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ANNUAL SCIENTIFIC REPORT

JANUARY - OCTOBER 1988

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BIOLOGICAL CONTROL OF PLANT DISEASES USING MICROORGANISMS GROWN ON AGRICULTURAL AND FORESTRY WASTES

BY

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CELL WALL COMPOSITION OF *FUSARIUM OXYSPORUM* f. sp. *VASINFECTUM* AND *F. OXYSPORUM* f. sp. *MELONIS* AS COMPARED WITH CELL WALLS OF *SCLEROTIUM ROLFSII* AND *RHIZOCTONIA SOLANI*.

ABSTRACT

The protein content of cell walls hydrolysates of *Fusarium oxysporum* was up to 115% and 194% higher than that of cell walls of *Sclerotium rolfsii* and *Rhizoctonia solani*, respectively. Similarly, cell walls of both forma speciales of *F. oxysporum* contained more hexosamines as compared to those of *S. rolfsii* and *R. solani*. However, cell walls of *S. rolfsii* and *R. solani* contained 58.6% and 57% neutral sugars, respectively, as compared with values of 19.5% and 37.9% in cell walls of *F. oxysporum* f. sp. *melonis* and *F. oxysporum* f. sp. *vasinfectum* , respectively. Of all tested fungal cell walls, those of *S. rolfsii* contained the highest amount of lipids (7.5%). Following 2h hydrolysis (2N H_2SO_4) the non hydrolyzed residue content of *F. oxysporum* cell walls was found to be much higher than that of *S. rolfsii* and *R. solani*.

INTRODUCTION

Fusarium spp. are spread all over the world and belong to the most devastating soil-borne plant pathogens, causing diversified symptoms. Forma speciales of *F. oxysporum* specifically induce vascular wilt diseases in many crops (MacHardy and Beckman, 1981).

The morphology, physiology and the ecology in soil of these pathogens was broadly reported (Nelson *et al.*, 1981). However, there are not sufficient data regarding the cell wall composition of Fusaria and, in particular, *F. oxysporum*. Fungal cell wall structure contributes to the unique hyphal shape and serves as the main barrier against microbial lysis (Potgieter and

Alexander, 1966 ;Chet et al., 1967 ; Van Eck, 1978).

In the present study we have carried out a chemical analysis of hyphal wall composition of F. oxysporum f. sp. melonis and F. oxysporum f. sp. vasinfectum in comparison with that of Rhizoctonia solani and Sclerotium rolfsii.

MATERIALS AND METHODS

Fungal culture conditions

Fusarium oxysporum Schlecht f. sp. melonis Snyd. and Hans. (F. o. melonis) and F. oxysporum f. sp. vasinfectum (Atk.) Snyd. and Hans. (F. o. vasinfectum) were cultured on a yeast extract-glucose medium (YM) containing (g/l distilled water) yeast extract (Difco Laboratories Detroit, Michigan, USA) 5 ; peptone (Difco) 5 ; glucose, 10 ; agar (Difco) 20, and were incubated at 27C.

Rhizoctonia solani Kuhn and Sclerotium rolfsii Sacc. were grown on a synthetic medium (SM ; Okon et al., 1973) and incubated at 30C.

Preparation of hyphal walls

Hyphal cell walls, produced according to Chet and Hutterman (1980). F. o. melonis, F. o. vasinfectum and R. solani, were grown in liquid YM while S. rolfsii was grown in liquid SM. All fungi were grown for 96h in Erlenmeyer flasks (250 ml). The mycelial mats were separated from the medium and thoroughly washed with distilled water and homogenized in an Ultra-Turrax, (Ika-Werk, W. Germany) for 5 min. Mycelial suspension was

centrifuged at 30,000xg, for 20 minutes, at 4C. The precipitate was re-suspended in distilled water and sonicated, intermittently, 3 times, for 5 minutes each, using a 'Heat Systems-Ultrasonics' (NY, USA) sonicator, at full amplitude. The suspension was centrifuged again at 800xg, for 20 minutes at 4C, to precipitate the coarse particles. The supernatant containing the remaining fine particles, was washed by centrifugation and homogenized intermittently until no residual glucose, protein and amino acids could be detected in the supernatant, as determined with glucose oxidase (Sigma), coomassie-brilliant blue (Sedmak and Grossberg, 1977) and nynhidrin (Cocking and Yemm, 1954) reagents, respectively. The precipitated walls were then deep-frozen, lyophilized and stored as a powder in a sealed container.

Chemical analysis of hyphal cell walls

Samples of hyphal cell wall preparations of F. o. melonis, F. o. vasinfectum, R. solani and S. rolfsii, of 20 mg each, were sealed in hydrolysis ampoules each of which contained 2 ml of 2N H_2SO_4 and heated in an air forced oven at 105 C for 2, 5, 12, 20 and 24h. This method resulted in the highest amount of glucose after hydrolysis of 5 h. The hydrolysates were then neutralized with NaOH and diluted to 25 ml. These diluted hydrolysates were assayed for total sugars and glucose by the glucose oxidase reagent and phenol sulphuric acid method (Dubois et al., 1956), respectively.

The hexosamine content was determined by the Elson-Horgan method (Elson and Horgan, 1933) in samples of the above mentioned cell wall hydrolizates, which were neutralized with hot Ba(OH)

and centrifuged at 30,000xg for 15 minutes, to remove the salt.

Amino acid determination was carried out using 20 mg samples which were hydrolysed in 6N HCl, for 24h, at 105 C, in an air forced oven. The hydrolysates were dried under vacuum in a rotovapor (Buchi, Switzerland) and tested, using a Biotronic, Model LC-7000 (W. Germany) amino acid analyzer. The protein content was calculated from the amino acid profile.

Lipids were extracted from 20 mg hyphal cell walls samples after shaking the samples in sealed 100 ml Erlenmeyer flasks each containing 10 ml chloroform-methnol (2:1;v/v) for 24h, at room temperature. The insoluble fraction was removed after centrifugation and the extracted lipids were determined gravimetrically in the separated chloroform fraction (Folch et al., 1957).

The ash content was determined gravimetrically after heating the hyphal wall samples at 650 C until no further weight decrease could be detected.

Infra-red (IR) spectra were obtained with an IR-spectrophotometer (Nicolet MX-S, USA) using 2 mg of fungal cell wall powder which was mixed with 90 mg of KBr and pressed into a pellet (Chet and Huttermann, 1980).

RESULTS

The results of acid hydrolysis of fungal cell walls (Table 1.) showed remarkable differences in almost each tested parameter. The hexosamine and protein content were found to be higher in cell walls of both fusaria when compared with R. solani

or with S. rolfsii. On the other hand, the total neutral sugar content was higher in hyphal cell walls of R. solani and S. rolfsii.

Seventy and 53 percent of total amino acids of F. o. melonis and F. o. vasinfectum, respectively, were found to be histidine.

The non-hydrolyzed residue content (after 5h hydrolysis of both fusaria cell walls) was up to 74% higher than those of R. solani and S. rolfsii. However, no differences in the non-hydrolyzed residue of the tested fungal walls could be detected after 24 h hydrolysis.

Similarly, no significant differences could be observed in the ash content among the hyphal cell walls.

The infra-red spectra of the tested hyphal cell walls showed a strong resemblance between cell walls of F. o. melonis and F. o. vasinfectum and those of R. solani and S. rolfsii. A comparison with the IR spectra of chitin and laminarin, showed that the proportion between these two polymers in cell wall of F. o. vasinfectum was different from that present in cell walls of S. rolfsii (e.g. higher amount of chitin) (Fig. 1). The main differences in the absorbance could be observed at wavenumbers ranging from 1100 to 1670. This part of the spectrum represents the chitin (Fig. 1,A) and is characterized by a high absorbance at wavenumbers: 1161.2, 1317, 1383, 1564 and 1666. The infra-red absorbance spectrum of chitin was found to be very similar to that of F. o. vasinfectum and F. o. melonis and different from those of cell wall of F. o. melonis and R. solani.

DISCUSSION

This study is a first report of chemical analysis of F. oxysporum cell walls and (in particular) of F. o. melonis and F. o. vasinfectum. The results of this analysis clearly point out the higher chitin content present in fusaria cell walls, when compared with those of R. solani or S. rolfsii. However, the total neutral sugar content was lower in cell walls of both fusaria. These results correlate with the report of Baaran and his co-workers (1975), whose work indicated that N-acetyl-glucosamine is the dominant monosaccharide present in hyphal cell walls of F. sulphureum (39%). Similarly, Skujins et al. (1965) showed an even higher content of this sugar (47%) in cell hyphal walls of F. solani f. sp. phaseoli. This fact may be of a great importance to mycoparasites which are unable to produce high levels of chitinase.

Data provided by the amino acid analyzer also revealed that the protein content of fusaria hyphal cell walls was up to 194% higher than the hyphal walls of R. solani. In the past, a high protein content was found in several other fusaria: 7.3% and 28% - in hyphal and chlamydospore cell walls of F. sulphureum, respectively (Baaran et al., 1975 ; Schneider et al., 1977). Similarly, Lamborda et al. (1974) showed a very high protein content (21%) in hyphal cell walls of F. culmorum. Apparently, a high content of protein in fungal cell walls may prevent their lysis by antagonistic soil microflora. This argument was also raised by Schneider et al. (1977), who suggested a linkage between the high content of protein in chlamydospore cell wall of F. sulphureum and their survival in soil as resting structures.

Cell walls of F. oxysporum. are more resistant to acid hydrolysis (2N H_2SO_4). This may explain the relatively high r' tstance of F. oxysporum to lytic enzymes (Sivan and Chet, unpubli .ed). After hydrolysis of 5h, the non-hydrolyzed residue content in cell walls of F. o. vasinfectum and F. o. melonis was 25.1% and 32.8%, compared with 8.4% and 12.2% in cell walls of R solani and S. rolfsii, respectively. Presumably, there are different or additional bonds in and/or between the polyssaccharides present in the cell walls of F. oxysporum. Indeed, Chu and Alexander (1970) argued that fusaria cell walls contain heterossaccharides which contribute to their resistance to lytic enzymes in soil.

The differences between the cell wall composition of F. oxysporum and those of R. solani and S. rolfsii were also exhibited by infra-red spectrophotometry. The spectral results clearly show that the chitin and laminarin content in cell walls of F. o. vasinfectum and F. o. melonis comprised different proportions (e.g. a higher chitin content) when compared with the spectra of R. solani and S. rolfsii. These results confirm the data obtained by the chemical analysis.

The uniqueness of the high content of chitin and protein of Fusarium hyphal cell walls, should be taken into consideration when screening antagonistic mycoparasites. Apparently, their lytic activity should consist mainly of chitinase and proteases as well as glucanase.

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Table 1. Chemical composition of hyphal cell walls of *F. oxysporum* f. sp. *vasinfectum*, *F. oxysporum* f. sp. *Melonis*, *R. solani* and *S. rolfsii*.

Cell wall component	Percentage of cell wall dry weight.			
	F. oxysporum f. sp. *vasinfectum*	*F. oxysporum* f. sp. *melonis*	*R. solani*	*S. rolfsii*
Neutral Sugars	19.5	37.9	57.0	58.6
Hexosamines	29.0	16.5	6.1	5.4
Protein (1)	3.5	5.6	1.9	2.6
Lypids	3.8	4.2	2.2	7.5
Ash	1.2	1.4	1.1	1.1
Non-hydrolyzed Residue (2)	32.8	25.1	8.4	12.2
Total	89.8	90.7	76.7	87.2

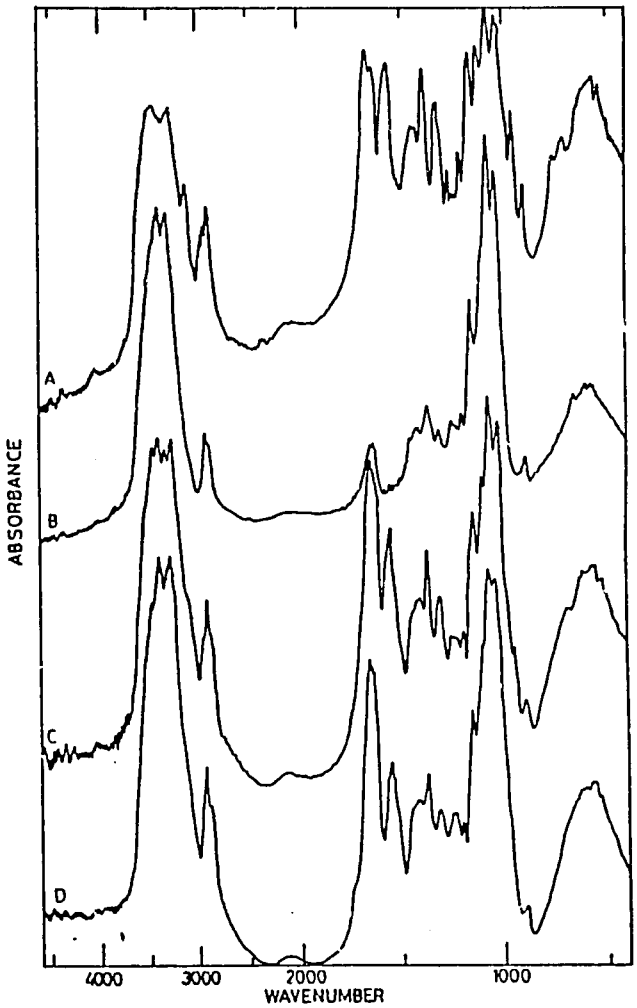
(1) Calculated from the amino acid composition obtained from the amino acid analyzer.

(2) 2N H_2SO_4 at 105 C, for 5h.

Table 2. Amino acid composition of hyphal cell walls of *F. oxysporum* f. sp. *vasinfectum*, *F. oxysporum* f.sp. *melonis*, *R. solani* and *S. rolfsii*.

Amino acids g/mg dry weight of hyphal cell walls.

F. oxysporum f. sp. *vasinfectum*		*F. oxysporum* f. sp. *melonis*		*R.solani*		*S.rolfsii*	
Amino acid	Conc.	Amino acid	Conc.	Amino acid	Conc.	Amino acid	Conc.
Aspartic acid	2.47	Aspartic acide	0.65	Aspartic acid	2.52	Aspartic acid	2.96
Threonine	3.16	Serin	0.8	Threonine	1.19	Threonine	1.5
Serine	1.52	Glutamic acid	0.75	Serine	1.21	Serine	1.64
Glutamic acid	3.52	Proline	0.96	Glutamic acid	0.25	Glutamic acid	3.3
Proline	2.74	Alanine	0.69	Glycine	0.11	Proline	1.34
Glycine	1.14	0.5 Cistine	1.46	Alanine	1.88	Glycine	1.34.
0.5 Cistine	0.27	Valine	1.87	Valine	2.39	Alanine	1.78
Valine	3.17	Methionine	1.08	Methionine	0.77	Valine	2.62
Methionine	0.77	Leucine	0.66	Leucine	1.07	Methionine	0.35
Leucine	1.32	Tyrosine	0.48	Tyrosine	0.73	Leucine	2.4
Tyrosine	1.18	Phenyl-alanine	0.73	Phenyl-alanine	1.25	Tyrosine	1.1
Phenyl- alanine	0.8	Hystidine	23.2	Hystidine	3.58	Phenyl-alanine	1.35
Hystidine	29.6			Lysine	0.74	Hystidine	1.16
Lysine	0.5			Arginine	1.15	Lysine	1.14
Arginine	1.16					Arginine	1.95


A
B
C
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ABSORBANCE
4000
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WAVENUMBER

LEGEND TO FIGURE

Fig. 1. Infra-red absorption spectra of chitin (A) and laminarin (B), as compared with hyphal cell walls of *F. oxysporum* f. sp. *vasinfectum* (C) and *S. rolfsii* (D).

Publication appeared until August 1988 from this project.

Ecology and Epidemiology

Possible Role of Competition for Nutrients in Biocontrol of Pythium Damping-Off by Bacteria

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ABSTRACT

Elad, Y., and Chet, I. 1987. Possible role of competition for nutrients in biocontrol of Pythium damping-off by bacteria. Phytopathology 77:190-195.

Of the 130 bacteria isolated from the rhizospheres of plants infested with *Pythium* spp., six were found to be efficient biocontrol agents of this pathogen under greenhouse conditions. No lytic enzymes were involved in in vitro interactions between the bacteria and *P. aphanidermatum*. Substances inhibiting growth of the fungus were produced by both the biocontrol agents and other rhizobacteria. On the other hand, competition for nutrients between germinating oospores of *P. aphanidermatum* and bacteria, which was unique to the biocontrol agents, significantly correlated with suppression of disease in the greenhouse. Oospore germination in rhizospheres of wheat, tomato, cucumber, melon, bean, and cotton plants was decreased in the presence of these bacteria. Broadcast application or seed coating with these bacteria reduced disease incidence in cucumbers planted in peat-vermiculite mixture or in soil by 60–75% compared with the untreated control. Damping-off caused by *P. ultimum* in cucumbers was reduced by up to 94%. Combining low levels of *Pseudomonas putida* or *P. cepacia* with the fungicide prothiocarb resulted in an additive effect. Bacteria applied to cucumber seeds became established along the roots. Populations were 100-fold higher at root tips than at their upper parts. On the other hand, except for the root tips, the total population of bacteria was decreased in the presence of the beneficial ones. Disease control was achieved in bean, pepper, melon, tomato, and cotton plants as well.

Rhizobacteria, by their interactions with various pathogens, play a major role in the biological equilibrium among microorganisms in the rhizosphere (1,23). *Pythium* spp. have a broad host range and cause preemergence and postemergence damping-off and root rots followed by considerable yield losses in many important crops.

Biological control of this pathogen with bacteria is not well established. Mitchell and Hurvitz (15) protected tomato seedlings against damping-off caused by *P. debaryanum* Hesse with a lytic strain of *Arthrobacter*, and Hadar et al (7) protected germinating cucumber seeds with *Enterobacter cloacae* (Jordan) Harm & Edwar from rots caused by *Pythium* spp. Howell and Stipanovic (9) isolated a strain of *Pseudomonas fluorescens* Migula that produces an antibiotic inhibiting growth of *Pythium ultimum* Trow on seeds.

Rhizobacteria have been the subject of several reports (11,12,16). It was suggested that the antibiotics or siderophores produced by these bacteria displace deleterious microorganisms, presumably minor pathogens (11,12). In such a case, competition with the native microflora should also be considered. The objectives of the present work were to isolate potential antagonistic rhizobacteria, apply them against Pythium damping-off, and investigate their antagonistic mechanism.

MATERIALS AND METHODS

Isolation and characterization of bacteria. One hundred thirty bacteria were isolated from roots of bean, cotton, radish, cucumber, and melon plants grown in soils infested with *Pythium* spp. or in peat-vermiculite growth media (PVM). Isolations were made on nutrient agar (NA) (Becton-Dickinson Corp., Cockeysville, MO) or King's medium B (KB) (10). Bacteria were identified by the Analytical Profile Index (API) system (La Balme les Grottes 38390, Montalieu, Vercieu, France). Identification was verified by *Bergey's Manual of Determinative Bacteriology*. The following strains were used: *Pseudomonas putida* (Trev.) Migula (805 and 310), *P. cepacia* Burk (808, 814, and Y11), *Alcaligens* Cast. & Chal. sp. (Rh2), and *Pseudomonas* (Migula) Doud & Pall. sp. (AA4, SS3, and 806). Cultures were maintained on NA for daily use. Pure cultures were grown on NA for 24 hr, washed from the agar surface with 10% skimmed milk, and freeze-dried.

Density of bacterial populations. Strains of the biocontrol agents resistant to rifampicin [3-(4-methylpiperazinyl-minomerhyl) rifamycin 5v) and nalidixic acid (Sigma) were selected from parent cultures by planting cell suspensions on NA containing 150 μg/ml rifampicin [3-(4-methylpiperazinyl-iminomethyl) rifamycin] and nalidixic acid and incubating them for 48 hr at 30 C. The antibiotic-resistant strains were similar to their parent cultures.

Antagonistic properties of selected bacteria. The cell wall-degrading enzymes—Exo-1,3-β-D-glucosidase (β-1,3-glucanase, EC 3.2.1.58); Exo-1,4-β-D-glucosidase (Cellulase, EC 3.2.1.74); and β-*N*-acetyl-D-glucosaminidase (Chitinase, EC 3.2.1.30)—were assayed for filtrates of bacteria grown on either laminarin (Sigma), chitin (Sigma), or cell walls or mycelium of *Pythium aphanidermatum* (Edson) Fitzp. as a sole carbon source. All enzymatic tests and preparation of fungal cell walls or mycelium were carried out according to previous publications (4,5).

For production of inhibitory substances, bacteria were grown for 40 hr in a potato-dextrose and nutrient broth (each 15 g/L) medium (PDNB). Bacterial cells were separated from the medium by centrifugation at 3,000 *g*, then the supernatant was sieved through a 0.45-μm pore size filter (Millipore Corp., Bedford, MA). The cellfree medium, diluted with a fresh, double concentration of PDNB, was seeded with a mycelial disk taken from a 48-hr-old culture of *P. aphanidermatum*. After 48 hr of incubation at 28 C, the mycelium was dried for 4 days at 60 C and weighed. Presence of antibiotics was also tested in solid NA.

Mats of mycelium removed from cultures of *P. aphanidermatum* grown for 48 hr in petri plates containing liquid PDNB were transferred into a fresh medium to check their growth in the presence of bacteria. Dry weight gained by the fungus during the

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interaction period was measured 48 hr later and compared with the average dry weight of the fungus before incubation with the bacteria.

Enrichment of soil with oospores. One hundred milliliters of carrot medium, in Roux bottles (18), was seeded with mycelial disks taken from the margin of a 48-hr culture of *P. aphanidermatum* and incubated for 2 mo at 30 C. Mycelial mats containing oospores were placed on four layers of cheesecloth, washed with running distilled water for 5 min, suspended in tap water, and homogenized in a Waring Blendor for 5 min. Oospores were separated on a 40-μm-mesh Nitex nylon screen (Tetko Inc., Elmsford, NY), resuspended in 100 ml of water, and added to 1 kg of air-dried, well-mixed loamy sand soil (pH 7.6) whose characteristics have been described previously (6). The soil was placed in plastic bags, incubated for 2 wk at 30 C, sieved through a 2-mm-mesh screen, and stored at 4 C until needed. This soil contained an average of 4.5×10^4 colony-forming units (cfu) of *P. aphanidermatum* per gram, as determined on a selective medium according to Schmitthenner (22). Microscopic observations revealed that colonies originated predominantly from oospores.

Oospore germination tests in soil. Bacterial strains that were grown on NA plates for 24 hr were collected, washed twice, and resuspended in distilled water to the desired concentration. Oospore-enriched soil samples (5 g each) were placed in a 10-ml test tube. Aliquots (0.1 ml) of glucose and asparagine solutions (to induce germination [19]) and bacterial suspensions were added to give the desired concentrations at 15% dry weight (−0.3 bar) water content. Soil was mixed thoroughly and incubated at 30 C for 24 hr. Three 0.5-g subsamples of each treatment were transferred to test tubes for processing, staining with Calcofluor New M2R (American Cyanamide Company, Bound Brook NJ) (20), and viewing at ×160 under a UV light microscope for fluorescence. One hundred oospores were counted for each of the three replicates per treatment.

Oospore germination in the rhizosphere was tested using the method developed by Elad and Baker (3). Oospore-enriched soil was mixed with bacteria and placed between two glass slides along with roots of pregerminated plants. Test plants were cucumber (*Cucumis sativus* L. 'Shimshon'), tomato (*Lycopersicon esculentum* L. 'Marmand Rehovot'), pepper (*Capsicum annuum* L. 'Maor'), melon (*Cucumis melo* L. 'Galia'), bean (*Phaseolus vulgaris* L. 'Brittlewax'), wheat (*Triticum aestivum* L. 'Scout'), and cotton (*Gossypium herbaceum* L. 'Pima U. SJ2'). Root exudates induced germination of oospores. Rhizosphere soil was separated from the roots after 48 hr of incubation, and oospore germination was assessed according to the procedure mentioned earlier.

Infestation of greenhouse growth media. *P. aphanidermatum* or *P. ultimum* were isolated from diseased cucumber seedlings on a selective medium for *Pythium* spp. (22) and identified according to Middleton (14) and Waterhouse (25). Growth medium contained 75% Canadian peat and 25% vermiculite no. 2. Oospores of *P. aphanidermatum* were added to PVM, after adjusting their concentration with a hemacytometer, by diluting the suspension with water to the desired level. The pH of PVM was 6.0.

In most experiments with both *Pythium* spp., infested peat was used as inoculum. Three or four repeated plantings with cucumbers, when 90–100% of the plants had died from damping-off, were used to increase inoculum. Inoculum was suspended in 500 ml of water to allow complete mix with the growth medium. The population of *Pythium*, as counted on Schmitthenner's selective medium (22), ranged from 5×10^3 to 5×10^4 cfu/g of dried infested medium.

Bacteria were applied either as water suspensions mixed with the total growth medium or by dipping seeds into a suspension of the candidate strain, drying at room temperature, and planting in the greenhouse.

Polypropylene boxes (7 × 19 × 14 cm) were filled with the infested media, planted with 10 seeds each, irrigated once a day, and the number of diseased seedlings recorded. All experiments were conducted under greenhouse conditions at 28–32 C and consisted of six replicates. Experiments were repeated at least twice. Severity of disease symptoms in cotton was calculated according to an index ranging from 0 for healthy, symptomless plants to 4 for plants covered with *Pythium* lesions over more than 80% of their hypocotyl. Preemergence damping-off, which occurred in each treatment, was calculated by deducting the rate of emerged plants in the pots of this treatment from the average emergence in the medium free of *Pythium*.

The fungicide prothiocarb (Previcur 50%) was mixed with PVM alone or combined with bacterial suspensions to control disease caused by *P. aphanidermatum* in cucumbers.

Population dynamics along cucumber roots were tested by the rhizosphere competence technique of Scher et al (21) as modified by Ahmed and Baker (*personal communication*). Two longitudinal halves of a 50-ml conical polypropylene tube (Falcon Div., Becton-Dickinson, Oxnard, CA) were filled with wet sandy loam soil (15%, −0.3 bar), and one seed coated with bacteria was placed between both parts, 1 cm from the top. The two parts were sealed with two rubber bands and incubated in a polyethylene plastic bag for 5 days at 30 C under artificial light without additional watering. At the end of the incubation period, the tube halves were separated and the plant gently removed to assess the rhizosphere population as mentioned earlier.

Counts of bacteria from soil or peat were carried out by plating the appropriate dilutions, using the drop plate method (17), on NA or KB medium. Results were expressed as colony-forming units per gram. Rhizosphere populations were assessed by separating the soil adhering to the specific root segments (20 min each), in water, by shaking them in a rotary shaker for 30 min at 100 rpm. Average dry weight of rhizosphere soil was calculated by measuring the weight of 20 samples of root segment that could be separated according to the distance from the base of the stem.

RESULTS

Isolation of potentially antagonistic bacteria. Bacteria were isolated from roots of bean, cotton, radish, cucumber, and melon plants. Selected plants were uprooted from soils naturally infested with *P. aphanidermatum*. Of the 130 bacterial isolates tested for their ability to suppress damping-off in cucumbers, the six that were superior in reducing disease incidence by 17–67% (Fig. 1) were selected for further experiments. These strains are *Pseudomonas putida* (805 and 310), *P. cepacia* (808, 814, and Y11), and *Alcaligens* sp. (Rh2).

Antagonistic activity of bacteria in culture. *Lytic enzymes.* None of the strains produced β-1,3-glucanase, chitinase, or cellulase when grown on laminarin, chitin, cellulose, or cell walls of *P. aphanidermatum*. Similarly, when placed in a liquid medium containing a mycelial mat of *P. aphanidermatum* as a sole carbon source, none of the bacteria degraded the mycelium.

Inhibitory substances. Bacteria were separated from the PDNB medium in which they were grown after 40 hr. The cellfree media, mixed with a double concentration of PDNB, were seeded with *P. aphanidermatum* and incubated for 48 hr for dry weight determination. Dry weight of mycelium grown in unamended control medium was 84.4 mg per plate. Bacterial isolates 805, 808, Rh2, and 310 inhibited growth of *P. aphanidermatum* by 87.9, 85.4, 49.8, and 39.0%, respectively, whereas isolates AA4, 806, and SS3, which did not control *Pythium*, reduced it by 76.5, 89.6, and 70.4%, respectively, compared with the control (dry weight 84.4 mg per plate) grown in unamended medium. Growth of *P. aphanidermatum* was inhibited by only 5.7% when grown in a medium that previously supported growth of the same fungus. No significant inhibition of growth of mycelium was observed on solid medium seeded with the test bacteria.

Competition. Competitive ability of both the bacteria and mycelium of *Pythium* was tested in dual cultures. *P. aphanidermatum* was grown in liquid PDNB for 48 hr and transferred into fresh medium along with different bacterial isolates. Strains 805, 808, Y11, Rh2, 310, and 814 inhibited fungal growth by 50, 45, 60, 98, 14, and 62%, respectively, compared with the control culture (dry weight 63.5 mg per plate) grown in bacteria-free medium. However, other bacteria, when grown together in liquid culture, also inhibited growth of *P.*

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aphanidermatum.

Inhibition of oospore germination by bacteria. Bacteria were introduced into raw soil enriched with oospores of *P aphanidermatum*, and glucose and asparagine were added. Oospore germination, determined after 24 hr of incubation, was inhibited by up to 57% by the biocontrol agents (2×10^8 cfu/g of soil) compared with only 13–20% inhibition by bacteria not effective in disease control under greenhouse conditions (Fig. 2). Similarly, when the initial population level of the bacteria was reduced to 2×10^7 cfu/g of soil, germinability of oospores was inhibited by 20–50%.

A significant correlation at $P = 0.05$ or 0.01 was found between the ability of the various bacteria (applied by broadcast or seed coating) to reduce Pythium damping-off in cucumbers grown under greenhouse conditions and their ability to inhibit oospore germination in soil enriched with 150 or 250 μg/g glucose or PVM.

TABLE 1. Influence of bacteria of germination of oospores of *Pythium aphanidermatum* rhizospheres of various crops[1]

Bacterial isolate	Oospore germination (%)						
	Wheat	Tomato	Melon	Cucumber	Bean	Cotton	Av.
805	7 a[2]	10 a	8 a	18 a	21 a	15 a	13
808	31 b	11 a	12 a	19 a	19 a	12 a	17
AA4	41 c	35 b	41 b	46 b	57 b	27 b	41
Untreated control	64 d	37 b	46 b	57 b	65 b	36 b	51

[1]Seeds of various crops were germinated in moist bags and placed on soil enriched with oospores, between two glass slides. Germination was calculated, after 3 days of incubation, by the Calcofluor staining method (20).

[2]Numbers in each column followed by the same letters are not significantly different according to Duncan's multiple range test ($P = 0.05$).

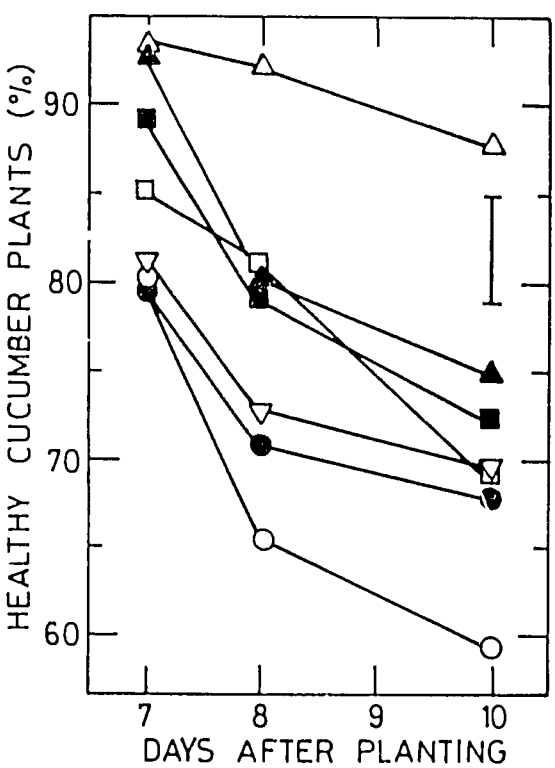

Fig. 1. Influence of bacterial suspensions on the percentage of healthy cucumber plants planted in peat-vermiculite medium infested with *Pythium aphanidermatum*. Treatments were control (o) and bacterial isolates 805 (▲), 808 (△), 310 (▽), Rh2 (•), Y11 (■), and 814 (□). Bar = $LSD_{0.05}$.

To determine whether the bacteria produce inhibitory substances affecting oospore germination, cellfree supernatants of 24-hr-old cultures of the different bacteria were added to the oospore-enriched soil. No significant reduction in oospore germinability was observed unless bacterial cells were present in the soil.

Germination of oospores in rhizospheres of wheat, tomato, melon, cucumber, bean, or cotton in the presence of bacteria was compared with their germination in the untreated control (Table 1). Average inhibitions of germination in all plant crops by isolates 805 and 808 were 72.3 and 66.4%, whereas isolate AA4, which is not capable of controlling *Pythium* in the greenhouse, inhibited germination by only 5–36%.

Biological control of damping-off in cucumbers. *Effects of bacterial concentrations.* Bacteria were suspended in water and mixed with PVM to give two initial population levels of 10^7 and 10^8 cfu/cm^3. Isolates 805 and 808 were the most effective in control of *Pythium* when applied in concentrations of 10^8 cfu/cm^3 (Table 2). Relatively high percentages of healthy cucumber plants (up to 62% reduction of disease) were obtained when bacteria were applied in concentrations of 10^7 cfu/cm^3. Cells of bacterial isolates 805 and 808 were suspended in three low concentrations to give final counts of 4×10^7, 8×10^6, and 2×10^6 cfu/cm^3 of PVM. The concentration of cells of isolate 808 influenced the efficacy of disease control, whereas differences between concentrations of applied isolate 805 were not reflected in marked differences in disease control (Fig. 2). Preemergence damping-off in untreated control was 71.6%, whereas it was reduced to 21.1–26.3 and 2.1–10.5% by treatments with isolates 808 and 805, respectively.

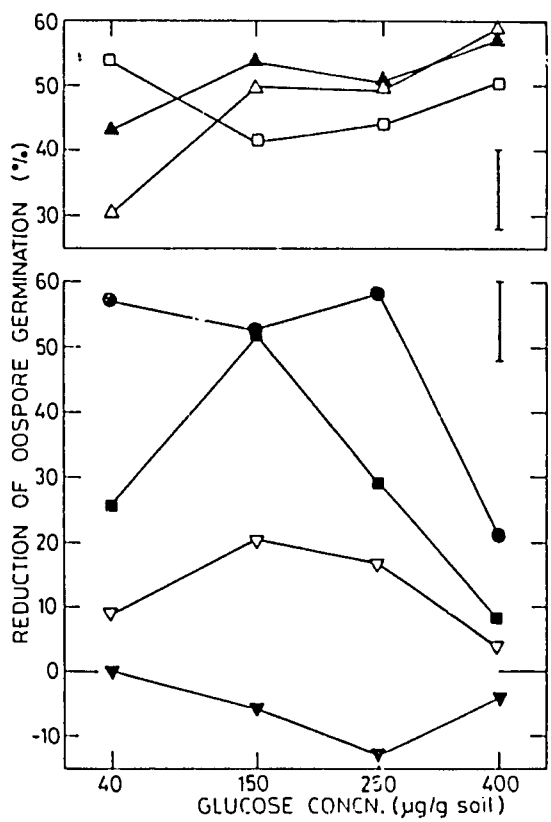

Fig. 2. Effects of suspensions of bacterial biocontrol agents 805 (▲), 808 (△), 814 (□), Rh2 (•), and Y11 (■) and other isolates, AA4 (▽) and SS3 (▼) on the percentage of reduction of oospore germination in soil amended with glucose and asparagine (5:1). Bar = $LSD_{0.05}$. Reduction was calculated according to the following formula: $[1 - (A/B)] \times 100$, where B = percentage of germination in the control without bacteria and A = percentage of germination in bacterial treatments.

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Effects of bacterial seed coating and integrated control. Cucumber seeds were dipped in bacterial suspensions (8×10^8 cfu/ml) and planted in PVM. Damping-off caused by *P. aphanidermatum* was recorded during 12 days after sowing. The most efficient isolates were 805, 808, and Rh2, which reduced disease incidence by 57–67%, compared with isolates 814 and 310, which reduced disease incidence by 38–42% (Fig. 4). Preemergence damping-off, 27.5% in the untreated control, was reduced to 0–12.5% by the different bacterial isolates.

Bacterial isolates, at the low rate of 3×10^6 cfu/cm^3 of PVM, were combined with prothiocarb at the rate of 0.25 ml/L of PVM. Incidence of damping-off caused by *P. aphanidermatum* in the untreated control was 89%. Isolates 805 and 808, alone, decreased Pythium damping-off by only 9–15%, because the bacterial level was low. A significant decrease in disease incidence, 34%, was obtained by the chemical treatment. Combination of prothiocarb with strain 805 resulted in 46% disease reduction; however, combination of prothiocarb with strain 808 was significantly superior, yielding an 80% reduction in disease incidence.

Effects of bacteria on disease buildup during successive replanting. Cucumber seeds were planted five times at 10-day intervals in PVM mixed with suspensions of isolates 805 or 808, and the percentages of healthy plants were recorded at the end of each growth cycle. In the untreated control, disease incidence of *Pythium*, which was 93% at the end of the first cycle, stabilized at 50% between the third and fifth growth cycles. Disease incidence in bacteria-treated media was 12–45% during the five successive plantings and was 12 and 27% at the end of the fifth cycle in treatments of strains 808 and 805, respectively.

Control of damping-off caused by P. ultimum in cucumbers. Control of *P. ultimum* in cucumbers was tested in PVM amended with each of the bacterial isolates. Disease incidence was drastically reduced by all isolates in the first growth cycle (Fig. 5). However, after 2 wk of the second growth cycle, there were 7, 18, 51, 42, 4, 71, and 17% healthy plants in the untreated control and treatments with isolates 808, 805, 310, 814, Rh2, and Y11, respectively.

Biological control of Pythium damping-off in pepper, melon, bean, tomato, and cotton. Isolates 805 and 808 were mixed with PVM, and their influence on disease caused by *Pythium* in several crops was examined in the greenhouse. Both isolates reduced disease incidence in melons, tomatoes, peppers, and beans (Fig. 6).

TABLE 2. Influence of bacterial concentration[y] in peat-vermiculite medium on ability of strains to control *Pythium aphanidermatum* in cucumber

Bacterial isolate	Emergence (%) 7 days after planting		Healthy plants (%) 11 days after planting	
	10^7 cfu/cm^3	10^8 cfu/cm^3	10^7 cfu/cm^3	10^8 cfu/cm^3
Rh2	18 a[z]	42 b	12 a	27 b
805	60 bc	83 d	48 c	70 d
Y11	37 ab	48 b	18 c	37 bc
808	77 cd	72 c	60 d	68 d
814	37 ab	51 b	10 a	32 b
310	42 b	53 b	19 ab	41 bc

[y]Suspensions of 24-hr-old bacteria were mixed with artificially infested peat-vermiculite container medium to give 10^7 or 10^8 cfu/cm^3.

[z]Numbers in each parameter followed by the same letters are not significantly different according to Duncan's multiple range test ($P = 0.05$).

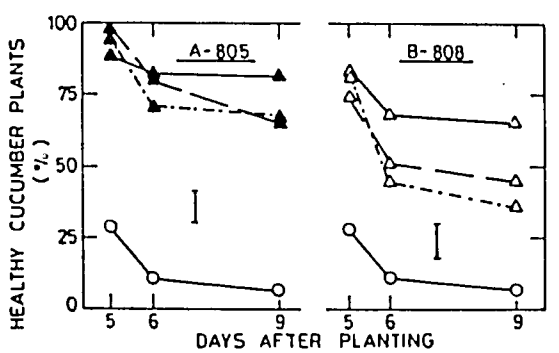

Fig. 3. Effects of isolates A, 805 and B, 808 applied at concentrations of 2×10^6 (–·–), 8×10^6 (--), and 4×10^7 (——) cfu/cm^3 (o = untreated control) on percentage of healthy cucumber plants planted in peat-vermiculite infested with *Pythium aphanidermatum*. Bars = $LSD_{0.05}$.

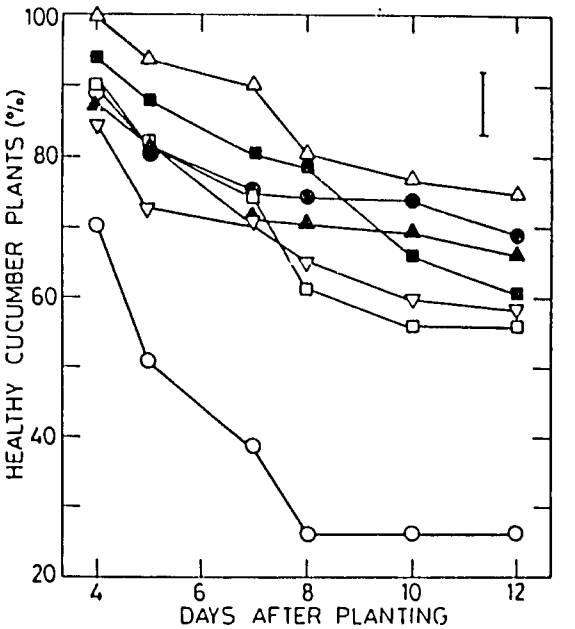

Fig. 4. Influence of seed coating with bacterial isolates 805 (▲), 808 (△), Rh2 (•), Y11 (■), 310 (▽), and 814 (□) (o = untreated control) on the percentage of healthy cucumber seedlings planted in peat-vermiculite medium infested with *Pythium aphanidermatum*. Bars = $LSD_{0.05}$.

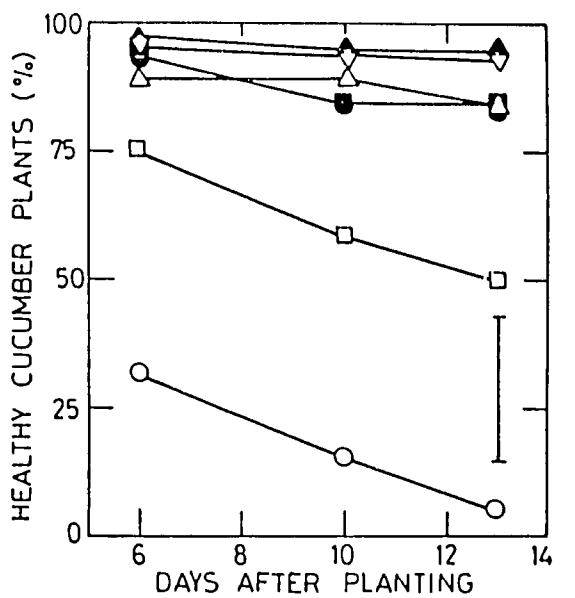

Fig. 5. Stand of cucumber plants in peat-vermiculite medium infested with *Pythium ultimum* and treated with isolates 805 (▲, 808 (△), Rh2 (•), Y11 (■), 310 (▽), 814 (□), or untreated (o).

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Disease indexes in cotton, calculated according to root coverage by *Pythium* lesions, were 2.17, 0.83, and 1.0, respectively, in the untreated control and in treatments with isolates 805 and 808. The respective percentages of disease incidence were 92, 50, and 62. Disease incidence was decreased by 19–53% when pepper, bean, and tomato plants were replanted in the original growth mixture.

Competitive root colonization by antibiotic-resistant biocontrol agents: general populations of bacteria and *Pythium*. Isolates 805 and 808 were selected for resistance to rifampicin and nalidixic acid. Both decreased disease incidence caused by *Pythium* in cucumbers by 50% when applied to soil. Efficient recovery of the resistant bacteria was obtained on medium supplemented with both antibiotics at a rate of 150 μg/ml.

Cucumber seeds were coated with suspensions of isolates 805, 808, 814, Y11, or Rh2. Two longitudinal halves of plastic tubes were filled with soil infested with *Pythium* and sealed together with one coated seed in each tube. The roots, exposed after 5 days, were cut into segments 0–1, 2–3, 4–5, and 6–7 cm from the stem base. General populations along the untreated roots ranged between 2.0×10^6 and 2.2×10^5 cfu/g of soil in the segments 0–1 and 6–7 cm (Fig. 7). Application of isolates 814, Y11, and Rh2 to the seeds decreased the general bacterial populations to 4.0×10^1–2.3×10^5 cfu/g of soil in segments 0–1 and 4–5 cm, whereas isolates 805 and 808 decreased bacterial numbers to 10^2 cfu/g in segments 2–3 and 4–5 cm. However, the general populations at the tips of treated roots did not differ from those of the untreated ones.

The specific populations of bacteria applied to seeds before incubation were 2.2×10^1–2.0×10^6 cfu/g of rhizosphere soil (Fig. 7). Except for isolate Y11, bacterial counts at root tips were up to 100 times higher than at the upper root parts. There was a positive correlation between the levels of general population and those of the biocontrol agents in all root segments except the tips. Colonization of cucumber roots by *P. aphanidermatum* was reduced by 50–100% in the presence of the biocontrol agents.

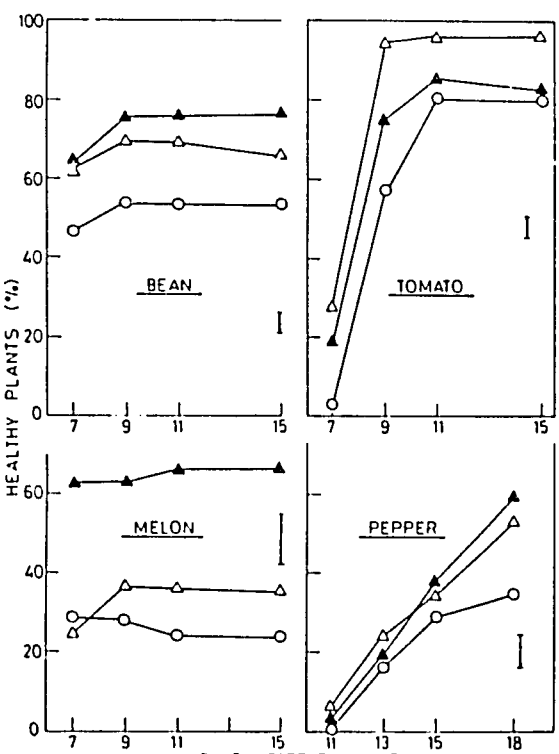

Fig. 6. Percentage of healthy plants in peat-vermiculite medium infested with *Pythium aphanidermatum* and treated with isolates 805 (▲) or 808 (△) or untreated (o).

DISCUSSION

A major objective of this study was to isolate potential bacteria and characterize their activity in controlling *Pythium* spp. The ability of six bacteria applied to PVM or soil to reduce the incidence of damping-off in the greenhouse was tested. About 60–90% of disease reduction was obtained under growth conditions prevailing in our experiments. Although cucumber was the major test plant throughout this work, disease control was also obtained in other crops susceptible to *P. aphanidermatum*, e.g., pepper, melon, bean, tomato, and cotton (Fig. 6). The bacteria were also most effective in controlling *P. ultimum* (Fig. 6). One application of the biocontrol agents was sufficient to prevent disease buildup in replanted cucumbers. The additive control achieved by combining bacterial application, at a low rate to achieve disease control, with the fungicide prothiocarb (commonly used against *Pythium*) reduced disease incidence 80%.

Our results indicate that the presence of bacteria along roots of susceptible hosts reduced the establishment of *Pythium* along the roots, which are the potential sites for its attack.

Damping-off, induced by *Pythium* spp., has been controlled by coating seeds with a variety of antagonistic microorganisms (7–9,13,24). In this work, seed coating was as effective as direct application of bacteria into the soil (Fig. 4). Seed treatments are an attractive method for introducing bacteria to the soil-plant environment, because in this way, the bacteria have an opportunity of being the first colonizers of the roots. It was evident that bacteria, introduced as seed treatments, moved along the roots. Weller (26) showed the distribution of *Pseudomonas fluorescens* introduced via seeds on seminal roots of winter wheat. Similarly, we have counted higher populations at root tips. Moreover, the lower counts of indigenous bacteria obtained in wheat (26) and cucumber (Fig. 7) in the presence of the introduced strains indicate the obvious competition between them. Populations of the indigenous bacteria generally were greatest where populations of the biocontrol agents were great (Fig. 7), probably because both

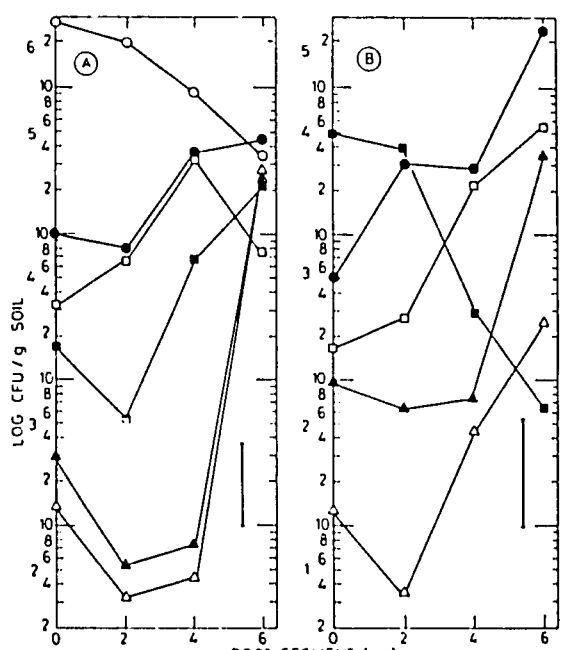

Fig. 7. Densities of A, general bacteria and B, biocontrol agents in the rhizosphere of 6-day-old cucumber plants. Isolates 805 (▲), 808 (△), Rh2 (•), Y11 (■), and 814 (□) (o = untreated control) were applied to seeds planted between two longitudinal halves of plastic tubes filled with soil. Bar = $LSD_{0.05}$.

were stimulated by the same factor—rhizosphere nutrients. Intense competition for resources such as nutrients among rhizosphere bacteria is very probable. Seed coating places the bacteria where they are needed the most, at the most important site of interaction with the plant-rotting *Pythium* spp. The ability of a bacterial strain to colonize or establisb a large population in the rhizosphere is a crucial factor that determines the importance of rhizobacteria as root associates.

Significant positive correlation was observed between inhibition of oospore germination and disease reduction induced by the same bacteria. Moreover, inhibition of germination of oospores in the rhizospheres of several plants was achieved by the biocontrol agents. On the other hand, there was no direct observable interaction between spores and bacteria. Siderophores may also be excluded as a possible mechanism for control of *Pythium*, because the pH of the PVM was calibrated to 6.0. In this case, iron is available in high concentration. This may not be tne case in *Pythium*-infested soil where pH is about 7.6, as was shown in the interaction of *Fusarium oxysporum* and *Pseudomonas* sp. (3).

Oospore germination has already been shown to be affected by exogenous nutrients (9). Nutrients supplied by root exudates stimulate oospore germination in the rhizosphere. It therefore appears that bacteria may compete with germinating oospores for available carbon or nitrogen sources, and by eliminating these resources, the bacteria reduce the percentage of oospore germination. Similar results were obtained recently with chlamydospores of *Fusarium* spp. (3). Moreover, the reduction in the general population of bacteria along the roots caused by the biocontrol agents is probably due to competition (Fig. 7). Although lysis was suggested as a mechanism in the control of *Pythium* sp. (15) when cell wall lytic bacteria are added to soil, such heterolytic activity was not confirmed in vitro in our studies. Production of inhibitory substances may be a potential means by which the bacteria affect the plant pathogen. *Pythium* is indeed known to be sensitive to such inhibitory effects. However, our in vitro experiments have shown that bacteria, unable to induce suppressiveness in soil, effectively inhibited mycelium of the pathogen. This may suggest that inhibitory substances or antibiotics do not play a major role in our systems.

Broadbent et al (2) added *Bacillus subtilis* (Fren.) Cohn to soil treated by aerated steam. *P. ultimum* was controlled on *Antirrhinum*, possibly by decreasing infection rather than by antibiosis. Hadar et al (7) protected seedlings from disease caused by *Pythium* by application of *Enterobacter cloacae*. Howell and Stipanovic (9) concluded that a strain of *Pseudomonas fluorescens* was antagonistic to *P. ultimum* because of the production of an antibiotic (pyoluteorin) by the bacterium.

Our results demonstrate the potential of bacteria to control *Pythium*. Further research is needed to develop the formulation of bacterial biocontrol agents and exploit them in agriculture.

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THE POSSIBLE ROLE OF COMPETITION BETWEEN *TRICHODERMA HARZIANUM* AND *FUSARIUM OXYSPORUM* ON RHIZOSPHERE COLONIZATION.

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ABSTRACT

Sivan, A., and Chet, I. 1987. The possible role of competition between *Trichoderma harzianum* and *Fusarium oxysporum* on rhizosphere colonization. Phytopathology

Soil was enriched with chlamydospores of *Fusarium oxysporum* f. sp. *vasinfectum* and *F. oxysporum* f. sp. *melonis*, and amended with increasing concentrations of glucose and asparagine. Maximal germination of chlamydospores was obtained in soil amended with 0.4 and 0.08 mg glucose and asparagine per g soil, respectively. Addition of conidia of the biocontrol agent *T. harzianum* (T-35) significantly (P=0.05) reduced the chlamydospore germination rate

of both Fusaria. However, in soils amended with higher concentrations (0.3 and 0.06 mg/g soil) of glucose and asparagine, respectively, the inhibition was nullified. Chlamydospore germination of F. O. melonis and of F. O. vasinfectum in melon and cotton rhizosphere soil were significantly inhibited after soil or seed application with T-35. As in the case of the glucose and asparagine, addition of an excess of seedling exudate increased the germination rate and eliminated the inhibition. Moreover, a continuous application of germinating cotton seed exudate to a soil infested with F. O. vasinfectum planted with cotton and treated with T-35, significantly reduced the disease control capability of the antagonist.

A seed treatment with T-35, in a constantly humid soil, resulted in high population densities of the antagonist on the developing rhizosphere. Plants grown from seeds treated with T-35 had roots with lower levels of Fusarium spp. in their rhizosphere than roots from plants from non-treated seeds. The greatest density and the largest reduction in Fusarium levels were detected on the lower 4 cm of the roots. Numbers of Fusarium in the rhizosphere were reversely proportional to the number of conidia of T-35 applied to soil. On the other hand as the concentration of the pathogen in soil increased, T-35 counts on root segments decreased. Trichoderma had a little effect on the survival of Fusarium spp. in non-rhizosphere soil. Inhibition

of germination may therefore have resulted from competition.

Plant roots growing in soils are a major source of carbon and energy to microorganisms in the form of root exudates. Cells detached from old parts of the root, or the root itself, after plant death (7). Barber and Martin, (3) estimated that 3-9% of assimilated carbon by wheat and barley is exuded by root by-products. Whipps and Lynch (25), in a similar experiment showed that the total carbon released from cereals was 25-30% of the total fixed carbon.

Competition for nutrients, primarily carbon and nitrogen, may result in biological control of soil-borne plant pathogens. In many cases where the amount of these nutrients become limiting for the pathogen due to intensive microbial activity, the soil becomes suppressive (7).

Cook and Schroth (8) identified carbon and nitrogen compounds required for chlamydospore germination of F. solani f. sp. phaseoli. Similarly, Sneh et al (23) demonstrated the role of glucose and asparagine in stimulating chlamydospore germination of F. oxysporum f. sp. lini. Recently, Elad and Baker (9) and Elad and Chet (10) reported that carbon sources, either provided by synthetic substances or excreted by plant rhizosphere, might be involved in the chlamydospore and oospore germination of F. oxysporum and Pythium aphanidermatum. Proliferation along the developing rhizosphere is one of the most important traits for antagonists applied to seeds (7).

However, most studies in this field have been shown by antagonistic rhizobacteria (5,12,24) and there is relatively little information involving fungi. Recently, Ordentlich, et al (15), showed the potential of chitinolytic Serratia marcescens in colonizing bean rhizosphere after its application to soil surrounding the germinating seed.

Chao et al (6) reported the unsuccessful establishment of Trichoderma harzianum in pea rhizosphere. The failure of Trichoderma to establish in rhizosphere of pea and bean was also reported by Papavizas (16). Recently, Ahmad and Baker (1) demonstrated that a benomyl resistant strain of T. harzianum, colonized the rhizosphere of several crops when applied to seeds. Wild types failed to establish in the rhizosphere.

The objectives of the present study were to evaluate the role of competition in the biological control of Fusarium wilt of melon and cotton and Fusarium crown rot of tomatoe by T. harzianum strain T-35 (20,21). We investigated the potential of this strain to inhibit chlamydospore germination in nutrient-amended soils. We also examined the rhizosphere colonization of T-35 and its effect on rhizosphere populations of F. oxysporum.

MATERIALS AND METHODS

Fungal isolates. F. oxysporum f. sp. melonis Snyd. and Hans. (F. o. melonis), F. oxysporum f. sp. vasinfectum (Atk.) Snyd. and Hans. and F. oxysporum f. sp. radicis-lycopersici Jarvis and Shoemaker were isolated from infected plants on the Fusarium

selective medium Nash and Snyder (13). After isolation, these fungi were cultured at 27C on a yeast extract -glucose medium (YM) containing 5 g yeast extract (Difco Laboratories, Detroit, Michigan, USA) 5 g peptone (Difco) 10 g glucose, and 20g agar (Difco) per liter. An isolate of T. harzianum obtained from a Fusarium-wilted cotton plant and designated T-35 (22), was cultured on a potato dextrose agar (PDA, Difco) or in a liquid synthetic medium (SM;16). Its isolation and properties have been described elsewhere (20).

Greenhouse experiments. Experiments were carried out in artificially-infested sandy loam soil (pH 7.1) consisting of 82.3% sand, 2.3% silt, 15% clay, 0.3% organic matter, 0.02% N, 0.067% K, 0.01% P, and 0.003% extractable Fe. Its moisture at field capacity was 12.2.% (w/w). Soil was infested with F. oxysporum with a microconidial suspension (20). Erlenmeyer flasks (250 ml) each containing 50 ml of liquid YM were seeded with mycelial disks from 72hr-old cultures of the pathogen. Flasks were incubated at 27 C in a rotary shaker at 120 rpm for 4 days. Microconidia were then separated from the mycelium by filtration through eight layers of cheesecloth. The conidia were washed three times by centrifugation at 9150x g for 30 min.at 4 C. Ten ml of this supension, adjusted to $2x10^7$ microconidia per ml, were mixed with sandy loam soil in an electrical soil mixer.

The test plants were: cotton (Gossypium barbardense L. 'Pima') and melon (Cucumis melo L. '56').

Experiments, comprising six replicates, were set up in plastic pots (9 x 4 x 10 cm) each containing 0.5 kg soil sown with 10 seeds of the test plant; seeds were not subjected to any chemical treatment. *T. harzianum* (T-35) was applied in one of the following formulations: (a) conidial suspension - conidia were collected from cultures grown in Erlenmeyer flasks containing 20 ml of PDA. The suspension was washed three times in sterile tap water by centrifugation (15,000xg) and adjusted to $1x10^6$ conidia/ml. (b) Seed coating - conidia were collected as described above, adjusted to $5x10^9$ conidia/ml and supplemented with 0.015% (v/v) of Nu-film-17 (Miller Chemicals, Penn. USA) as an adhesive. Four ml of this suspension were used to coat 20 g of melon and cotton seeds or 10 g of tomato seeds. The number of *Trichoderma* populations on seed surfaces were counted by shaking 5 g of seeds in 50 ml of sterile tap water, for 1 hr, in 250 ml Erlenmeyer flasks in a rotary shaker at 200 rpm. Serial dilutions of the suspension were plated on a *Trichoderma* selective medium (TSM) (11). (c) Wheat-bran peat preparation (22) - Wheat bran/peat mixture (1:1, v/v) 10% moisture was amended with 40 ml of tap water per 100 g mixture. The mixture was then autoclaved in autoclavable polyethylene bags (50 x 50 cm) for 1 hr at 121 C on three successive days. Homogenized mycelium from 48 hr-old cultures of *T. harzianum* grown in liquid SM, was transferred to the wheat bran-peat mixture and incubated in an illuminated chamber for 14 days at 30 C. This preparation, containing $5x10^9$ colony forming units (cfu)/g, was mixed with soil at a concentration of 5 g/kg soil.

Soil enrichment with chlamydospores of Fusarium was performed according to Sneh et al (23). Mycelial mats of F. oxysporum f. sp. melonis (F. o. melonis) and F. oxysporum f. sp. vasinfectum (F. o. vasinfectum) were grown in liquid YM, in 'Roux' bottles at 27 C. After 7 days, mycelial mats were separated, placed in four layers of cheesecloth and washed under running distilled water for 5 min. Twelve mats of each fungus were blended for 1 min and centrifuged for 10 min at 3,000 x g. The pellet was resuspended in 150 ml of water and added to 1 kg of soil. The soil was mixed, placed in glass bottles and incubated at 27 C for 4 wk then air-dried to 10% moisture, sieved through a 2 mm screen, mixed well and stored at 4 C until use. The soil contained an average of $5x10^4$ cfu/g of F. oxysporum as determined after soil dilutions on Fusarium selective medium.

Chlamydospore germination tests in soil were carried out according to Sneh et al (23). Samples of 1 g of chlamydospore-enriched soil were placed in 10 ml test tubes. Aliquots (0.1 ml) of glucose and asparagine (5:1, w/w) were added to reach the desired concentrations of nutrients at 15% water content. These compounds were often used to induce germination of Fusarium spp. chlamydospores (9,23). T-35 was added as a pre-germinated conidial suspension (in water solution of 0.01% glucose). Soil was thoroughly mixed and incubated at 27 C for 22 hr. After the incubation, the soil in each test tube was stained with 1 ml aliquots of 0.3% Calcofluor New M2R (American Cyanamid Company,

Bound Brook, NJ) solution according to Scher and Baker (19). After 5 min, the excess solution, was removed and replaced with 1 ml of distilled water. Soil was suspended and a drop of the suspension taken for fluorescent observation under an epifluorescent light microscope under ultraviolet light (Olympus, Japan) at x 400. Three test tubes were replicated for each treatment and 100 chlamydospores were counted at each observation.

Chlamydospore germination in the rhizosphere was tested according to Sneh et al (23). Cotton and melon seeds were disinfested for 3 min in 4% sodium hypochlorite and placed between four layers of wet No. 1 chromatography paper which were wrapped in plastic bags and incubated at 30 C for 24 hr. The germinated seeds were then transferred to 5 g of chlamydospore enriched soil, some samples were also amended with T-35, and the mixture was placed between two microscope glass slides secured with two rubber bands. The slides were slightly slanted in moist soil in plastic pots (9 x 9 x 10 cm), wrapped in a plastic bag and incubated at 27 C for the desired period. The slides were then recovered from soil, the rubber bands and germinated seedlings were carefully removed and the soil adhering to roots, transferred to a 10 ml test tube for microscopic observation, as described above.

In both chlamydospore germination tests (e.g. rhizosphere and non-rhizosphere soil), T-35 was also applied as a seed coating. Three germinated seeds were replicated per treatment and 100 chlamydospores were counted at each observation.

Production and collection of plant exudates

Cotton and melon seeds were disinfested with 4% sodium hypochlorite for 10 min. The efficacy of disinfestation was tested by placing samples of the treated seeds on PDA or nutrient agar (Difco) plates. Eight hundred seeds of each crop, were placed in a glass column (80 x 7 cm) containing 1 L of sterile tap water. The lower part of the column was connected to an air compressor and air was forced inside through a sterile glass fibre filter at a pressure which thoroughly agitated the suspended seeds. At the upper part of the column an air outlet containing an additional glass fibre filter, was mounted. The column with the germinating seeds was incubated at 27 C for 4 days. The resulting seed exudate mixture was then collected in a sterile container, frozen at -30 C, lyophilized and kept at -30 C until use.

Rhizosphere colonization tests. Rhizosphere population dynamics of T. harzianum and Fusarium spp. were studied according to the method developed by Scher et al (19) as modified by Ahmad and Baker (1) and Elad and Chet (10). A seed of the test plant was placed at the upper part of two longitudinal halves of 50 ml polypropylene tubes measuring 11.5 x 2.8 cm (Falcon Div. Becton-Dickinson Co., Oxnard, CA, USA) filled with sandy loam soil with 15% (w/w) moisture content. T-35 was applied to soil as a seed coating or as a conidial suspension, while F. oxysporum was introduced to soil as a microconidial suspension (10^3

microconidia/g soil). One seed of cotton, melon or tomato (Lycopersicum esculentum L.) was placed on the half tube 1 cm below the rim. The unseeded half tube was carefully placed on the first half and secured with rubber bands. Tubes of all treatments were completely randomized and placed in plastic pots (9x9x10 cm) containing sandy loam soil with the same water content. The pots were covered with polyethylene bags to maintain the same soil water content and placed in an illuminated chamber, at 28 C.

In an additional experiment, aimed at studying the population dynamics of T-35 in the rhizosphere of irrigated plants, the soil was daily irrigated with 3 ml of tap water. After 8 days of incubation, tubes were removed from the pots and their two halves carefully separated. The roots with adhering soil were cut, starting from the crown into 2 cm segments. Corresponding segments from six plants were combined and shaken in a rotary shaker for 1 hr in 250 ml Erlenmeyer flasks each containing 30 ml of sterilized tap water. Serial dilutions were plated on TSM and Nash and Snyder medium for Trichoderma and Fusarium spp. counts, respectively. The counts of each fungus were expressed as cfu/g root including the adhering soil (dry weight).

Each colony of Trichoderma was transferred from TSM to PDA containing cycloheximide (100 mg/L) to distinguish between the resident Trichoderma soil populations and from T-35 which is very resistant to this substance (Pe'er and Chet, unpublished).

All tests were performed at least twice. Statistics were carried out using Duncan's Multiple Range Test with a significance level of P=0.05.

RESULTS

Chlamydospore germination in soil amended with glucose and asparagine. Without the addition of nutrients to soil 19% to 30% of added chlamydospore of F. o. melonis or F. o. vasinfectum germinated. The first germinating chlamydospores were observed after 18 hr, while maximal germination occurred 22 hr after incubation.

Addition of pre-germinated conidia of T-35 to soils reduced germination of chlamydospores of F. o. melonis and F. o. vasinfectum (Fig. 1, Table 1). Levels of nutrients amended to soil were reversely related to the ability of T-35 to reduce germination. At 0.3 and 0.06 mg/g soil of glucose and asparagine, respectively (the highest level amended) T-35 had almost no effect (Fig. 1). T-35 had a similar effect when applied as a seed treatment (Table 1). However, when (in another experiment) T-35 was added as ungerminated conidia the inhibitory effect was not significant.

Chlamydospore germination in rhizosphere soil. In rhizosphere soil of melon and cotton seedlings previously enriched with chlamydospores of F. o. melonis and F. o. vasinfectum, and planted with melon and cotton seeds, respectively, the maximal germination rate of chlamydospores (45 % and 56%, respectively) was observed after 60 hr incubation (Fig. 2).

The inhibitory effect, of T-35 treatments, on chlamydospore germination in cotton or melon rhizosphere soil was nullified after the addition of an excess of germinating cotton and melon seed exudates (Table 2). Similarly, under greenhouse conditions, a continuous external addition of these exudates to soil infested with F. o. vasinfectum, and planted with cotton, reduced the disease control obtained by T-35, after its application as either seed coating or a conidial suspension (Table 2).

Rhizosphere colonization by T. harzianum and F. oxysporum. After its application as a seed treatment, T-35 successfully colonized the rhizospheres of developing melon, cotton and tomato roots, up to 12 cm from the crown. In irrigated soil the rhizosphere population of T. harzianum, resistant to 100 mg/L of cycloheximide (e.g. T-35), was much higher than that counted in non-irrigated soil (Fig. 3). In both cases the maximal counts of T-35 were monitored on root segments which included the crown and the root tip.

In soil infested with F. o. vasinfectum (10^3 microcoidia/g soil) the colonization of cotton rhizosphere by T-35 resulted in a decline in Fusarium spp. cfu on the respective root segments (Fig. 4). A reduction of more than one order of magnitude was recorded on root segments at distances of 8-12 cm from the crown. A similar effect was obtain in the rhizosphere of melon as a consequence of addition of T-35.

The amount of colonization with T-35 of melon rhizosphere was dependent on the inoculum concentration of F. o. melonis.

Soil infested with increasing numbers of microconidia of either F. o. melonis or F. o. vasinfectum reduced the establishment of T-35 in the rhizosphere by up to two orders of magnitude on the root tip (Fig. 5). Similarly, establishment of F. o. melonis in melon rhizosphere was affected by the number of conidia of T-35 introduced in the soil. In untreated soil the counts of Fusarium spp. in rhizosphere ranged between 1.5×10^5 and 8.8×10^4 cfu/g root. However, T-35 treatment with 10^6 conidia/g soil reduced the establishment of both pathogens up to 6.3×10^2 cfu/g of melon root and rhizosphere soil (Fig. 6).

In order to determine the colonization of rhizosphere and non-rhizosphere soils, infested with F. o. melonis and treated with T-35, serial dilutions from these soils were carried out (Table 3). After a seed coating treatment, T. harzianum effectively colonized the rhizosphere soil but failed to establish in non-rhizosphere soil. Application of T-35 as a conidial suspension or a wheat bran-peat preparation, resulted in high colonization of both rhizosphere and non-rhizosphere soils. All Trichoderma treatments had little effect on the recovery of Fusarium spp. from the non-rhizosphere soil. However, Trichoderma treatments reduced (by up to 3 orders of magnitude) numbers of Fusaria in rhizosphere soil (Table 3).

DISCUSSION

Currently, there are two indirect approaches to test competition as a mechanism in biological control of soil-borne plant pathogens. The first is based on the hypothesis that biological control resulting from competition has little or no effect on the viability of the pathogen (2). The second involves the use of limiting factors for which (by definition) microorganisms may compete. Addition of an excess of the candidate factor should nullify the antagonistic effect. The present study, using those approaches, raises the question whether competition for nutrients and rhizosphere colonization are involved in the biocontrol of F. oxysporum obtained with T-35 (20,21).

One of the most sensitive stages for nutrient competition in the life cycle of Fusarium is chlamydospore germination (2). In soil infested with chlamydospores of F. oxysporum the germination rate ranged between 20 and 30%. These levels of germination could result from reserve materials in the chlamydospore, or might be due to residual organic matter in the raw soil. The major sources of nutrients for soil microorganisms are nutrients derived from seeds or roots. In our study we used glucose and asparagaine to simulate the organic carbon and nitrogen components of plant exudates as stimulants of chlamydospore germination. Soil amendment with increasing amounts of glucose and asparagine enhanced the germination rate to 62%. However,

the addition of higher concentrations of these nutrients did not result in further increase in germination. The failure of the remaining chlamydospores to germinate is probably a result of their continuous dormancy or may be due to utilization of most of the amended nutrients by the resident soil microflora (7).

Although the role of plant exudates is an important nutritional factor for rhizosphere microorganisms is well established, their role in biocontrol is still unclear. In the present study we evaluated the effect of plant exudates on the inhibition of chlamydospore germination in vitro and on the suppression of Fusarium wilt of cotton in vivo obtained with T-35 when soil was amended with low concentrations of glucose and asparagine (0.05 and 0.01mg/g soil, respectively) or with plant exudates, pre-germinated conidia of T-35 were found to inhibit the germination of chlamydospores. However, at concentrations higher than 0.3 and 0.06 mg/g soil of amended glucose and asparagine, respectively, or when an excess of exudates was added the inhibition was nullified. Similarly, in the presence of an excess of exudates the reduction in Fusarium wilt of cotton, by T-35 was completely neutralized. This indicates that competition for these or similar nutrients may occur. At low levels nutrients are limiting and competitive effects are evident, resulting in significant reduction in chlamydospore germination. Indeed, Rovira and his co-workers (197) have already mentioned that plant root exudates contain lower amounts of carbon and energy than those required by rhizosphere microflora. Similar

phenomena have already been described (9,23), in the inhibition of F. oxysporum f. sp. cucumerinum chlamydospore germination by pseudomonads. Chlamydospores of F. o. vasinfectum and F.o. melonis in the rhizosphere of cotton and melon, respectively, germinated at higher levels compared with the germination obtained in non-rhizosphere soil. The same also occurred with chalmydsopores of F. solani f. sp. phaseoli and F. oxysporum f. sp. cucumerinum, when exposed to bean and cucumber exudates (23). Inhibition of chlamydospore germination of F. oxysporum could be obtained even when the antagonist was applied as a seed coating. Apparently, effective utilization of root exudates may enable antagonists applied to seeds to proliferate along the developing rhizosphere and interfere with seed and root pathogens (7). After application of T-35 as a seed treatment in soil without irrigation, T-35 was recovered from the rhizosphere indicating the active proliferation of this strain along the developing roots. The greatest counts of T-35 in the rhizosphere were detected on root segments which included the root base and tip. Since the population dynamics of T-35 were recorded from rhizosphere portions which included roots and soil. It is not clear whether T-35 colonized the rhizoplane, the rhizosphere soil or both. The population of T-35 could be distinguished from the resident soil population of Trichoderma spp. by using the natural resistance of this strain to relatively high concentrations of cycloheximide (Pe'er and Chet, unpublished). Similar findings were demonstrated by Ahmad and Baker (1) with a benomyl resistant

mutant of T. harzianum. The relatively high population levels of antagonists recovered from root tips were also demonstrated with bacteria (10,15,19).

Most of the exudates present in the rhizosphere are excreted from the root tips. Thus, colonization of this region in the rhizosphere might reduce infection by Fusarium-like pathotypes which penetrate the vascular system of their hosts through the undifferentiated xylem at the root tip (7). Rhizosphere colonization of cotton and melon, by T-35, was accompanied by a simultaneous decline in Fusarium spp. Reduction in the pathogen population in the rhizosphere should lead to a decrease in the infection rate. The level of rhizosphere colonization by T-35 was affected by the inoculum density of the pathogen in soil, and counts decreased in soils inoculated with high inoculum levels of both Fusaria. On the other hand, increasing the conidia concentration of T-35, in soil, resulted in a decrease in rhizosphere coloniztion by F. oxysporum.

In soil infested with F. o. melonis, Trichoderma treatments had only a slight effect on the survival of F. oxysporum in non-rhizosphere soil. However, application of T-35 as a wheat bran-peat preparation, conidial suspension or seed coating, significantly reduced the pathogen counts in thizosphere soil of melon and cotton. The minimal effect of T-35 on population densities of F. oxysporum in non-rhizosphere soil further suggest a competitive mechanism in biocontrol (2). These findings are in

correlation with the lack of mycoparasitic and antibiotic interactions between T-35 and F. oxysporum in vitro (Sivan and Chet, unpublished). The great reduction of the pathogen population densities in the rhizosphere soil could be a result of a lower proliferation rate of the pathogen in a rhizosphere already colonized by the antagonist. However, this does not exclude other biocontrol mechanisms from being involved in the rhizosphere.

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LEGEND TO FIGURES

Fig. 1. The effect of pre-germinated conidia of Trichoderma harzianum on chlamydospore germination rate of Fusarium oxysporum f. sp. melonis, after 22 hr of incubation in soil enriched with chlamydospores and amended with glucose and asparagine (5:1 ; w/w). Conidia of T. harzianum were applied at concentrations of 10^3 ( ) and 10^6 ( ) conidia per g soil and compared with the untreated control ( ). Germination values, at each level of nutrients marked with an asterik are not significantly different from the control (P=0.05).

Fig. 2. Chlamydospore germination of Fusarium oxsysporum f. sp. vasinfectum A and F. oxysporum f. sp. melonis B in cotton and melon rhizosphere soil ( ), respectively, as compared with the germination rate in non-rhizosphere soil ( ). Brackets indicate the S.E.

Fig. 3. Rhizosphere colonization, of several crops, by Trichoderma harzianum (T-35), applied as a seed coating, in A, irrigated and B, non-irrigated soil.

Fig. 4. The effect of cotton seed coating with Trichoderma harzianum (T-35) on A, rhizosphere soil population of T-35 and B, Fusarium spp. (B) after 8 days of incubation in soil inoculated with microconidia of F. oxysporum f. sp.vasinfectum (10^3/g soil).

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Fig. 5. The effect of microconidial concentration, in soil, of Fusarium oxysporum f. sp. melonis on colonization of melon rhizosphere by Trichoderma harzianum after its application as a seed coating.

Fig. 6. The effect of Trichoderma harzianum (T-35) conidial suspension concentration on colonization of melon rhizosphere by Fusarium oxysporum f. sp. melonis.

TABLE 1. Effect of addition of *Trichoderma harzianum* on chlamydospore germination of *Fusarium oxysporum* f. sp. *melonis* (FOM) and *F. oxysporum* f. sp. *vasinfectum* (FOV) in rhizosphere and non-rhizosphere soil

	Clamydospore germination (%) (u)			
	Rhizosphere soil (v)		Non-rhizosphere soil (w)	
Trichoderma treatments	FOM	FOV	FOM	FOV
None	43.5 a (x)	50.3 a	39.1 a	25.1 a
Seed coating (y)	25.3 b	33.1 b	19.3 b	12.1 b
Conidial suspension (z)	28.1 b	34.5 b	15.8 b	7.5 c

(u) Counted under U.V. light microscope using Calcofluor.

(v) Melon and cotton seeds were germinated for 48 hr in soil enriched with chlamydospores of *F. o. melonis* and *F. o. vasinfectum*, respectively.

(w) Soil enriched with chlamydospores of *F. oxysporum* and amended with 0.05 and 0.01 mg glucose and asparagine per g soil, respectively.

(x) Values of each column, followed by the same letter, do not differ significantly (*P*=0.05) according to Duncan's multiple range test.

(y) 7.5×10^4 and 5×10^4 conidia of T-35 per each cotton and melon seed, respectively.

(z) 1×10^6 pre-germinated conidia of T-35 per g soil.

TABLE 2. Effect of germinating seed exudates of cotton and melon on the inhibitory effect of *T. harzianum* on chlamydospore germination of *F. oxysporum* and on the disease control of Fusarium wilt of cotton.

Trichoderma treatments	(v) Exudates	Chlamydospore germination (%) (u) FOV	FOM	Wilted cotton plants (w)
None	Not added	24.5 ab (x)	23.8 ab	73.8 ab
	Added	29.8 a	27.1 a	85.9 a
(y) Conidial suspension	Not added	12.4 c	10.1 c	33.6 c
	Added	22.1 b	19.3 b	68.9b
(z) Seed coating	Not added	14.7 c	13.1 c	39.1 c
	Added	23.4 b	25.6 a	75.1 ab

(u) Counted under U.V. light microscope using Calcofluor

(v) Exudates were produced from 800 germinating seeds. The calculated amount of exudates produced by 10 seeds was added to each replicate (0.5 Kg soil sown with 10 seeds) at sowing date and once every 3 days, until the end of the experiment (twenty days after sowing).

A. Calculated amount of exudates produced by 3 cotton or melon seeds were mixed with 50 g of soil enriched with chlamydospores of *F. o. vasinfectum* or *F. o. melonis*, respectively.

(w) Under greenhouse conditions in soil infested with microconidia of *F. o. vasinfectum* (2×10^6/kg soil).

(x) Values of each column followed by the same letter, are not significantly different (P=0.05) according to Duncan's multiple range test.

(y) 1×10^6 conidia of T-35 per g. soil.

(z) 7.5×10^4 and 5×10^4 conidia of T-35 per each cotton or melon seed, respectively.

TABLE 3. Effect of *T. harzianum* on *Fusarium* spp. population in melon rhizosphere and non-rhizosphere soil[r].

	Colony forming units/g soil x 10^2			
	Trichoderma spp.[s]		*Fusarium* spp.[t]	
Trichoderma treatments	Rhizosphere soil[u]	Non-rhizosphere soil[v]	Rhizosphere soil	Non-rhizosphere soil
None	4.2 c[w]	1.5 d	1900 a	920 a
Seed coating[x]	480 b	9 c	35 b	750 a
Conidial suspension[y]	51000 a	20000 b	41 b	30 b
Preparation[z]	55000 a	55000 a	43 b	35 b

(r) The soil was inoculated with $2x10^8$ microconidia/g soil, planted with melon seeds and placed in the greenhouse for 30 days.

(s) Counted on TSM (12)

(t) Counted on Fusarium selective medium (15) amended with 2 mg/L of benomyl.

(u) The plants were gently uprooted and the soil adhering to the roots was collected.

(v) Combined soil samples from distances greater than 1 cm from the roots.

(w) Values of each column followed by the same letter are not significantly different (P=0.05) according to Duncan's multiple range test.

(x) $7.5x10^4$ conidia of T-35 per seed.

(y) $5x10^6$ conidia of T-35 per g soil.

(z) Wheat bran-peat (1:1; v/v) preparation of T-35 consisting of $5x10^6$ cfu/g (d. wt.) was introduced into soil at a rate of 5 g/kg soil.

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