vitro. At pH levels lower than 6.5, the linear growth rate of *T. harzianum* was significantly higher relative to that of *R. solani*. Similarly, maximal conidiophore formation and conidium germination of *T. harzianum* were found at pH values lower than 6 and 5.2, respectively. These findings explain the observation of Liu and Baker (16) that acidity accelerated the generation of suppressiveness in their radish monoculture system.

Based on the ability of *T. harzianum* hyphae to coil around and to lyse hyphae of *R. solani,* the mechanism of antagonism appeared to be parasitism. No antibiotic activity could be detected. *T. harzianum* released highly active β-(1-3) glucanase and chitinase into the culture supernatant when cell walls of *R. solani* were the sole carbon source. These enzymes are known as lytic enzymes and are capable of degrading fungal cell walls (2,17). These findings corroborate those of Hadar et al (6) who found that another isolate of *T. harzianum* was capable of degrading cell walls of *R. solani*. The lytic activity of the Fort Collins isolate was slightly higher than that of their isolate when tested on the cell walls of *R. solani* as a substrate. Even though some competition may occur between *T. harzianum* and *R. solani* the results support the idea that the most important mechanism involved in this host-parasite relationship is parasitism followed by lysis. Similarly, Jones and Watson (13) found that β-(1-3) glucanase of *T. viride* solubilized the hyphae of *Sclerotinia sclerotiorum*. The optimal pH of the lytic enzymes β-(1-3) glucanase and chitinase is 4.5 and 5.3, respectively, thus supporting the important role of acidic pH in the antagonistic activity of *T. harzianum*. In naturally infested soil, suppressiveness was slowly generated and the CI decreased from 0.77 to 0.42 in weekly radish monoculture for 8 wk (Fig. 5). Most of the propagules of *R. solani* were large and this explains the relatively low decrease, especially in alkaline soils, in CI after monoculture growth of radish. However, when 10^6 conidia per gram of *T. harzianum* soil were added to the infested soil, rapid induction of suppressiveness was observed. The *T. harzianum* conidia apparently did not change the resistance of the plants; seedlings washed free of suppressive soil and transplanted to naturally infested soil were 90% diseased after 14 days (Fig. 6). When similar seedling were transferred with the relatively large amounts of soil surrounding them and transplanted, however, only 40% damping off was recorded after the same period. These findings suggest the possibility of applying the biocontrol agent in small quantities around the seedling in the nursery. A similar phenomenon also was reported in carnation (9).

The survival units of *R. solani* recovered from field soil were mostly in the form of large propagules. Thus, for practical biocontrol of this pathogen, antagonists must be effective against these propagules. In previous work (23), however, radish monoculture did not induce suppressiveness in soils containing large propagules. This problem was solved by acidification of soil containing large propagules. Ultimately, this resulted in suppressiveness being induced when various hosts were grown in monoculture (Fig. 1). Suppressiveness in soil containing large propagules also was induced by adding the biocontrol agent *(Trichoderma)* at propagule levels found in suppressive soil.

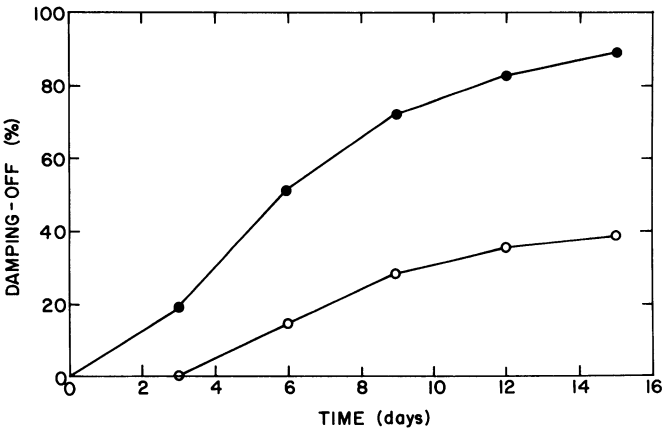

Fig. 6. The effect of transferring radish seedlings from nursery soil treated with *Trichoderma harzianum* to naturally infested soil with (—•—) or without (—o—) the surrounding soil.

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