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Biological control of plant diseases using microorganisms grown on agricultural forestry wastes

by

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Table of Contents

	Page
Chapter I	
Studies in Israel and Cooperation	1
The role of competition for nutrients in biocontrol of *Pythium* damping off by bacteria	3
Chapter II	
Studies undertaken in Ghana	32
Chapter III	
Studies undertaken in Israel	52
The control of *Rosellinia necratix* in soil and in apple orchard by solarization and *Trichoderma harzianum*	52
The possible role of competition between *T. harzianum* and *Fusarium oxysporum* on rhizosphere colonization	73

Project Title: Biological Control of Plant Diseases Using Microorganisms Grown on Agricultural and Forestry Wastes.

Objectives of Project

The objectives of the project are as follows:

- To isolate potential biocontrol agents such as *Trichoderma* spp. or bacteria from local soils and to test their antagonistic ability against plant pathogenic fungi.
- To grow the biocontrol agents on various agricultural wastes such as baggase or cassava, wood waste such as sawdust, chips and bark fortified with nitrogen and phosphorus.
- To determine the most economic and appropriate methods of application of these antagonists either to soil or on seeds of the plant.
- To scale up the system in a semi-solid fermentation and to test the spectrum of effectiveness under field conditions against the most common plant diseases.

Chapter I
Studies in Israel and cooperation

Isolation of potential biocontrol agents

Soil samples were sent to Israel for isolation of biocontrol agents. Four strins of *Trichoderma* spp. and one bacterium were isolated from these soils.

Work started in our laboratory for the quantitative estimation of Trichoderma spp. in some cultivated soils in Ghana. So far isolations have been made from soils on which tomato, cowpeas and garden eggs have been cultivated. The Trichoderma selective medium (TSM) is being used for the isolations. Isolates obtained from this study will be screened for antagonistic properties against plant pathogenic fungi.

Incidence of Rhizoctonia solani and Sclerotium rolfsi Diseases in selected crops

A study was initiated to determine the incidence of R. solani and S. rolfsi diseases in tomato, cowpea and garden eggs in some nurseries and fields.

The investigation will give information on the magnitude of the problem and also provide strins of the fungi for the study of antagonism between them Trichoderma spp. and other biocontrol agents.

Antagonism between Trichoderma spp. and some soil borne plant pathogens

Work started on the antogonism between Ghanaian and the Israel isolatesof Trichoderma spp. and a strain of Sclerotium rolfsi isolated from cowpeas. The test is being conducted using tomato as the indicator crop.

STUDIES UNDERTAKEN IN ISRAEL

THE ROLE OF COMPETITION FOR NUTRIENTS IN BIOCONTROL OF *PYTHIUM* DAMPING OFF BY BACTERIA

ABSTRACT

Of the 130 bacteria isolated from the rhizoshpere of *Pythium* infested plants, 6 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 *P. aphanidermatum* oospores 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 soil by 60-75%. P. ultimum damping off in cucumbers was reduced by up to 94%. Integrating low amounts of bacteria with the fungicide Previcur 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.

INTRODUCTION

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

Biological control, by means of bacteria, of this pathogen is not well developed. Mitchell and Hurvitz (15) protected tomato seedlings against damping off caused by P. debaryanum, with a lytic strain of Arthrobacter. Howell and Stipanovic (9) isolated a strain of Pseudomonas fluorescens, which produces an antibiotic inhibiting growth of P. ultimum on seeds.

Rhizobacteria have been the subject of several reports (11,12,16). It was suggested, that the antibiotics produced by these bacteria displace deleterious microorganisms, presumably minor pathogens (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 to investigate their antagonistic mechanism.

MATERIALS AND METHODS

Isolation and characterization of bacteria. About 130 bacteria were isolated from roots of bean, cotton, radish, cucumber and melon plants, grown in Pythium spp. infested soils or peat vermiculite growth media. Isolations were made on nutrient agar (NA) (Becton-Dickinson Corp. Cockeysville, MO 21030) or King's Agar (KB) (10). Bacteria were identified by the API system (La Balme les Grottes 38390, Montalieu Vercieu, France). The following isolates were: Pseudomonas putida (805,310), P. Cepacia (808, 814, Y11) and Alcaligens sp. (Rh2) and Pseudomonas 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.

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 b-N-acetyl-D-glucosaminidase (Chitinase, E.C. 3.2.1.30), were tested in filtrates of bacteria, grown on either laminarin (Sigma Chemical Co., St. Louis, MO 63178), chitin (Sigma), cell walls or mycelium of

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Pythium aphanidermatum, 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 3000 g after which the supernatant was sieved through a 0.45 u millipore. The cell free medium, diluted with fresh, double concentration of PDNB, was inoculated with a mycelium disk taken from a 48 hr old culture of P. aphanidermatum. After 48 hr incubation at 28 C, the mycelium was dried for 4 days at 60 C and weighed.

Mycelium mats removed from P. aphanidermatum cultures, 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 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. Roux bottles, each containing 100 ml of carrot medium (18), were inoculated with mycelial disks, taken from the margin of a 48 hr culture of P. aphanidermatum, and incubated for two months at 30 C. Mycelial mats, containing oospores, were placed on four layers of cheesecloth, washed with running distilled water for 5 min to remove the medium, suspended in tap water and homogenized in a Waring blender for 5 min. Oospores, separated on a 40 um Nitex nylon screen (Tetko Inc., Elmsford NY 10513), were resuspended in 100 ml of H_2O and added to 1 kg air-dried,

well-mixed loamy sand soil whose characteristics have been described by Hadar et al., (6). The soil was then placed in plastic bags, incubated for 2 weeks at 30 C, sieved through a 2 mm screen and stored at 4 C until needed. This soil contained an average of 4.5 x 10 colony forming units (cfu) of P. aphanidermatum per gram, as determined on a selective medium according to Schmitthenner (22). Colonies originated predominantly from oospores.

Oospore germination tests in soil. Bacterial isolates, 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% (-0.3 bar) water content. Soil was thoroughly mixed 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 08805) according to Scher and Baker (20) and viewing at x 160 under a UV light microscope (Zeiss, W. Germany) for fluorescence observation. 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 cv 'shimshon'), tomato (Lycopersicum esculentum L cv 'Marmand Rehovot'), pepper (Capiscum annum L cv 'Maor'), melon

(Cucumis melon L cv '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 oospsore germination was assessed according to the procedure mentioned above.

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

In most experiments with both Pythium spp., infested peat was used as inoculum, after replantings were carried out to give 100% of diseased cucumbers. Inoculum was suspended in 500 ml H_2O to allow fine mix with the growth medium. Average level of Pythium, as counted on Schmitthenner's selective medium (22), ranged from $5 \times 10^3 - 5 \times 10^4$ cfu/g 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 tested isolates, drying at room temperature and planting in the greenhouse.

Polypropylene boxes (7 x 19 x 14 cm) were filled with the infested media, planted with 10 seeds each, irrigated once a day and

the number of diseased seedlings was recorded. All experiments were conducted under greenhouse conditions at 28 - 32 C, consisted of six replicates and were repeated at least twice. Severity of disese symptoms in cotton was calculated according to an index ranging from 0 for healthy symptomless plants to 4 for plants covered with Pythium scars over more than 80% of their hypocotile. Preemergence damping-off was calculated by deducting the rate of emerged plants in a certain treatment from the average emergence in Pythium free medium.

The fungicide prothiocarb (Previcur, S-ethyl N-(3-dimethylaminopropyl) thiocarbonate (50%) (Schering, A.G., Berlin, W. Germany) was mixed with PVM alone or combined with bacteria suspensions to control diseases caused by P. aphanidermatum in cucumbers.

Density counts and bacterial populations. Strains of the biocontrol agents resistant to Rifampicin (3-(4-methylpiperazinyl-minomerhyl) rifamycin 5v) and nalidixic acid (Sigma) were derived from parent cultures by planting cell suspensions on NA containing 150 µg/ml Rifampicin and nalidixic acid and incubating them for 48 hr at 30 C.

Counts of bacteria from soil or peat were carried out by plating the appropriate dilutions, using the drop plate method (17), on agar medium. Results were expressed as colony forming units per g dry soil (cfu/g). Rhizosphere populations were assessed by separating the soil, adhering to the specific root segments, in water, after shaking them in a rotary shaker for 30 min at 100 rpm. Average dry weight of rhizosphere soil was measured in 20 samples of each root segment according to the distance from the root neck. Population dynamics

along cucumber roots were tested by the rhizosphere competence technique of Scher et al (21) as modified by Jaleed and Baker (personal communication). Two longitudinal halves of a 50 ml conical polypropylene tube (Falcon Div. Becton Dickinson and Co., Oxnerd, CA. 17030) were filled with wet sandy loam soil (15% - 0.3% bar), and one with bacteria coated seeds was placed between both parts, 1 cm away from the top. The two parts were sealed with 2 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 above.

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 *Pythium aphanidermatum*. Of the 130 bacterial isolates tested for their ability to suppress damping-off in cucumbers, the six which were superior, reducing disease incidence by 17-67% (Fig. 1), were selected for further experiments.

Antagonistic activity of bacteria in culture

Bacteria were tested for the following antagonistic activities: Lytic enzymes - All bacterial isolates produced neither β-1,3-glucanase, chitinase, nor cellululase when grown on laminarin, chitin, cellulose or cell walls of *P. aphanidermatum*. Similarly, when

inoculated into liquid medium containing a mycelium mat of P. aphanidermatum as a sole carbon source, none of the bacteria degraded the mycelium.

Inhibitory substances - Bacteria were separated from the potato dextrose nutrient broth (PDNB) medium, in which they were grown, after 40 hr. The cell free media, mixed with a double concentration of PDNB, inoculated with P. aphanidermatum and incubated for 48 hr for dry weight determination. Dry weight of mycelium grown in non-amended control medium was 84.4 mg per plate. Bacterial Isolates No. 805, 808 Rh2 and 310 inhibited P. aphanidermatum growth by 87.9, 85.4, 49.8 and 39.0% respectively, while isolates AA4, 806 and SS3, which are not biocontrol agents of Pythium, reduced it by 76.5, 89.6 and 70.4%, respectively, as compared with the control (dry weight 84.4 mg/plate), grown in non-amended medium. P. aphanidermatum growth was inhibited by only 5.7% when grown in a medium which previously supported growth of the same fungus.

Competition - Competitive ability of both the bacteria and Pythium mycelium 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. Isolates 805, 808, Y11, Rh2, 310 and 814 inhibited fungal growth by 50,45,60,98, 14 and 62%, respectively, compared with the control (dry weight 63.5 mg/plate), grown in bacteria-free medium. However, other bacteria when grown together in liquid culture also inhibited P. aphanidermatum growth.

Inhibition of oospore germination by bacteria.

Bacteria were introduced into raw soil enriched with oospores of P. aphanidermatum. Oospore germination, determined after 24 h of incubation, was inhibited by up to 57% by the biocontrol agents (2 x 10^8 cfu/g soil) compared with only 13-20% inhibition by bacteria not effective in disease control under greenhouse conditions (Fig. 2). These results reduced by 20-50% by lowering the initial population level of the bacteria to 2 x 10^7 cfu/g soil.

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

To determine whether the bacteria produce inhibitory substances affecting oospore germination, cell-free supernatant of the different bacteria cultures 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 were compared with their germination in 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 3.3%.

Biological control of damping-off in cucumbers

Effect of bacterial concentrations. Bacteria were suspended in water and mixed with PVM to give two high initial population levels: 10^7 and

10^8 cfu/cm^3 Isolates 805 and 808 were the most effective in Pythium control 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. As shown in fig. 3 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. 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.

Effect of bacterial seed coating and intergrated control - Cucumber seeds were dipped in bacterial suspensions (8×10^9 cfu/ml) and planted in PVM. Damping off caused by P. aphanidermatum, was recorded during 12 days post sowing. The most efficient isolates - 805, 808 and Rh2, reduced disease incidence by 57-67% compared with 38-42% by isolates 814 and 310 (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 Previcur, at the rate of 0.25 ml per liter PVM. Isolates 805 and 808, alone, decreased Pythium damping-off by 9-15%, due to the low bacterial level in the growth medium (Table 3). A significant decrease in disease incidence, 34%, was obtained by the chemical treatment. However, the combination of Previcur with isolate

808 was signficantly superior, yielding an 80% reduction in disease incidence.

Effect of bacteria on disease build up 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, Pythium disease incidence which was 93% at the end of the first cycle, stabilized on 50% between the third and fifth growth cycles (Fig. 5). Disease incidence in bacteria-treated media was 12-45% during the five successive plantings.

Biological control of P. ultimum damping-off 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 on the first growth cycle (Fig. 7). However, after two weeks 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 Pythium disease in several crops was examined in the greenhouse. Both isolates reduced disease incidence in melons, tomatoes, peppers and beans (Fig. 6). Disease indexes in cotton, calculated according to root coverage by Pythium scars 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, selected for their tolerance to 150 ug/ml of the antibiotics rifampicin and nalidixic acids, were applied to soil. Both decreased Pythium in cucumbers by 50%. Efficient recovery 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 Pythium infested soil 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 root neck. Serial dilutions were performed for assessment of bacterial population in the rhizosphere. General population, along the untreated roots, graduated between 2.0×10^6 - 2.2×10^5 cfu/g soil in the 0-1 and 6-7 cm segments (Fig. 8). Application of isolates 814, Y11 and Rh2 to the seeds decreased the general bacterial population to 4×10^3 - 2.3×10^5 cfu/g soil in segments 0-1 - 4-5cm, whereas isolates 805 and 808 decreased it to 10 cfu/g soil in segments 2-3 and 4-5 cm. However the general population of the tips of treated roots did not differ from that of untreated ones.

The specific population of bacteria applied to seeds before incubation was 2.2×10^1 - 2.0×10^6 cfu/g of rhizosphere soil (Fig. 8). 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 level of general population and that of the biocontrol agents in all the root segments, except for the tips.

Colonization of cucumber roots by P. aphanidermatum was reduced by 50-100% in the presence of the biocontrol agents.

DISCUSSION

A major objective of the present study was to isolate potential bacteria and characterize their activity in controlling Pythium spp. The ability of six bacteria applied into peat vermiculite mixture 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). Moreover, the bacteria were most effective in controlling P. ultimum (Fig. 7). One application of the biocontrol agents was sufficient to prevent disease build up in replanted cucumbers (Fig. 5). The additive control achieved by combining bacterial application, at a rate too low to achieve disease control, with the fungicide previcur (commonly used against Pythium), reduced disease incidence by 80% (Table 3).

Our results indicate that the presence of bacteria along roots of susceptible hosts reduced the establishment of the pathogen in its potential sites of attack.

Damping-off, induced by Pythium spp., was successfully controlled by coating seeds with a variety of antagonistic microorganisms (7,8,9,13,24). In this work, seed coating was as effective as direct application of bacteria into the soil (Fig. 4). Nevertheless, seed treatment is an attractive method for introducing bacteria to the

soil-plant environment, since in this way, the bacteria have an opportunity of being the first colonizers of roots (Fig. 8). It was evident that bacteria, introduced as seed treatments, moved along the roots. Weller (26) showed the distribution of Pseduomonas fluorescens introduced via seeds on seminal roots of winter wheat . Similarly, we have counted higher populations near the seed or at root tips. Moreover, the lower counts of endogenous bacteria obtained in wheat (26) and cucumber (Fig. 8), in the presence of the introduced strains indicate the obvious competition between them. Intense competition for resources such as nutrients or space between 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 establish a large population in the rhizosphere is a crucial factor which determines the importance of rhizobacteria as root associates.

Competition for carbon or nitrogen between the efffective bacteria and germinating oospores of P. aphanidermatum may be the mechanism involved in disease control. 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 rhizosphere of several plants was achieved by the biocontrol agents.

Oospore germination has already been shown to be affected by exogenous nutrients (9). Nutrients supplied by root exudates play the same role, as exogenous glucose, on oospore germinability in the rhizosphere. It, therefore, appears that bacteria compete with

germinating oospores for available carbon or nitrogen sources and by eliminating these resources, the bacteria reduce the percent of oospore germination. Similar results were recently obtained with chlamydospores of Fusarium spp. (3). Moreover the reduction in the general poulation of bacteria along the roots, caused by the biocontrol agents, is probably due to competition (Fig. 8).

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.

According to Baker (1), bacteriazation is not a very successful method of biological control in non sterilized soils. One of the reasons for this is the fact that microorganisms present around roots and showing antibiosis in vitro, were not necessarily those effecting biological control in soil. Broadbent et al. (2) added B. subtilis to soil treated by aerated steam. P. ultimum was controlled on Antirrhinum possibly by hindering infection rather than by antibiosis. Hadar et al (7) protected seedlings from Pythium disease by application of Enterobacter cloacae. Howell and Stipanovic (9) concluded that a strain of P. fluorescence was antagonistic to P. ultimum due to the production of an antibiotic - pyoluteorin - by the bacterium.

We have shown here the potential of bacteria to control Pythium. The bacteria applied as a seed coating or mixed with the growth medium

compete with the pathogen along the roots. Further research is needed to develop the formulation of bacterial biocontrol agents and exploit them in agriculture.

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Table 1: Inhibition of oospore germination (%) by bacteria in rhizosphere of various crops*

Bacterium Isolate	CROP					
	Wheat	Tomato	Melon	Cucumber	Bean	Cotton
805	89a[y]	71a	83a	66a	68a	59a
808	52b	70a	74a	67a	71a	67a
AA4	36c	5b	11b	20b	12b	25b
Percent of germination in untreated control	64	37	46	57	65	36

* Seeds of the 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.

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

All percentages of germination in treatments of isolates 805 or 808 differed significantly from the non-amended control.

Table 2: Influence of bacterial concentration, in peat vermiculite medium, on their ability to control P. aphanidermatum in cucumber

Bacterium 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	10^7 cfu/cm^3
Untreated control	25		16	
Rh2	18	42	12	27
805	60	83	48	70
Y11	37	48	18	37
808	77	72	60	68
814	37	51	10	32
310	42	53	19	41

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.

Table 3: Effect of low population of bacteria combined with previcur on percentage of damping-off of cucumbers, induced by *P. aphanidermatum*.

Previcur treatment *	Bacterial isolate		
	None	805	808
-	89a[y]	81a	76a
+	59b	48b	18c

* Previcur was applied at a dose of 0.25 ml/l PVM.

Bacteria were applied at a rate of 3×10^6 cfu/cm^3 PVM.

y Numbers followed by a common letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

LEGENDS TO FIGURES

Fig. 1. The influence of bacterial suspensions on the percentage of healthy cucumber plants planted in P. aphanidermatum-infested PVM. Treatments were: control (o) and bacterial isolates 805 (▲), 808 (△), 310 (▽), Rh2 (●), Y11 (■) and 814 (□).

Fig. 2. The effect 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).

Reduction was calculated according to the following formula: $(1-\frac{A}{B})\ 100$ where B = percent of germination in the control without bacteria and A = percent of germination in bacterial treatments.

Fig. 3. The effect of isolates 805 (A) and 808 (B), applied at concentrations of -.- 2×10^6, -.- " 8×10^6 and -- 4 x 10 cfu/cm on percentage of healthy cucumber plants planted in PVM infested with P. aphanidermatum.

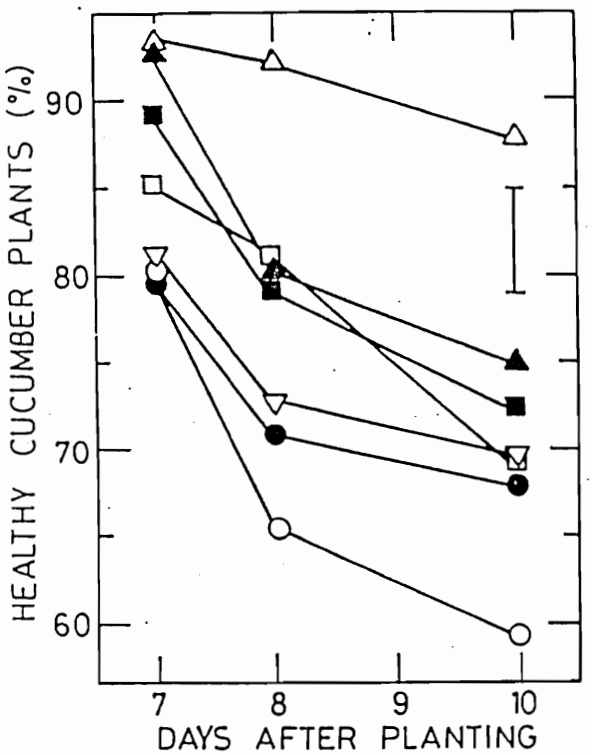
Fig. 4. Influence of seed coating with bacterial isolates 808 (△) 805 (▲), Rh2 (●), Y11 (■), 310 (▽) and 814 (□) on the percentage of healthy cucumber seedlings planted in P. aphanidermatum-infested PVM.

Fig. 5. Stand of cucumber plants, 14 days following each of 5 successive replants in PVM infested with P. aphanidermatum and treated once with isolates 805 (▲), 808 (△) or non-treated (O).

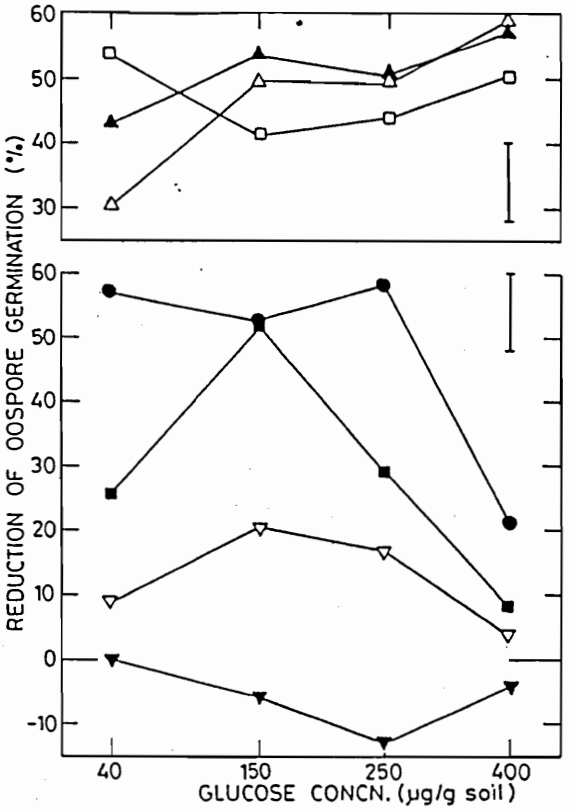
Fig. 6. Percent of healthy plants in PVM infested with P. aphanidermatum and treated with isolates 805 (▲), 808 (△) or not treated (O).

Fig. 7. Stand of cucumber plants in P. ultimum infested PVM treated with isolates 805 (▲), 808 (△), Rh2 (●) Y11 (■), 310 (▽), 814 (□) or non treated (O).

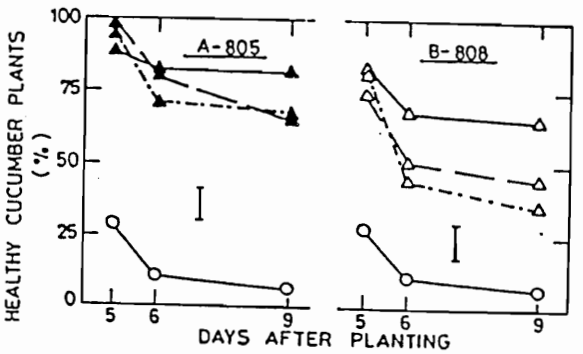
Fig. 8. Densities of general bacteria (A) and biocontrol agents (B) in the rhizosphere of 6 day old cucumber plants. Isolate 805 (▲), 808 (△), Rh2 (●), Y11 (■) and 814 (□) were applied to seeds planted between two longitudinal halves of plastic tubes filled with soil . (O - control).


HEALTHY CUCUMBER PLANTS (%)
90
80
70
60
7
8
9
10
DAYS AFTER PLANTING

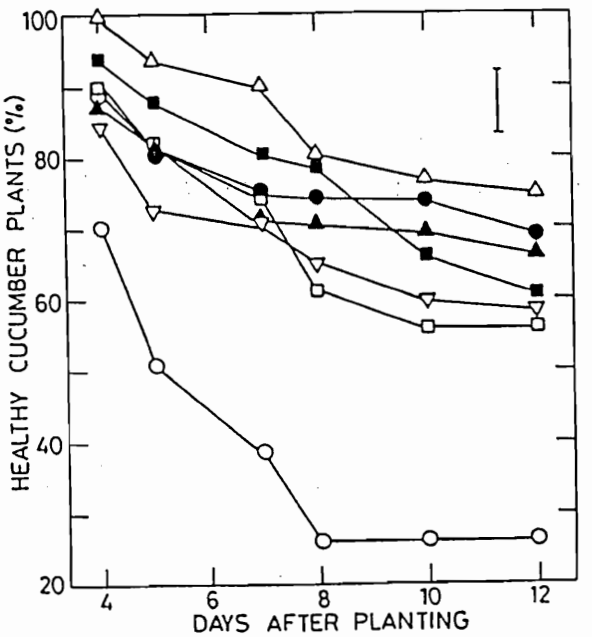
2


REDUCTION OF OOSPORE GERMINATION (%)
60
50
40
30
20
10
0
-10
40
150
250
400
GLUCOSE CONCN. (µg/g soil)

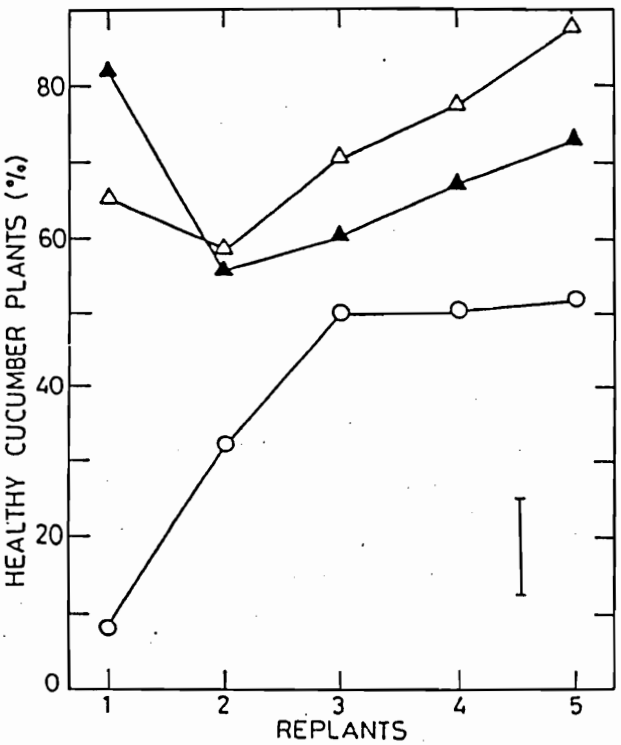
3


HEALTHY CUCUMBER PLANTS (%)
100
75
50
25
0
A-805
B-808
5
6
9
DAYS AFTER PLANTING

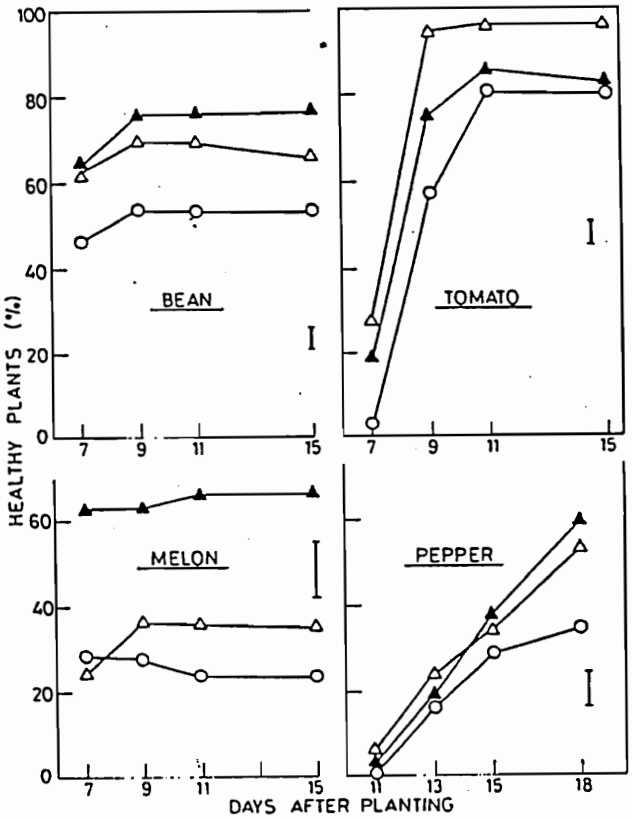
4


HEALTHY CUCUMBER PLANTS (%)
100
80
60
40
20
4
6
8
10
12
DAYS AFTER PLANTING

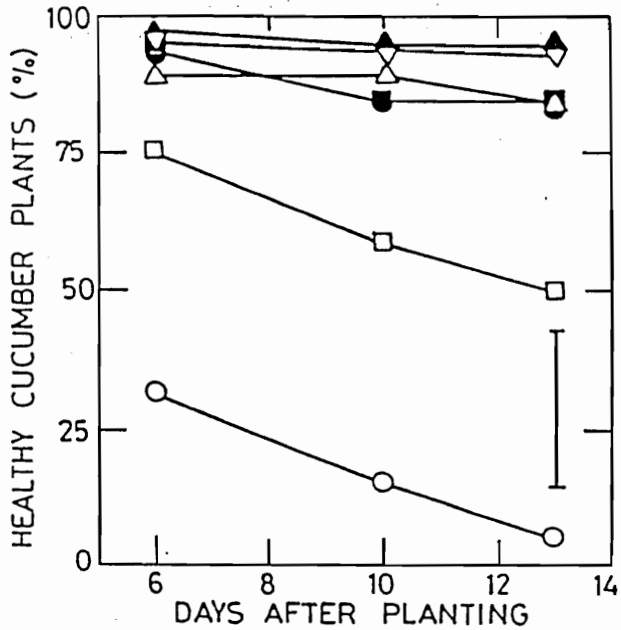
5


HEALTHY CUCUMBER PLANTS (%)
REPLANTS

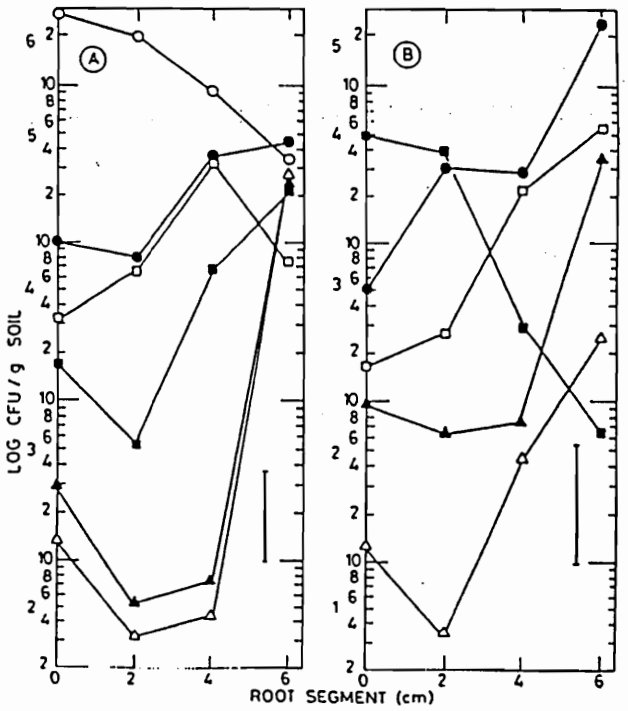
6


HEALTHY PLANTS (%)
BEAN
TOMATO
MELON
PEPPER
DAYS AFTER PLANTING

7


HEALTHY CUCUMBER PLANTS (%)
DAYS AFTER PLANTING

8


A
B
LOG CFU/g SOIL
ROOT SEGMENT (cm)

Visits

Prof. Ilan Chet paid a visit to Ghana from 4th to 10th April 1986.

During the period he gave a seminar to final year B.Sc. students in Biological Sciences, Agriculture, Forestry and Biochemistry on Opportunities in Agricultural Biotechnology A case study of Biocontrol using Trichoderma spp.

He held discussions with the Ghanaian team on programing, control, financing and procurement of equipment and supplies for the joint project.

He also gave a final seminar to the staff of the Forest Products Research Institute, the Institute for Renewable Natural Resources and Faculty of Agriculture on financing of research in Israel.

Dr. Ofosu-Asiedu paid a reciprocal visit to the Israel laboratory from November 14th to December 3rd 1986.

He discussed with Prof. Chet's team procedures and methodologies for conducting experiments in biological control. He was taken through methods of screening antagonistic micro-organisms, isolation of antagonists and preparation of bulk inoculum of Trichoderma spp. for laboratory and field experiments in biocontrol of plant pathogenic fungi.

Dr. Ofosu-Asiedu gave a seminar on "Nutrient Changes in Soils planted to Pure Culture inoculated Pines" to graduate students and staff of the Plant Pathology and Microbiology Department of the Faculty of Agriculture.

Prof. Chet and Dr. Ofosu-Asiedu held discussions again on the budget and procurement of equipment and supplies for the project.

He was shown laboratory experiments in progress and visited many places of interest in Israel.

Chapter II

During the last two years soils were brought from Ghana to Israel. Trichoderma spp. were isolated and tested for their antagonism to plant pathogens.

Mr. O. Kleifeld travelled to Ghana and spent several weeks in Dr. Ofosu-Asiedu's laboratory. He worked with students and technicians in Kumasi, conducting experiments on biological control.

During the last year, Dr. Ofosu-Asiedu visited in Israel and studied some techniques. He also reported on the current work of his group.

It is planned that in 1988 Professor Chet will visit the Institute in Kumasi, present lectures and teach microbiological techniques regarding microbial interactions and mycoparasitism.

The enclosed report summarizes the work carried out in both countries accordingly.

STUDIES UNDERTAKEN IN GHANA

Evaluation of diseases caused by Rhizoctonia, Sclerotium and Pythium spp. in which there was some crop detection of damping-off and root rots in tomato.

This study was conducted to:

a) Assess the damage caused by damping-off and root rots in tomato raised in various soils.

b) Isolate fungi from the diseased plants and

c) Correlate the incidence of disease with soil type.

Three soils supporting different vegetations Forest, Grass and Crops were collected for the study and are described as soil types. Plastic boxes measuring 22.50 x 12.50cm were filled with the soil. Twenty-eight seeds of the tomato var. Heinz were sown per box, at a spacing of 1x1cm and watered daily. Each treatment was replicated four times. The plants were observed daily for damping-off. Diseased plants were removed, washed, sterilised and plated on cassava-dextrose agar (C.D.A.). The plates were incubated at 30°C and any fungi growing on them were sub-cultured on CDA and then later identified.

There was a high survival of plants in all the soils and little damping-off occurred 14 days after the emergence of the seedlings. Of the total of 112 seedlings per treatment 93% survival was recorded in the forest soil, 90% survival in the grass soil and 87.5% survival in cultivated soil. Fungi isolated from the diseased seedlings were mainly *Sclerotium rolfsii*. Damping-off could mainly be caused in these soils by this fungus.

Survival of plants was closely correlated with the soil type on which the seedlings were raised.

Incidence of disease in tomato transplants grown on continuously cropped soil

Since there was low disease incidence in the tomato seedlings raised in the three soils, the experiment was continued by transplanting seedlings from the 3 soils into sandy loam soil continuously cropped to tomato and with a history of tomato root rot. An attempt was made to monitor the behaviour of the transplants on this soil.

Black polyethylene bags were filled to 3/4 full with the continuously cropped soil and 14 days old seedlings with a ball of soil were transplanted into them. Six seedlings were planted in each polyethylene bag. Each treatment was replicated eight times. The experiment was arranged in a complete randomised design. Transplants were watered daily and observed for disease incidence. At each inspection period diseased plants were roqued out, counted and isolations made from them.

Disease appeared in the plants seven days after transplanting but it was at its peak at flowering stage (Fig.1) when the experiment was terminated because all the plants were showing symptoms of the disease.

Transplants from forest soil(Table 1) were least attacked giving a high percentage survival. On the other hand transplants from cultivated soil were heavily diseased whilst those from grass soil were intermediate between the two.

Isolation of fungi from diseased tomato transplants

From each treatment 50 apparently healthy and 50 diseased plants were selected at random for the isolation of fungi.

The plants were thoroughly washed in running tap water, cut into 1-2cm portions of stem, root collar and root, washed in 3% solution of detergent(teepol) and immersed for 2 to 3 minutes in 50% ethanol. The portions were rinsed in sterile distilled water, plated out on CDA, potato dextrose agar (PDA) and nutrient agar(NA) and incubated at 30°C for 10 days.

Colonies appearing on the media were counted, sub-

cultured on C.D.A. and identified.

There were no differences in the number of colonies on the different media(Table II). Only a single colony grew out of the stem portions but most of the colonies came from the root portions.

Apart from a few isolates of bacteria all the colonies were identified as Sclerotium rolfsii of which 69.23% were isolated from the root portions of the plant. Root rot in the tomato transplants was caused mainly by S. rolfsii.

Populations of Trichoderma spp in soils used for detection of diseases

Population of Trichoderma spp in the four soils used for detecting diseases were counted on the Trichoderma selective medium (TSM) and correlated with the survival of tomato transplants in the continuously cropped soil.

One gram of soil was weighed and put into 10ml of water. Serial dilutions of 10^{-1}. 10^{-2}, 10^{-3}, 10^{-4} were prepared for each soil type. One ml aliquots were dispensed into 9cm petri dishes containing TSM. Each dilution was replicated 3 times. The plates were incubated at 30°C and colonies appearing on the medium were counted on the 7th day of incubation using a colony counter.
The results were analysed using the analysis of variance model for a complete randomised design. The relationship between Trichoderma counts and the survival of transplants on continuously cropped soil was determined using a simple correlation model.

T.S.M. did not only support Trichoderma spp but also other fungi like Fusarium spp and Aspergillus spp appeared on it. At the dilution of 10^{-1} there were too many colonies on the medium which made counting difficult. However, because of the low population of Trichoderma in the continuously cropped soil population count was possible at a dilution of 10^{-1}.

High Trichoderma counts (Table III) were obtained from forest soil followed by grass and cultivated soil. The difference between the populations was significant at 5% level of significance. As cultivation increases the population of Trichoderma spp in the soil appears to decrease.

The correlation between Trichoderma population in the soils and percentage survival of tomato transplants in continuously cropped soil (Table IV) was determined using the correlation

equation $$r = \frac{N\Sigma xy - \Sigma x \times \Sigma y}{\sqrt{(N\Sigma x^2 - (\Sigma x)^2 (N\Sigma y^2 - (\Sigma y)^2)}}$$

where r = correlation coefficient

N = number of treatments

K = independent variable, total Trichoderma count

Y = dependent variable: % survival.

There was a high positive correlation between Trichoderma population in the soil and plant survival in the continuously cropped soil (Table IV) with a correlation coefficient of r = 0.97. Where the original soil for raising seedlings had a high population of Trichoderma spp

the transplants appeared to survive better on the highly infested continuously cropped soil. It is likely that the soil adhering to the transplants carry with them a protective ball of Trichoderma infested soil which probably protected them for a period.

Test of antagonism between Trichoderma spp and strains of Sclerotium rolfsii on different media

This study was to test the antagonism between two species of Trichoderma, hamatum from Ghana and harzianum from Israel and two strains of S. rolfsii from Israel and Ghana on different nutrient media.

The antagonist Trichoderma and the pathogen Sclerotium were inoculated at the opposite sides of 9cm petri dishes containing either dehydrated potato dextrose agar (PDA I) fresh potato extrose agar (PDA II) or cassava dextrose agar(CDA). The inocula were 5mm disks from well grown colonies. Each pairing was replicated three times. The plates were incubated at 30°C and examined for antagonism after 5 and 10 days. The features examined were the rate of growth of the antagonist and the pathogen, formation of inhibition zone and colour production at the boundary between the pathogen and the antagonist.

Table V gives the summary of the development of antagonism between the two organisms.

The paired fungi behaved differently depending upon the medium on which they were paired but generally the antagonist grew faster than the pathogen especially on fresh potato dextrose agar, where even the pathogen outgrew the

antagonist in the pairing between T. hamatum and S. rolfsii from Israel.

No clear zone of inhibition was produced when the organisms were paired on any of the media except with T. harzianum and S. rolfsii on fresh potato dextrose agar.

Whenever the antagonist and the pathogen met a colour was produced. However when T. hamatum was paired with isolates of S. rolfsii(Israel) on dehydrated potato dextrose agar no colour was produced. Also the pair between T. harzianum and the Ghana isolate of S. rolfsii did not produce any colour on CDA and PDA II.

Antagonistic activity depended to a large extent on the medium on which the test was conducted and was not uniform between the paired fungi. The antagonist and the pathogen from Israel exhibited strong antagonism when paired on PDA(1) but not on CDA. However fresh potato dextrose agar appeared to be the best medium for testing antagonism for all the fungi.

Mass production of Biocontrol agents on agricultural and forest wastes

The study was conducted to find the most suitable medium for the mass production of Trichoderma inoculum, determine the best combination of sawdust and other amendments such as maize bran, corn cobs, wheat bran, rice bran and assess the requirements for nitrogen in the growth medium.

Sawdust alone, combinations of sawdust and the amendments were prepared at the ratio of 1:0.25, 1:0:50, 1:0:75, 0:1 by weight. They were packed into 9cm petri dishes and autoclaved at 15lb for 30 minutes after adjusting their moisture levels by adding 15ml of water to the sawdust. There were 3 replicates for each treatment. Radial growth of the mycellium were measured on the 5th, 12th and 18th day after incubation.

Sawdust and maize bran proved to be the most suitable substrate and even in the lower additions to sawdust the fungus grew well and fruited continuously. Wheat bran combined with sawdust gave good growth of the fungus especially at higher ratios. The fungus could, however, not grow on wawa sawdust alone or on combinations of wawa sawdust with rice bran. The addition of maize bran or wheat bran improved the quality of the substrate even at low levels.

Heavy fruiting was observed on maize bran and wheat bran amended sawdust a feature that was most predominant in the higher proportion of the two amendments. The green colour of Trichoderma appeared earlier on maize bran amendments and the myceluim formed a heavy mat on the substrate.

In a second trial ground corn cobs, inexpensive agricultural waste, was compared to maize bran as an amendment to sawdust. High and low ratios were prepared and seeded with T. harzianum.

After 18 days incubation radial growth on corn cob amended sawdust was similar to maize bran amended substrates (Table VII). At low proportions of amendments to sawdust Corn cob appeared to support better growth. Initial growth was generally faster on the maize bran than on the corn cobs.

Fruiting started later on corn cobs but by the end of the experiment fruiting intensity was similar on both substrates.

Addition of 0.062 gram of corn cobs or maize bran is sufficient to induce good growth and development on the substrate since the quality of the medium was substantially improved.

Corn cobs could therefore be used as substitute for maize bran in the preparation of the substrate for mass production of *Trichoderma hamatum* inoculum.

Bio control of *S. rolfsii* infection in tomato by *Trichoderma harzianum*

The experiment was conducted to control *S. rolfsii* attack of tomato with *T. harzianum* and determine the rate of development of the disease.

Eight untreated seeds of the tomato variety Ayalon from Israel were sown in black polyethylene bags measuring 15x7.5cm filled with sterilized and unsterilized sandy loam soil. The soil was sterilized by autoclaving for 24 hours at 121°C on two consecutive days.

One set of the untreated soil was inoculated with a peat preparation of T. harzianum at 5gm per kilogram of soil. Another set of sterilised soil was inoculated with S. rolfsii at 50mg of sclerotia per kilogram of soil. A third set of sterilised soil was inoculated with both T. harzianum and S. rolfsii at the same dosages. In all there were four treatments and each treatment was replicated five times.

The plants were observed for 30 days from seedling emergence. Diseased plants were roqued out at every inspection, counted and expressed as percentage infection.

T. harzianum reduced the attack of S. rolfsii in both the unsterilized and artificially inoculated soil from 80% to 37.5% and 95% to 27.5%(Fig.2). Infection was lower in the naturally infested soil probably because of low inoculum potential of the pathogen. On the other hand the control of the disease in the naturally infested soil was poorer than in the artificially inoculated soil.

The disease developed faster initially in plants raised on the naturally infested soil but in the end there were more diseased plants in the artificially inoculated soil.

T. harzianum controlled to some extent the attack of tomato by S. rolfsii in sterilized and unsterilized soil.

Mode of Application of biocontrol agents

Application of *T. harzianum* as a biocontrol agent of *S. rolfsii* in tomato

The study was conducted to find the best method for applying *T. harzianum* for the control of *S. rolfsii* in tomato.

In this connection 2 methods of applying the biocontrol fungus were tested. These were:

1) Seed coating with conidial suspension and
2) Direct inoculation of soil with a peat preparation.

Ten grams of tomato seeds were coated with 3ml of conidial suspension containing 5 x 10^6/ml of *T.harzianum* conidia. The seeds were immediately dried in warm draft. Direct soil inoculation was done by applying 5g of peat preparation to 1Kg of soil.

S. rolfsii was inoculated at a concentration of 50mg of sclerotia to 1Kg of soil.

Seeds of Ayalon variety of tomato were used for the experiment. The soil was sterilized by autoclaving for 2 hours at 121°C in two consecutive days. Eight seeds were sown in a polythene bag full of soil. The plants were watered daily.

The following treatments were applied:

1. Control
2. Inoculation with *S. rolfsii*

3. Direct inoculation with T. harzianum
4. Seeds coated with T. harzianum
5. Direct inoculation of T. harzianum and S. rolfsii.
6. Seeds coated with T. harzianum and soil inoculated with S. rolfsii

Eash treatment was replicated five times.

Efficacy of the mode of application was assessed by emergence of seedling and dry weitht of the plants.

Coating of the seeds with a conidial suspension of T. harzianum was more effective in controlling pre-emergence damping off(Fig 4) than inoculating the soil directly with a peat preparation of T. harzianum. It decreased pre-emergence damping-off by 21% whilst direct inoculation reduced the disease by 11% in sterilised soil. In unsterilised soil seed coating with spore suspension reduced pre-emergence damping-off by 18%. In unsterilized and sterilized soil coating the seeds with spore suspension greatly increased the germinability of the seeds.

Directly inoculated plants produced heavier seedlings than seed coated plants (Table IX) even in treatments where the plants were also inoculated with the disease fungus.

Exchange of visits

During the reporting year exchange of visits were made between the two collaborating laboratories.

Mr. Offer Kliefeld from the Laboratory of Fungal Physiology of the Faculty of Agriculture, Hebrew University visited our Laboratory in Ghana. He spent a month conducting joint field and laboratory experiment with the Ghanaian group. He held general discussions on the project especially on the availability of minor items which facilitated and speeded up our research. He finally gave a lecture on the general concept of biological control.

Dr. Ofosu-Asiedu paid a reciprocal visit to the Israel Laboratory from mid September to mid October 1987 during which time he discussed progress of work with Prof. Chet and his group and the conduct of the field experiments in Ghana. He also learnt new methods in bio-control.

Table 1: Survival of tomato transplants on continuously cropped soil

Substrate	Total No.of seedlings	Number of seedlings surviving	% surviving
Forest soil	48	31	65
Cultivated soil	48	17	35
Grass soil	48	20	42

Table II: Number of fungal colonies from different parts of the plant on different nutrient media.

Part of plant	Colony count on media		
	CDA	PDA	NA
Stem	1	0	0
Collar	17	18	16
Root	39	40	38

Table III: Populations of <u>Trichoderma</u> spp in different soil dilutions.

Dilutions	Populations of Trichdorma in soils			
	Forest	Grass	Garden	Continously
10^{-1}	-	-	-	16
10^{-2}	112	93	83	11
10^{-3}	100	65	65	9
10^{-4}	99	57	57	6

Table IV. Total populations of *Trichoderma* spp in the different soils and transplant survival in the continuously cropped soil.

Soil type	Total *Trichoderma* population	Plant survival %
Forest	41	65
Grass	28	42
Garden	25	35
Continuously cultivated	21	18

Table V: Interaction between antagonist and pathogen on different media

Parings	C.D.A.			PDA II			PDA I		
	Interaction								
	R	I	C	R	I	C	R	I	C
Antagonist + *Pathogen*									
T. hamatum + *S. rolfsii*(G)	e	a	y	f	a	b	e	a	n
T. hamatum + *S. rolfsii*(II)	f	a	y	f	a	b	s	a	n
T. harzianum + *S. rolfsii*(G)	f	a	n	f	a	n	s	a	b
T.harizianum + S. rolfsii(I)	s	a	y	f	p	y	f	a	b

Legend R = rate of growth of anaagonist,(f) fast(3)slow (e) equal

I = Zone of inhibition, (p) present,(a)Absent

C = Colour at boundary (y) Yellow, (b) Brown No colour

Table VI. Development of *T. hamatum* on combinations of wawa sawdust and cereal brans.

Substrate	1:0	1:0.25	1:0.50	1.0.75	1:1	1:1
	Radial growth (mm)					
Sawdust + maize bran	0	85	87.50	88.50	90.00	88.50
Sawdust + Wheat bran	0	75	80.00	87.00	90.00	88.50
Sawdust + Rice bran	0	0	0	0	0	0

Table VII: Development of *T.hamatum* on combinations of wawa sawdust, maize bran and ground corn cobs

Substrates	Corn cobs + sawdust	Maize bran + sawdust
Ratios	Diameter (mm)	
1:0.031	50	45
1:0.062	71	80
1:0:125	85	90
1:0. 25	87	85
1:0. 50	90	87
1:0. 75	80	88
0:1	90	90
1:1	70	90
1:0	0	0

Table VIII: Incidence of *S. rolfsii* attack of tomato in sterilized and unsterilized soil inoculated with or without *T. harzianum*.

Days	% infection in uninoculated soil	% infection in uninoculated soil	% infection in sterilized soil inoculated with *S. rolfsii*	% infection in sterilized soil inoculated with *S. rolfsii* and *T. harzianum*
0	0	0	0	0
5	0	0	17.5	5.0
10	37.5	12.5	17.5	5.0
15	50.0	17.5	72.5	12.5
20	75.0	32.5	87.5	17.5
25	80.0	32.5	92.5	27.5
30	80	37.5	95.0	27.5

Table IX: Dry weight of seedlings of tomato from different treatments.

Treatments	Shoot dry weight(g)	Root dry weight(g)	Total dry weight(g)
1 Control	2.04	0.31	2.35
2 Inoculation with *S. rolfsii*	1.03	0.29	1.32
3 Direct inoculation with *T. harzianum*	3.00	0.87	3.87
4 Seeds coated with *T. harzianum*	2.56	0.51	3.07
5 Direct *T. harzianum* + *S. rolfsii*	2.81	0.62	3.43
6. Seed coated + *S. rolfsii*	2.03	0.39	2.42

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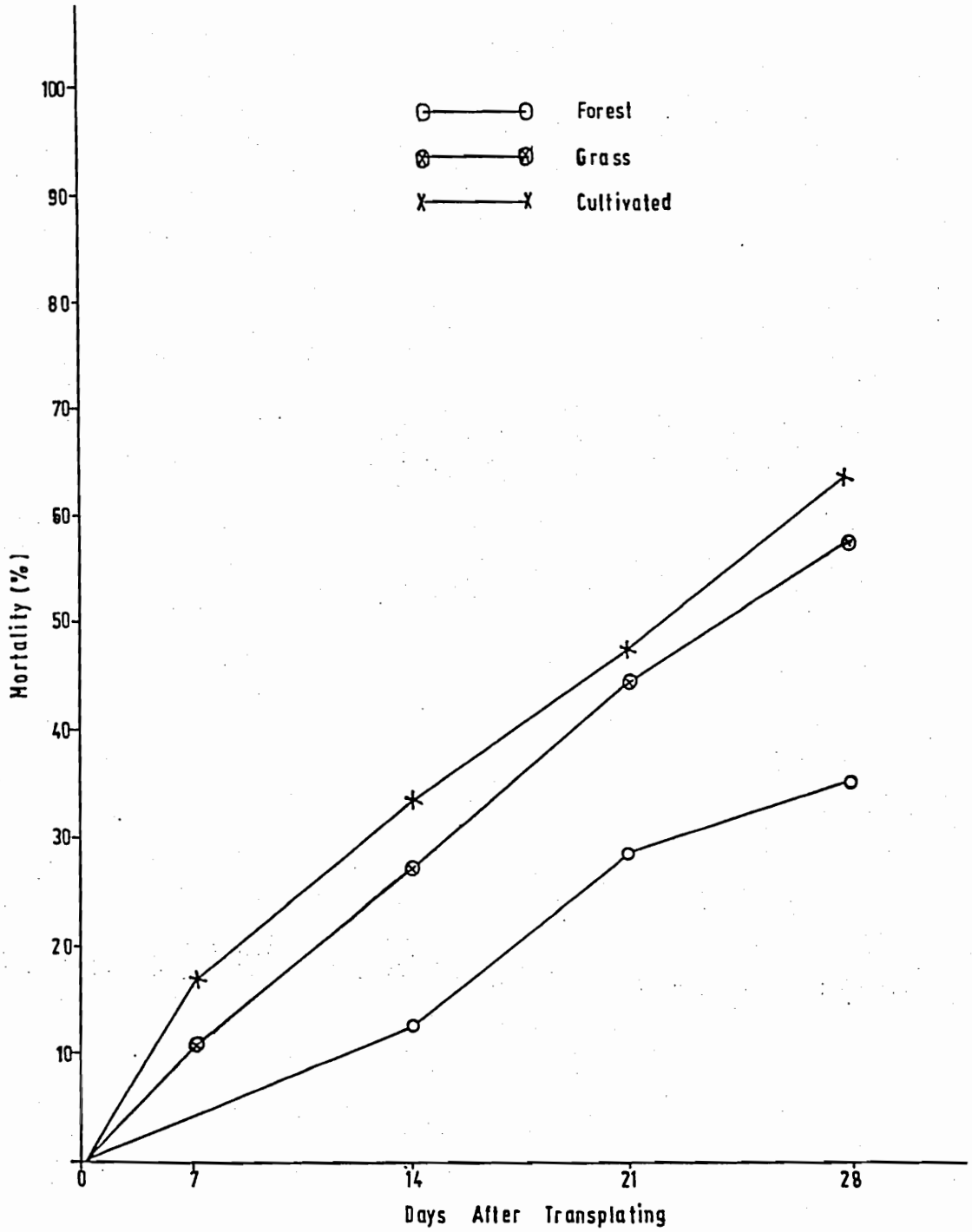

FIG 1 : DEVELOPMENT OF ROOT ROT IN TOMATO TRANSPLANTS IN CONTINUOUSLY CROPPED SOIL

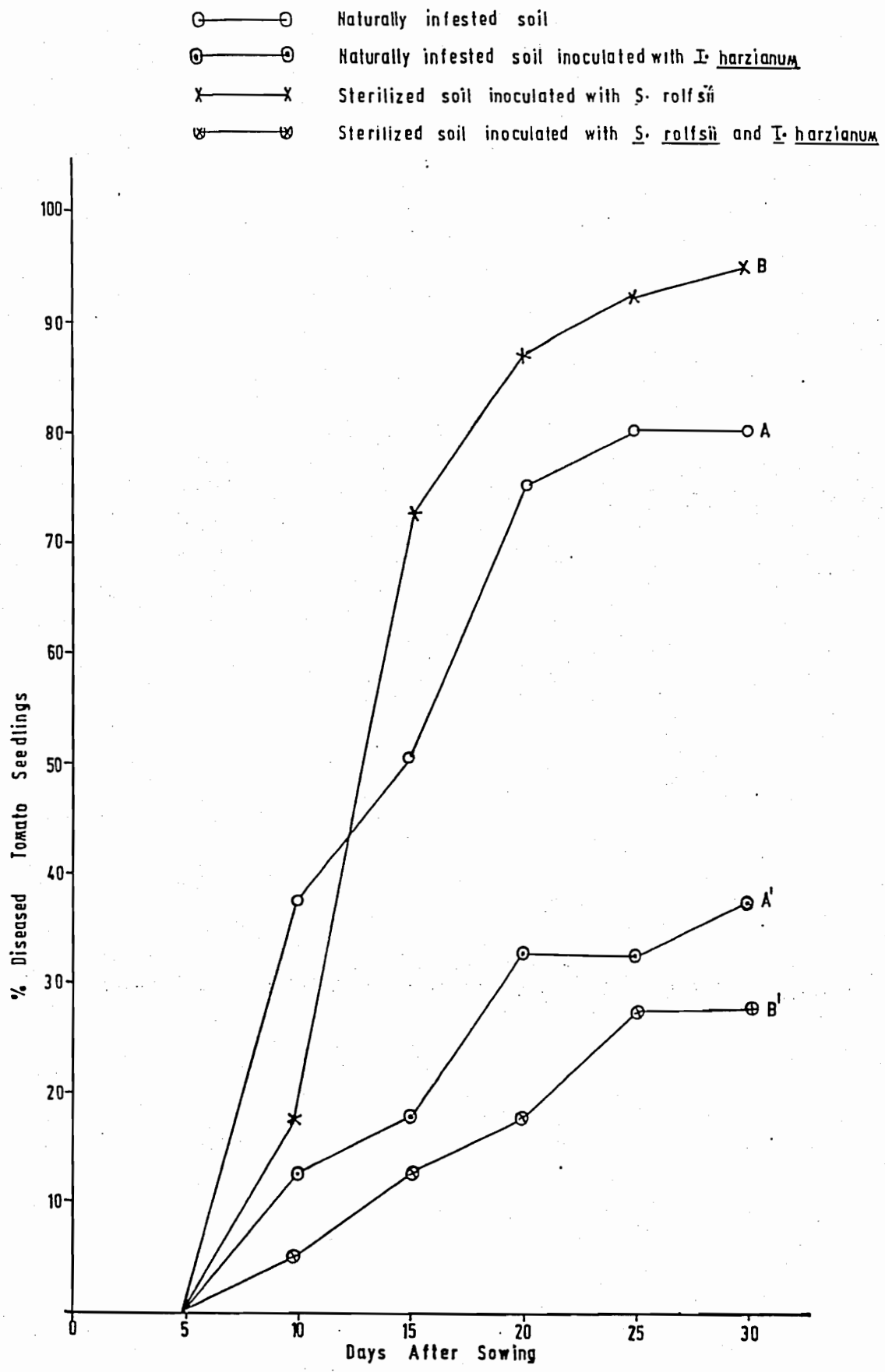

FIG 2 DEVELOPMENT OF ROOT ROT OF TOMATO IN TREATED AND UNTREATED SOILS INOCULATED WITH T. HARZIANUM AND S. ROLFSII

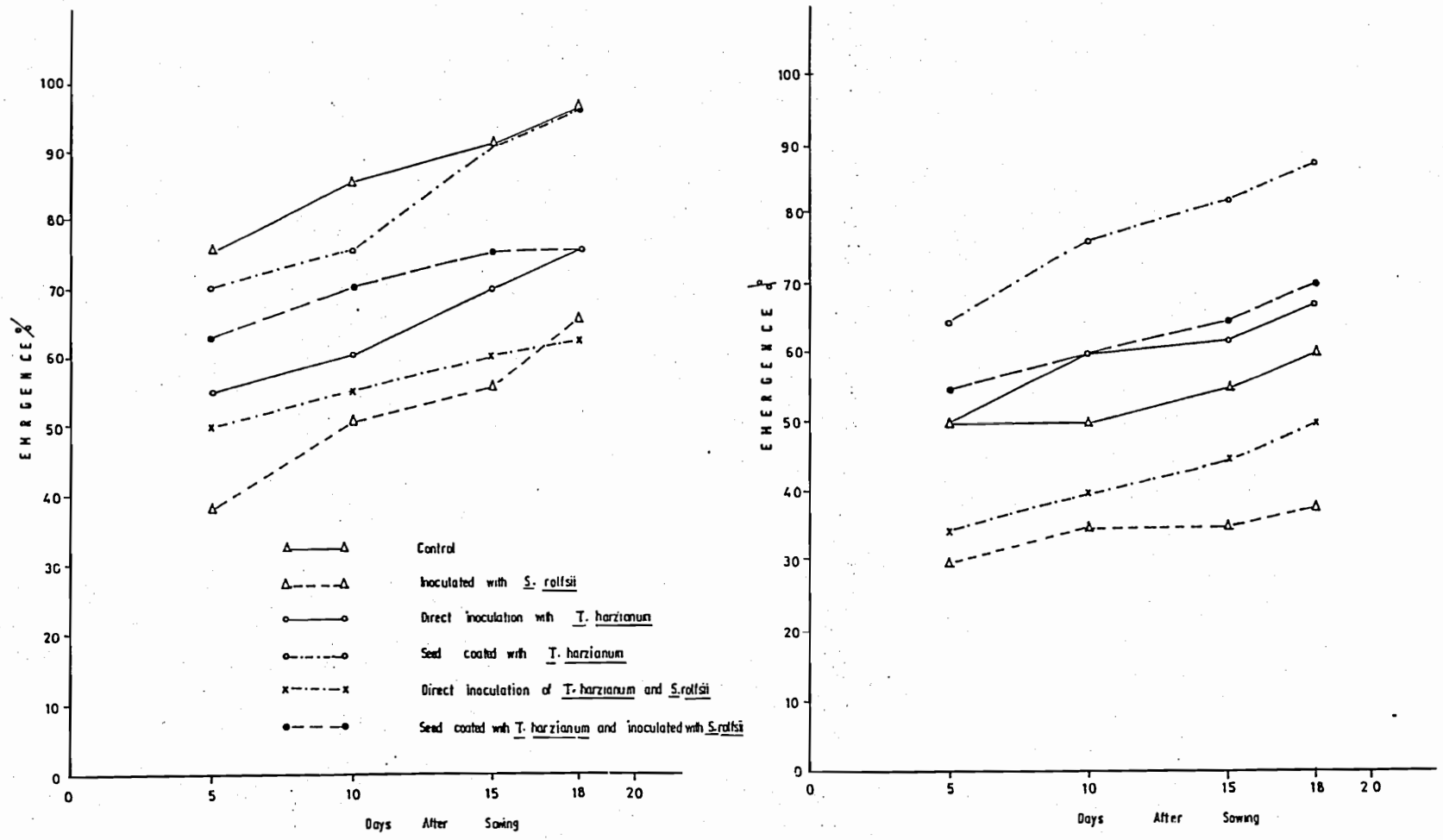

FIG 3. DEVELOPMENT OF PRE - EMERGENCE DAMPING OFF IN TOMATO INOCULATED WITH T. HARZIANUM AS SEED

Chapter III

STUDIES UNDERTAKEN IN ISRAEL

1. The control of Rosellinia necratix in soil and in apple orchard by solarization and Trichoderma harzianum.

Rosellinia necatrix Prill. (anamorph: Dematophora necatrix Hartig) is the causal agent of the white root rot disease of many plants, particularly of fruit trees such as apple, pear and avocado (13,21). The symptoms are rotting of roots, yellowing of leaves followed by leaf fall, wilting and death of the tree. Usually the infected roots are covered with white mycelium.

Various approaches that might be followed for the control of R. necatrix are: 1) eradication of the fungus in infested soils by soil disinfestation in order to prevent spread of the pathogen to adjacent fields; 2) suppression of the pathogen in soil and, therefore, protection of the plant from infection and 3) treatment of the soil in an existing orchard where trees are diseased, in order to prevent further infection. Both solarization i.e. solar heating of the moistened soil by tarping with transparent polyethylene (12), and the antagonistic fungus Trichoderma harzianum Rifai (6) are potential control measures for use against R. necatrix and other soilborne pathogens. The purpose of this work was to study the effectiveness of artificial heating of inocula, solarization and T. harzianum treatments, separately and combined, for control of R. necatrix under controlled and environmental conditions, in a field soil and in an existing orchard.

MATERIALS AND METHODS

Pathogen:- Rosellinia necatrix was isolated from roots of naturally infected apple (Malus sylvestris Mill. seedling rootstock) from Kibbutz En Zurim in the south and from naturally infected roots of avocado (Persea americana Mill.) from

Kibbutz Hanita in the north of Israel. In both cases the trees showed symptoms typical of the disease, i.e. chlorosis, leaf fall and dieback.

Antagonist:- *Trichoderma harzianum* was isolated from roots of an apple tree naturally infected by *R. necatrix* from Kibbutz En Zurim. The fungus was grown for 2 wk on a wheat bran/peat (1:1,^{v}/v) preparation (19). Various quantities (1g and 5g) of this preparation were mixed with naturally infested En Zurim soil, Vertisol, (3.7% silt, 46.3% clay, and 50% sand; pH 7.6) or Hanita soil, Terra rosa, (17.5% silt, 55% clay and 27.5% sand; pH 7.8) to a *T. harzianum* population of 10^8 colony forming units per g dry wt of soil mixture.

R. necatrix inoculum:- Inoculum was produced in wheat seeds which were soaked for 12 hr in 250 ml Erlenmyer flasks filled with distilled water. Each flask containing 100 ml seed was subsequently autoclaved after excess water had been drained off. After sterilization, three fungal discs of a 2 wk old culture of *R. necatrix* grown on malt extract agar (MEA), were placed aseptically in each flask. Flasks were then incubated at 25 C in light for a 2 wk period, unless otherwise stated, and shaken every 2-3 days to avoid clustering of seeds. The inoculum in each flask was then macerated in a Waring blendor under sterile conditions and a 4 g quantity was mixed with 1 kg sterile autoclaved En Zurim soil.

Inoculum level assessment:- The avocado leaf disc colonization method was used to determine inoculum levels of *R. necatrix* (23) in naturally and artificially infested soils. This method is reliable for assessing relative levels of the pathogen population and within certain limits, inoculum density and percentage of colonization are linearly related (6). The tested soil was placed in plastic containers (11 X 11 X 4 cm) each holding 250 g soil, with avocado leaf discs (1.6-cm-diameter) serving as traps for *R. necatrix*. These containers were incubated in light at 25 C for 12-14 days, whereafter leaf

colonization was assessed. The colonized discs developed characteristic, white mycelium and changed color to cream or light brown, while noncolonized discs were dark brown or remained green. All treatments were carried out in 4 replicates with 15 avocado leaf discs each. Percentage of reduction in colonization (C) was calculated by the following equation: $C = [(B-A)/B] \times 100$ where, A = % of colonized discs in the treatment and B = % of colonized discs in the nontreated control.

Heat treatment of *R. necatrix* inocula:- Quantities of 100 g soil either naturally infested or containing culturally produced inocula were placed in plastic bags (18 X 24 cm) and heated at constant temperatures in a plastic water bath. Inocula of *R. necatrix*, consisting of artificially infected wheat seeds at 2, 4, and 6 wk incubation periods and naturally infected apple root segments, were inserted into test tubes containing 10 ml sterile water. The test tubes were capped and incubated at various temperatures from 0-4 hr in a water bath to determine heat sensitivity of inocula of various ages and types. Following heat treatment, viability of the inocula was determined by monitoring mycelial growth from wheat seeds incubated 48-72 hr on MEA plates and from roots in moisture chambers at 25 C. Seed samples consisted of 40 seeds per treatment with 10 seeds per plate. Infected root samples consisted of 40 segments (1.5-2 cm) per treatment. Viability of inoculum in soil was assessed by the avocado leaf colonization method.

Control of *R. necatrix* under field conditions:- The field experiment was carried out in a 15-yr-old apple grove, in naturally infested soil at Kibbutz En Zurim. Treatments were: 1) soil solarization, 2) *T. harzianum*, 3) solarization combined with *T. harzianum*, and 4) a nontreated control. Twenty plots were selected randomly with treatments consisting of 5 replicate plots. Each plot (10 X 6 m) contained one established tree and the area of an adjacent tree, that had

been killed by R. necatrix and uprooted 2 months before the experiment was begun. The area directly under the canopy of the existing tree (ca. 2 m^2) which was not exposed to direct sunshine and was shaded during most of the day, is referred to as the shaded tarped site. Trees in the orchard were spaced 3.5 X 4 m apart.

The treated plots were sprinkler-irrigated to a depth of 90-120 cm. The soil of 10 plots, (5 solar and 5 integrated solar and T. harzianum-treated plots), was covered by 40 μm thick, transparent polyethylene sheeting on 4 July 1984. It was removed 8 weeks later. The solar-treated trees were situated 3 m from the edges of the tarped sheeting. Outer edges of the tarps were held in place by burying them in shallow trenches. The existing trees in the tarped plots were irrigated during and after solarization using a drip system.

Soil temperatures were recorded with Grant equipment (U.K.) by means of thermistors, for a period of 2 weeks, starting 12 days after solarization had begun, at depths of 10, 30, and 50 cm. in solarized and control plots.

T. harzianum preparation (60 g per 2 m^2 per tree) was incorporated into the soil of existing nonsolarized trees in July 1984 and to solarized ones after tarp removal.

Efficacy of treatments for control of R. necatrix was evaluated using three tests: 1) naturally infected root segments (1.5-2.0 cm long) were buried in groups of 20 in each plot in the soil at various depths ranging from 10 to 60 cm in the tarped and control sites, and at a 10 cm depth in soil in the T. harzianum plot, before solarization was started. Two, 4 and 8 weeks later, the segments were removed from the soil and incubated in moisture chambers for 1 wk to determine mortality of R. necatrix. The segments were in groups of 20 in nylon net bags attached to a nylon cord buried in the soil and were lifted without significantly affecting soil temperature. Control efficiency was

expressed as % mortality of the pathogen; 2) soil samples taken from solar and nonsolar treatments, immediately after and at 280 days after solarization had been terminated, were assessed for the presence of R. necatrix using the leaf colonization method; 3) two weeks after solarization had been terminated, 6-mo-old avocado plants were planted in the location of the uprooted trees to evaluate disease incidence. Each treatment consisted of 25 plants, with 5 samples per plot. Avocado plants in the two T. harzianum treatments were drenched with a 50 ml T. harzianum spore suspension each (10^8 conidia/ml) at planting time. Disease incidence was determined by assessing plant mortality over a period of 10 months following solarization.

Solarization in existing orchard:- Before commencing solarization, 14 and 25 mo later, the trees were rated with the following disease index: 0 = Healthy tree with a full canopy of foliage; 1 = Tree with mild chlorosis and a few dry branches; 2 = Tree with considerable chlorosis and many dry branches; 3 = Dead tree.

Induced suppressiveness to R. necatrix:- In the first approach, naturally infested En Zurim soil was mixed (1:1, w/w) with noninfested adjacent soil or with adjacent solarized soil. The inoculum level of R. necatrix in these soils, after various incubation periods, was determined by the avocado leaf colonization method. In the second approach (14), quantities of 25 g solarized and nonsolarized soils were placed in plates (9-cm-diameter). Boiled cellophane membranes with four MEA culture discs (4-mm-diameter) of R. necatrix were laid on the soil surface and incubated at 25 C for 24 hours. The mycelial growth on the discs was assessed microscopically. The length of five hyphal threads was measured in each of four microscopic fields per disc. Mycelial growth index was evaluated as average relative length of hyphae X number of hyphae within a microscopic field at 10 X 15 magnification.

Statistical analysis of the data was determined by using Duncan's multiple range test, factorial analysis or linear regression analysis as indicated, with a significance level of (P=0.05).

RESULTS

Heat sensitivity of inocula of R. necatrix:- Viability of the pathogen, expressed as percentage of avocado leaf colonization in a soil artificially infested with inoculated wheat seeds, was inversely related to increasing temperatures (from 34 - 38 C) or exposure time (Fig. 1). Inoculum in inoculated wheat seeds was much more sensitive to heat treatment than artificially infested soil. Heat sensitivity of R. necatrix was affected by inoculum quality since sensitivity decreased with aging of artificially inoculated wheat seeds (Fig. 2A, B). In comparison, naturally infected roots were the least heat-sensitive at 33 C and the most sensitive at 38 C.

Effect of combined physical and biological treatments on R. necatrix viability:- Heating naturally infested En Zurim soil or treating it with T. harzianum, reduced leaf colonization (Fig. 3). Combining the treatments further reduced colonization. These treatments were less effective for control of the pathogen in another naturally infested soil (Hanita), where the natural inoculum was at a higher density and was less heat-sensitive. Sensitivity of the inocula in these two naturally infested soils to heat treatment was higher than that of artificially infested soil (Fig. 1).

Pathogen and disease control by soil solarization and T. harzianum:- Maximum soil temperatures are presented in Table 1. Temperatures were highest in the solarized plots and decreasing with soil depth. Tarping the soil in solarized plots increased soil temperatures by 5 to 11 C above the respective nonsolarized

controls. Soil temperatures in the tarped shaded plots were only slightly higher than those in the nonsolarized plots. Soil temperatures at 60 cm depth were not recorded but the calculated maximum temperatures using suitable equations (25) were 29.5, 35.2 and 31.1 C for nonsolarized, solarized and shaded tarped plots, respectively. Various approaches were followed to determine solarization efficacy for control of R. necatrix. Mortality rate of the surviving pathogen population in segments of naturally infected roots buried in the soil of the various plots, was determined periodically after tarping. Results (Fig. 4) show that the pathogen remained viable in the untreated and T. harzianum treated plots at all tested depths throughout the 8 week trial period. Solarization resulted in a decline of pathogen recovery which progressed with time and was most pronounced at the upper soil layers. After 28 days of solarization, the pathogen was completely eradicated at 10 to 30 cm soil depths. After 56 days of solarization, 75% pathogen mortality was recorded at the 60 cm depth. Pathogen mortality in the tarped shaded plots was less pronounced, resulting in kill percentages after 56 days of 38 and 12% at depths of 10 and 30 to 60 cm respectively.

The effect of solarization on pathogen population in naturally infested soil was determined by taking soil samples from 10 and 30 cm depths 56 days after tarping. The relative pathogen population was estimated with the avocado leaf colonization method. Colonization percentages in the untreated soil at depths of 10 and 30 cm were 58.3 and 33.3, respectively. Colonization declined to zero in both previously solarized and shaded tarped plots at 10 and 30 cm depths. Soil samples were also taken from the field 280 days after removal of the polyethylene sheets. Colonization pecentages were 35 and 13.3% in the untreated plots at 10 and 30 cm depths, respectively. The percentage of R.

necatrix colonization of soil from the solarized plots at 10 and 30 cm depths remained zero.

The effectiveness of solarization and T. harzianum treatments for disease control in the naturally infested soil, was also examined by planting avocado plants in this soil. Diseased plants in the untreated plots were first detected in April 1985, 7 mo after termination of the solarization treatment (Fig. 5). By the end of the experiment, 3 mo later, disease percentages in the control and T. harzianum plots were 52 and 40%, respectively. Both solarization treatments were completely effective in controlling the disease throughout the experimental period.

The effectiveness of solarization for control of the disease in existing trees in the orchard was examined on a limited scale. Results (Table 2) show that the two solarized treatments reduced the disease to negligible levels over 25 mo at least, while disease levels of trees in the untreated plots or treated with T. harzianum increased during the two years of the experiment. No apparent damage was observed in trees subjected to solarization.

Behaviour of R. necatrix in solarized soils:- Two approaches were followed for determining the fate of the pathogen introduced into previously solarized or untreated soils. Naturally infested soil served as the inoculum source and was mixed with either naturally noninfested field soil or with the comparable solarized soil and subsequently, the colonization percentage of avocado leaf discs by the pathogen was determined. Results (Fig. 6) show that the solarized soil suppressed pathogen activity, especially after an extended period of incubation. After 28 days of incubation, colonization in the solarized soil mixture declined to 7% as compared to 28% with the nonsolarized soil mixture.

Agar culture discs of the pathogen were laid on either nonsolarized or solarized soil and growth rate of the pathogen was determined after 24 hours of

incubation. Results from two typical experiments out of six (Table 3) show a similar trend, namely, that the solarized soil suppresses pathogen growth, thus, confirming the results with the same soils using the colonization approach (Fig. 6). Samples of solarized soil taken from the field 9 months after solarization and tested by the mycelial growth method, remained suppressive to R. necatrix.

DISCUSSION

Soil solarization is very effective in controlling the white root rot disease and in reducing the population or activity of R. necatrix in soil to the depth of at least 60 cm. This was shown by using various approaches for assessing levels of inocula of various types. The successful control of R. necatrix by solarization which lasted for at least 25 mo in certain cases, might be attributed to a variety of physical, chemical and biological mechanisms. R. necatrix is highly sensitive to heat, as shown in other studies (3), more so than Verticillium dahliae, which is a heat-sensitive fungus (18). The reduced heat sensitivity of the pathogen upon aging may be due to the formation of sclerotia and other melanin-containing structures (22). In the solarized soil, activity and growth of the pathogen were suppressed (Table 3, Fig. 6) indicating the existence of biological control processes. The pattern of mycelial growth suppression in the solarized soil (Table 3) which was evident for at least 9 months, is similar to that observed with Phytophthora cinnamomi in solarized soil (17) and with Rhizoctonia solani in suppressive soils (14). Similarly, incidence of diseases caused by Sclerotium rolfsii, Fusarium oxysporum f. sp. lycopersici and F. oxysporum f. sp. dianthi was lower in the solarized soil due to induced suppressiveness and enhanced antagonistic activity (7,9). Thus, it

appears that biological control in solarized soils is not an exceptional phenomenon. Inoculum of R. necatrix in a naturally infested soil was eradicated to a depth of 30 cm in a shaded tarped soil where soil temperatures were only slightly higher than those in the untarped soil. This might be due to the accumulation of volatiles under the polyethylene tarp (10). In a similar study, various nematode populations decreased in moist polyethylene-covered but shaded soil (20). A weakening effect by sublethal temperatures, which further facilitates biological control as shown with Armillaria mellea (16) and S. rolfsii (15), may also operate in soil during solarization. Thus, a combination of mechanisms are involved in the drastic reduction of inoculum in the solarized soil, finally leading to a long term effect of disease control, as described (11).

T. harzianum was not effective in the present study when applied in the field. This could be attributed, among other things, to an inadequate mode of application. Various studies have shown that combining solarization with other methods of control e.g. T. harzianum (2,4), vapam (5) or a crop rotation (11) improved pathogen control or extended it. Combining partial heating with reduced dosage of T. harzianum, resulted in an improved control of the pathogen in En Zurim soil (Fig. 3). The possibility that such a combination could lead to a more lasting control, should be further studied.

Soil solarization was effective in controlling R. necatrix in an existing orchard as also found for V. dahliae in pistachio (1) and olive groves (24). In the present case, soil solarization fulfills the requirements for a successful control of a soilborne pathogen in an existing orchard (1,10): The tree was not damaged, the inoculum was controlled to a considerable depth, soil reinfestation was delayed and in the tarped shaded area the inoculum was also reduced. Soil solarization is a promising method of soil disinfestation that can be applied as

a post-planting treatment. The apple rootstock survived the high temperatures prevailing during solarization although it is considered heat-sensitive (8). This may be due to a higher heat tolerance of this rootstock. Certain chemical treatments which reduce heat damage (8) should be considered, where necessary. As compared to annual crops, the control of soilborne pathogens in existing orchards presents difficulties since the pathogen has to be controlled to greater depths and for longer periods of time without damage to trees. Therefore, integrated methods of control should be given a high priority in such future research programs.

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LEGEND TO FIGURES

Fig. 1. Effect of heating on survival of Rosellinia necatrix in either artificially infested soil or in inoculated wheat seeds. Survival in artificially infested soil was determined using the leaf colonization method and in wheat seeds by determining percentage of seeds yielding the pathogen, after treatment. Numbers on the graph lines indicate temperature (C). Following a linear regression, all the coefficients ($-0.99 < r < -0.98$) are significant except for graph line indicating 36 C, and slopes are significantly different from zero (graph line indicating 25, 33 C) ($P=0.05$).

Fig. 2 A,B. Heat sensitivity of Rosellinia necatrix at two temperatures (A,B) in inoculated wheat seeds as affected by inoculum age. For comparison, naturally infected apple roots were subjected to the same heat treatments. Values at each temperature having a common letter are not significantly different ($P=0.05$).

Fig. 3. Effect of heating for 4 hours and Trichoderma harzianum, separately or combined with heating, in two naturally infested soils, on R. necatrix survival, assessed by the leaf colonization method. In both soils, following a factorial analysis, a significant interaction ($P=0.05$) existed between T. harzianum and temperature. A significant difference existed between all temperatures in the no T. harzianum treatments ($P=0.05$). An asterix denotes a significant effect ($P=0.05$) of T. harzianum at the respective temperatures.

Fig. 4. Effect of solarization and Trichoderma harzianum on the mortality of Rosellinia necatrix in an apple orchard. The inoculum consisting of naturally

infected apple root segments (1.5-2 cm) was buried in the soil at three depths, removed after various periods and tested for viability. Numbers on graph lines indicate soil depth (cm). Solar = solarized sites far from the shaded area; Shaded = solarized sites under the tree canopy; T. harzianum = sites treated by T. harzianum at 1g preparation/kg soil; Control = untreated sites. Mortality percentage remained zero throughout the tested period in T. harzianum treated and control soils at the tested depths. Following ln transformation of X coordinate, the linear regression coefficients ($0.95 < r < 0.98$) are significant except for graph line indicating shaded 10. All slopes are significantly different from zero (graph line indicating control and T. harzianum treatments) ($P=0.05$).

Fig. 5. Effectiveness of solarization and Trichoderma harzianum for control of R. necatrix in a naturally infested soil. Soil was tarped during July and August, 1984. Avocado plants were planted in October 1984 in the tested soil. Percentage of plants with white root rot symptoms was assessed at various times thereafter. Solar = plants in solarized sites; T. harzianum = plants treated with 50 ml T. harzianum spore suspension (10^8 conidia/ml); Control = plants in untreated sites. Following logit $\left(\ln \frac{x}{100-x}\right)$ transformation of Y coordinate, the linear regression coefficients ($0.94 < r < 0.97$) are significant. Slopes of the graph lines indicating nonsolar treatments are significantly different from the slope of graph line indicating solar treatments ($P=0.05$).

Fig.6. Effect of solarized soil on colonization capacity of Rosellinia necatrix. Naturally infested soil (D) served as an inoculum source and was mixed with either noninfested soil or the comparably solarized soil. The soils were

incubated at 25 C for 14 or 28 days before leaf colonization assessement. Values having a common letter are not significantly different (P=0.05). C = Noninfested soil. S = Solarized soil.

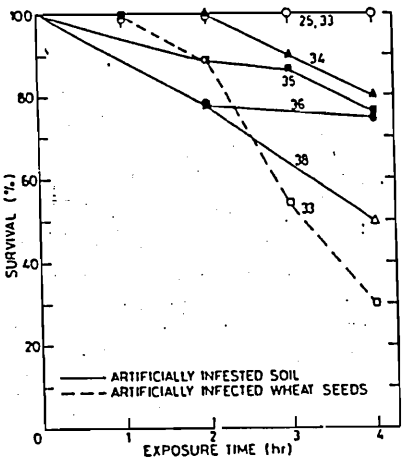


Fig. 1.

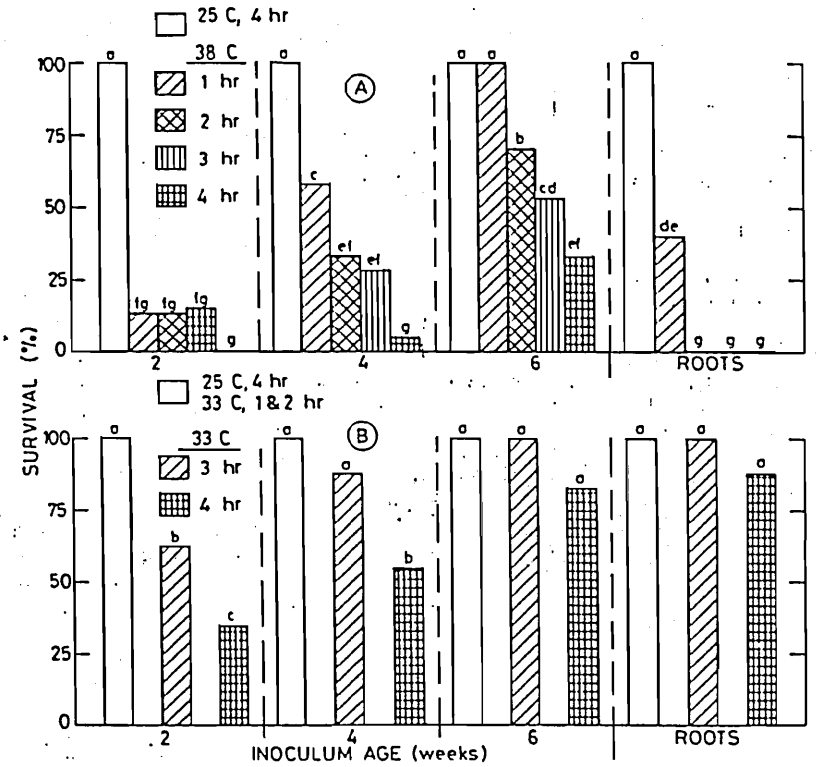
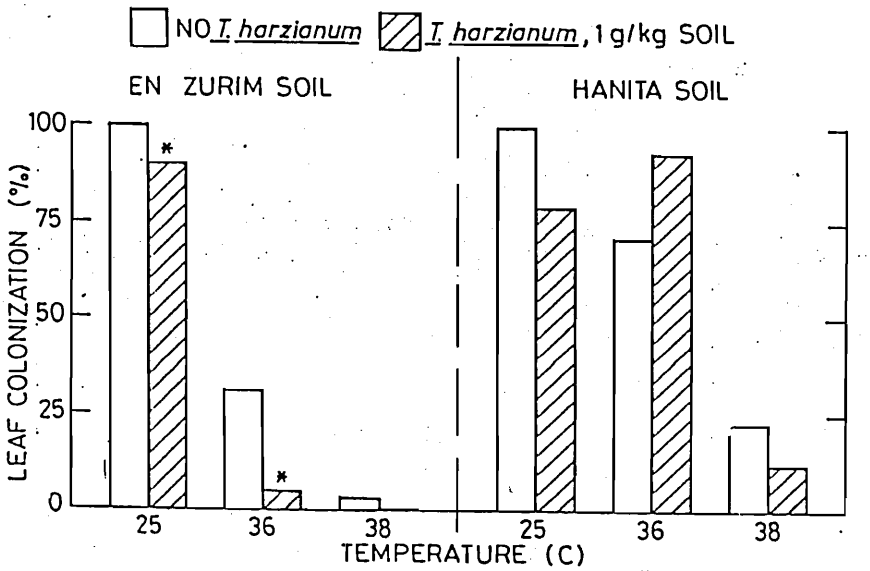


Fig. 2.



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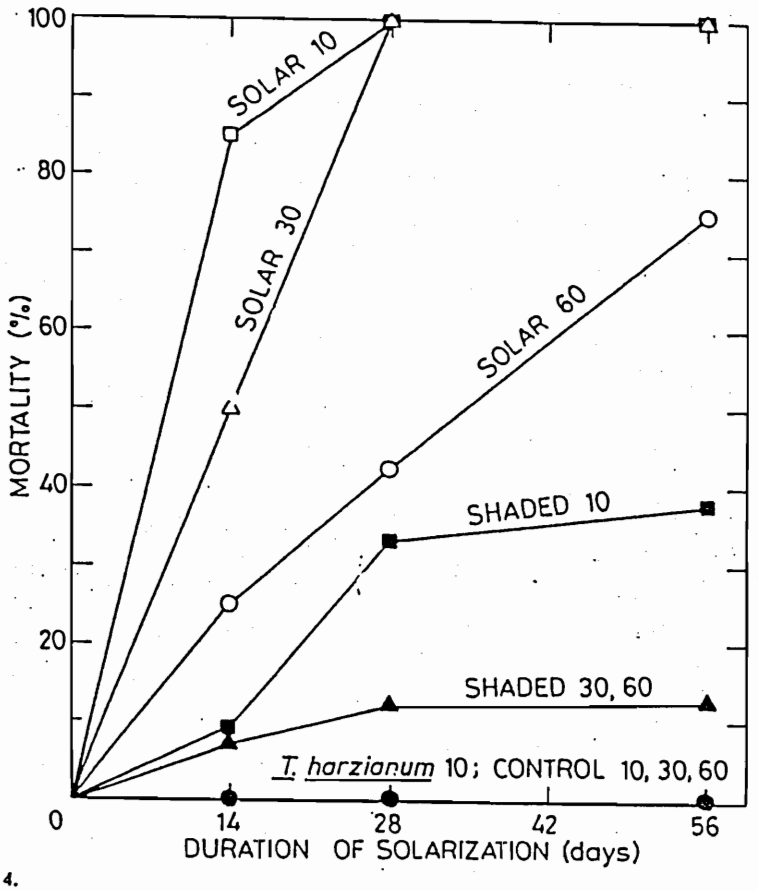

Fig. 4.

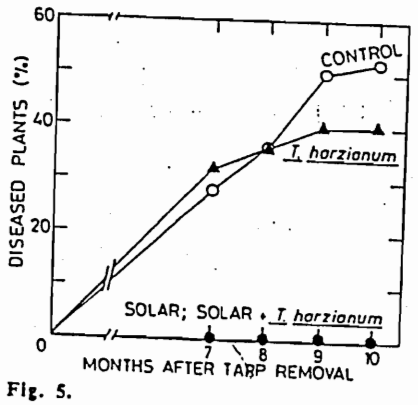

Fig. 5.

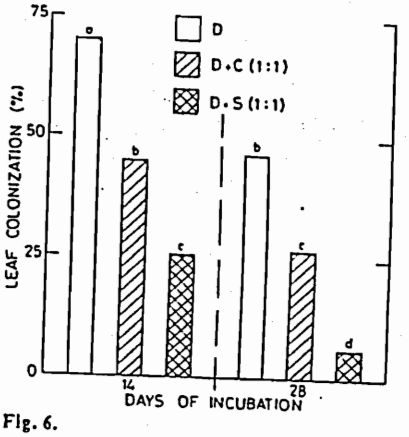

Fig. 6.

Table 1. Maximum soil temperatures recorded in a solarized apple orchard, during July, 1984 at En Zurim, Israel.

Soil depth (cm)	Maximum soil temperature (C)		
	Nonsolarized	Solarized	Solarized, shaded
10	35	46	37
30	33	38	33
50	31	37	32

Table 2. Effect of soil solarization and *Trichoderma harzianum* in an existing orchard on white root rot disease in apple trees. Disease rating was carried out before tarping in July 1984, in September 1985 and August 1986.

	Disease index [x]			
	(A)		(B)	
	July	Sept.	Aug.	
Treatment	1984	1985	1986	% Change [z]
Untreated	1.0 a [y]	2.0 a	2.5 a	+ 150
T. harzianum	1.2 a	2.2 a	3.0 a	+ 150
Solarization	1.2 a	0.2 b	0.04 b	— 97
Solarization + *T. harzianum*	0.4 a	0 b	0 b	— 100

[x] – Disease was rated on a scale; where 0 = healthy and 3 = dead tree.

[y] – Values in each year having a common letter are not significantly different (P=0.05).

[z] – % Change in disease incidence from July 1984 to Aug 1986 calculated as [(A–B/A)]X100.

able 3. Mycelial growth index [y] of Rosellinia necatrix in solarized and onsolarized En Zurim soil.

oil Treatment	Experiment 1	Experiment 2
Intreated	118.2 a [z]	140.0 A
olarized	39.6 b	56.0 B
Reduction	66.5	60.0

- Evaluated as average relative length of hyphae X number of hyphae within a icroscopic field at 10 X 15 magnification.

- Values having a common letter are not significantly different (P=0.05).

2. The possible role of competition between T. harzianum and Fusarium oxysporum on rhizosphere colonization.

INTRODUCTION

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 (8) Barber and Martin, (4) estimated that 3-9%

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of assimilated carbon by wheat and barley is contributed by root exudates.

Competition on nutrients favoring biological control of soil-borne plant pathogens, is mainly focused on limited amounts of carbon and nitrogen (5). In many cases where the amount cf these nutrients is insufficient due to intensive microbial activity, the soil becomes suppressive and no disease will develop (8).

Cook and Schroth (9) pointed out the carbon and nitrogen compounds needed for chlamydospore germination of F. solani f. sp. phaseoli. Similarly, Sneh et al. (25) showed the role of glucose and asparagine in stimulating chlamydospore germination of F. oxysporum f. sp. lini. They also reported the competitive ability of antagonistic rhizosphere Pseudomonads on these nutrients which resulted in significant inhibition of chlamydospore germination of this pathogen. Recently, Elad and Baker (10) and Elad and Chet (11) reported the importance of the carbon source, either provided by synthetic substances or excreted by plant rhizospheres, on the chlamydospore and oospore germination of F. oxysporum and Pythium aphanidermatum, respectively.

Most of the findings dealing with rhizosphere colonization by antagonists, mainly correspond to bacteria. Antagonistic rhizobacteria of this type such as P. fluorescence and P. putida were reported, in the past, as plant growth promotors of potatoes (6,14). Another Pseudomonas spp. isolated from rhizosphere of sugar-beet increased the plant weight and total

sucrose content, when applied as a seed treatment. Fluorescentic Pseudomonads, which are effective root colonizers and inhibit cultures of Gaeumannomyces graminis colonized, wheat roots in large numbers, in soil suppressive to this pathogen (26) Recently, Ordentlich, et al. (17), has shown the potential of chitinolytic Serratia marcescens in colonizing bean rhizosphere after its application to soil surrounding the germinating seed.

However, there are not many reports dealing with rhizosphere competence of fungi, including antagonists from the genus Trichoderma. Chao et al (7) showed the unsuccessful establishment of T. harzianum in pea rhizosphere. The failure of Trichoderma to establish in rhizosphere was also reported by Papavizas (18).

Recently, Ahmad and Baker (1) showed the potential of an ultra violet mutated strain of T. harzianum, which became benomyl tolerant, to colonize the rhizosphere of several crops, while the wild type failed to establish in the rhizosphere.

The objectives of the present study are to evaluate the role of competition in the biological control of Fusarium wilt of melon and cotton which has been demonstrated by T-35 (22, 23). We have therefore tested its potential to inhibit chlamydospore germination, when exposed to either synthetic nutrients or root exudates and have, moreover, studied the rhizosphere competence of this isolate and its effect on rhizosphere populations of F. oxysporum.

MATERIALS AND METHODS

Fungal isolates

Fusarium 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 a *Fusarium* selective medium (SQA;15). These fungi were cultured on a yeast extract -glucose medium 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 27 C. 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 synthetic medium (SM;16).

Greenhouse experiments

Experiments were carried out in artificially infested sandy loam soil (pH 7.8) 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, and having a moisture (at field capacity) of 12.2.%. Soil inoculation with *F. oxysporum* was performed using a microconidial suspension of the pathogen. Erlenmeyer flasks (250 ml) each containing 50 ml of liquid SM were seeded with mycelial disks from 72 h 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 conidial suspension was washed three times by centrifugation at 9150x g for 30 min.at 4 C. Ten ml of this supension, adjusted to $2x10^7$ microconidia/ml, were mixed with sandy loam soil using an electrical soil mixer.

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

Experiments, comprising 6 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. T. harzianum (T-35) was applied by three methods:

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, and 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 h, in 250 ml Erlenmeyer flasks in a rotary shaker at 200 rpm. Serial dilutions of the suspension were plated on a Trichoderma selective medium (12).

c) Wheat-bran peat preparation (24) - Wheat bran/peat mixture (1:1, v/v) adjusted to 40% moisture (w/w) was autoclaved in

autoclavable polyethylene bags (50 x 50 cm) for 1 h at 121 C on three successive days. The substrate mixture was inoculated with homogenized mycelium from 48 h cultures of T. harzianum grown in liquid SM, and incubated in an illuminated chamber for 14 days at 30 C. This preparation containing $5x10^9$ colony forming units (CFU), was mixed with soil at a rate of 5 g/kg soil.

Soil enrichment with chlamydospores of Fusarium was performed according to Sneh et. al. (25). Mycelial disks of F. o. melonis and F. o. vasinfectum were grown in liquid SM, in 'Roux' bottles at 27 C. After 7 days of incubation, mycelial mats were separated, placed in 4 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 well mixed, placed in glass bottles and incubated at 27 C for 4 wk and 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 of F. oxysporum per gram, as determined after soil dilutions on SQA medium.

Chlamydospore germination tests in soil were carried out according to Sneh et. al. (25). 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) and of pre-germinated T-35 conidial suspension (in water solution of 0.01% glucose) were

added to reach the desired concentrations of nutrients and antagonist, at 15% (-0.3 bar) water content. Soil was thoroughly mixed and incubated at 27 C for 22 h. 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 (1984). 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 UV light microscope (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. (25). Cotton and melon seeds were disinfested for 3 min in 4% sodium hypochlorite and placed between 4 layers of wet No. 1 chromatography paper, wrapped in plastic bags, at 30 C for 24 h. The germinated seeds were then transferred to 5 gr of chlamydospore enriched soil, treated or not with T-35, 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 microscopical 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 5% sodium hypochlorite for 10 min. Eight hundred seeds of each crop, were placed in a round glass column (80 x 7 cm) containing 1 l sterile tap water. The lower part of the column was connected to an air compressor and air was forced inside through a 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 liquid, containing the germinated seed exudates, was then collected in a sterile container, frozen at -30 C, lyophilized and kept in a deep freeze until use.

Rhizosphere competence tests.

Rhizosphere population dynamics of T. harzianum and Fusarium spp. was studied according to the method developed by Scher et al. (21) as modified by Ahmad and Baker (1) and Elad and Chet (11). A seed of the test plant was placed at the upper part of two longitudinal halves of 50 ml polypropylene tubes (Falcon Div. Becton-Dickinson Co., Oxnerd, CA, USA) filled with sandy loam soil with 15% moisture content (-0.3 bars). T. harzianum was applied 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 (e.g. -0.3 bars). Unless otherwise mentioned, the pots were covered with polyethylene bags to maintain the same soil matric potential, leaving enough space for plants to grow and placed in an illuminated chamber, at 28 C. 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,to 2 cm segments. Segments of 6 plants, representing the same distance from the crown, were shaken in a rotary shaker, for 1 h, in 250 ml Erlenmeir flasks each containing 30 ml of sterilized tap water. Serial dilutions were plated on TSM and SQA for Trichoderma and Fusarium spp. counts, respectively. The counts of each fungus were expressed as cfu/g root (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 originating from T-35 which is very resistant to this substance (Peer and Chet, unpublished).

RESULTS

Chlamydospore germination in soil amended with glucose and asparagine.

Without the addition of nutrients to soil enriched with chlamydospore of F. o. melonis or F. o. vasinfectum, the chlamydospores germination rate of both Fusaria ranged between 19 and 30%. The first germinating chlamydospores were observed after 18 h, while maximal germination occurred 22 h after incubation. The addition of glucose and asparagine (5:1;w/w) enhanced germination up to 51% at concentrations of 0.3 and 0.06 mg glucose and asparagine per g soil, repectively. (Fig. 1). Higher amounts of these nutrients did not result in further germination. When applied at concentrations of 10^6 per g soil, treated with 0.05 and 0.01 mg glucose and asparagine per g soil, respectively. conidia of T. harzianum (T-35) induced the maximal inhibition of chlamydospores (43%). However, at concentrations higher than 0.3 and 0.06 mg/soil of glucose and asparagine, respectively, the inhibitory effect was diminished. The same inhibition in chlamydospore germination rate was also obtained after application of T-35 as a seed treatment.

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, the maximal germination rate of chlamydospores (45 % and 56%, respectively) was observed after 60 h of seed germination (Fig. 2).

In soil amended with glucose and asparagine solutions, no difference in chlamydospore germination of both Fusaria, could be found between these two application methods of T-35 (e.g. seed coating and conidial suspension ; Table 1).

The addition of excess from cotton or melon germinating seed exudates completely nullified the inhibitory effect (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 antagonistic effect of T-35, after its application as either seed coating or a conidial suspension (Table 2).

Rhizosphere competition tests between T. harzianum and F. oxysporum.

After its application as a seed treatment, T-35 successfully colonized the rhizosphere soils of developing melon, cotton and tomato roots, up to 12 cm from the crown. In irrigated sandy loam soil the rhizosphere soil 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 inoculated with microconidia of F. o. vasinfectum (10^3 /g soil) the colonization of cotton rhizosphere by T-35 resulted in a simultaneous decline in Fusarium spp. counts 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 where the same application of T-35 was carried out.

The rate of colonization with T-35 of melon soil rhizosphere was dependent on the inoculum concentration, in soil, of F. o. melonis. Soil inoculation with increasing amounts of microconidia of either F. o. melonis or vasinfectum, reduced the establishment of T-35 in the rhizosphere by up to two orders of magnitude on segments included in the root tip (Fig. 5).

Establishment of F. o. melonis in melon rhizosphere was greatly affected by the amount of conidia of T-35 introduced in the soil. In untreated soil the counts of Fusarium spp. in rhizosphere ranged between $1.5x10^5$ and $8.8x10^4$ cfu/g root. However, T-35 treatment with 10^6 coinidia/g soil reduced the establishment of both pathogens up to $6.3x10^2$ cfu/g of melon root (Fig. 6).

Serial dilutions from rhizosphere and non-rhizosphere soils, inoculated with F. o. melonis and treated with T-35, 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 bran-peat preparation, resulted in high colonization of both, rhizosphere and non-rhizosphere soils. All Trichoderma treatments had minimal effect on the recovery of Fusarium spp. from the non-rhizosphere soil. However, when tested in rhizosphere soil, Trichoderma treatments resulted in

significant reduction (up to $3.5x10^3$ cfu/g soil) in counts of Fusarium spp., compared with $1.9x10^5$ cfu/g soil in the control (Table 3).

DISCUSSION

During this study we have shown the potential of Trichoderma harzianum to compete effectively with Fusarium oxysporum on nutrients and rhizosphere colonization.

One of the most sensitive stages to competition on nutrients, in the life cycle of Fusarium, is the chlamydospore germination (3). Chlamydospores of F. solani f. sp. phaseoli easily germinate in culture, even without the addition of nutrients, while in wet row soil only 1-2% germinate (13). However, with the addition of carbon and nitrogen sources to soil, 30-50% of these chlamydospores germinate (9).

In sandy loam soil enriched with chlamydospores of F. o. vasinfectum and F. o. melonis the germination rate, of both Fusaria ranged between 20 and 30%. These high germination levels could result from reserve materials in the chlamydospore, or may be due to residual available organic matter in the row soil. Soil amendment with increasing amounts of glucose and asparagine enhanced the germination rate up 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 stage or may be due to utilization of most of

the amended nutrients by the resident soil microflora (8).

In soil amended with low concentrations of glucose and asparagine (0.05 and 0.01 mg/g soil, respectively) pre-germinated conidia of Trichoderma harzianum (T-35), were found to inhibit the germination of chlamydospores. Germination was reduced even when the soil was not amended with these nutrients which may result from utilization of the available small amounts of nutrients, present in soil, by the antagonist.

However, at concentrations higher than 0.3 and 0.06 mg/g soil of glucose and asparagine, respectively, inhibition was nullified, indicating that competition on these nutrients did take place. At low levels of nutrients their utilization by the antagonist is more effective and resulted in a significant reduction in chlamydospore germination. Addition of excess glucose and asparagine, to soil, increased the availability of these nutrients for both the antagonist and the pathogen (e.g. T-35 and F. oxysporum, respectively). Similar phenomena were also described by Sneh et al. (25) and Elad and Baker (10), regarding the inhibitory effect on chlamydospore germination of F. oxysporum f. sp. cucumurinum induced by Pseudomonas spp.

Chlamydospores of F. o. vasinfectum and F. o. melonis in rhizosphere soil of cotton and melon, respectively, germinated at higher rates than the germination levels observed in non-rhizosphere soil. The same also occurred with chlamydospores of F. solani f. sp. phaseoli and F. oxysporum f. sp. cucumerinum, when exposed to bean and cucumber seedling exudates (25).

In our study it has been shown that inhibition of chlamydospore germination of F. oxysporum can be achieved even when the antagonist is applied as a seed coating. Probably, the germinating conidia of T-35 may utilize the germinating seed exudates making them unavailable to the pathogen.

Similar to glucose and asparagine, addition of an excess of exudates, significantly diminished the inhibition of chlamydospore germination induced by T-35. Apparently, plant root exudates produced during the plant growth are not sufficient for supplying the required nutrients for germination by both chlamydospores of F. oxysporum and conidia of T. harzianum. However, the competitive saprophytic activity of T-35 is presumably higher than that of F. oxysporum and thus inhibition of chlamydospore germination occurs. Indeed, Rovira et al. (19) have already mentioned that plant root exudates contain lower amounts of carbon and energy than those required by rhizosphere microflora.

The population of T-35 can easily be distinguished from the resident soil population of Trichoderma spp. by using the natural resistance of this isolate to relatively high concentrations of cycloheximide (Pe'er and Chet, unpublished). The major counts of T-35 in the rhizosphere were detected on root segments which included the root base and tip. Similar findings were reported by Ahmad and Baker (1) with a benomyl resistant mutated strain of T. harzianum. The relatively high population levels of antagonists recovered from the root tips were also demonstrated with bacteria (11, 17, 21).

Most of the exudates are excreted from the root tips. Thus, colonization of this region of the rhizosphere might result in significant biological control of Fusarium which penetrate the vascular system through the undifferentiated xylem at the root tip (8). Rhizosphere colonization of cotton and melon by T-35 was accompanied by a simultaneous decline in Fusarium spp. rhizosphere populations which was mainly observed on the last 4 cm of root segments.

Data provided, herein, indicate that rhizosphere competition takes place between T-35 and F. oxysporum. The rate 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, the conidia concentration of T-35 in soil increased, as the counts of F. oxysporum rhizosphere population decreased.

Biological control resulting from competition has a little or no effect on the viability of the pathogen in soil (3). In the present study, we have shown that soil application of T. harzianum had a only a slight effect on the survival of F. oxysporum in non-rhizosphere soil. However, application of T-35 as a bran-peat preparation, conidial suspension or seed coating, significantly reduced the pathogen counts in rhizosphere soils of melon and cotton. The different effects of T-35 on Fusarium population in rhizosphere soil as compared with non-rhizosphere soil possibly result from competition between the antagonist and the pathogen on root exudates. This type of competition probably

prevents the germination of propagules of the pathogen and thus decreasing the population density of the latter. On the other hand, in non-rhizosphere soil the permanent starvation confronting the soil microflora (2) and the lack of lytic or antibiotic mechanisms induced by T-35 against Fusaria (Sivan and Chet, unpublished) enables the pathogen to survive.

These results raise the question whether this competition plays any role in the biological control of F. oxysporum obtained with this isolate of T. harzianum (22, 23) and in order to answer this we produced large quantities of germinating cotton seed exudates. A continuous amendment of these exudates to soil inoculated with F. o. vasinfectum, completely reduced the controlling effect of Fusarium wilt obtained by T-35 treatments. The potential of plant root exudates, when applied in large quantities, to prevent biological control, supports the ypothesis that competition on nutrients plays a major role in \s biological control process.

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Table 1. Effect of *T. harzianum* on chlamydospore germination of *F. oxysporum* in rhizosphere and non-rhizosphere soil.

	Clamydospore germination (%)[1]			
	Rhizosphere soil		Non-rhizosphere soil	
Trichoderma treatments	*F. oxysporum* f. sp. *melonis*	*F. oxysporum* f. sp. *vasinfectum*	*F. oxysporum* f. sp. *melonis*	*F. oxysporum* f. sp. *vasinfectum*
None	43.5 a[2]	50.3 a	39.1 a	25.1 a
Seed coating[3]	25.3 b	33.1 b	19.3 b	12.1 b
Conidial suspension[4]	28.1 b	34.5 b	15.8 b	7.5 c

(1) Melon or cotton seeds were germinated in soil, enriched with chlamydospores of *F. oxysporum* and amended with glucose and asparagine (0.05 and 0.01 mg/g soil). The soil was placed on glass slides. After 48 h the germinated seedlings were removed and the soil was taken for counting germinated chlamydospores under U.V. microscope, using Calcofluor.

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

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

(5) 1×10^6 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*.

Trichoderma treatments	(2) Exudates	Chlamydospore germination (%) of *F. oxysporum* f. sp. (1)		Cotton wilted plants (%)
		vasinfectum	*melonis*	
None	Not added	24.5 ab (3)	23.8 ab	73.8 ab
	Added	29.8 a	27.1 a	85.9 a
(4) Conidial suspension	Not added	12.4 c	10.1 c	33.6 c
	Added	22.1 b	19.3 b	68.9b
(5) Seed coating	Not added	14.7 c	13.1 c	39.1 c
	Added	23.4 b	25.6 a	75.1 ab

(1) Counted after 48h under U.V. microscope using Calcofluor.

(2) Eight hundred external disinfested seeds were germinated in a water column (1 L) at 27C, for 4 days. The calculated volume of exudates, produced by 10 seeds was added to each replicate (0.5 Kg. soil sown with 10 seeds) at sowing date and each consecutive 3 days, until the end of the experiment, twenty days after sowing.

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

(4) $1x10^6$ conidia of T-35 per Kg. soil.

(5) $7.5x10^4$ and $5x10^4$ conidia of T-35 per each cotton or melon seed, respectively.

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Table 3. Effect of *T. harzianum* on *Fusarium* spp. population in melon rhizosphere and non-rhizosphere soil.[1]

CFU/g soil x 10^2

Trichoderma treatments	*Trichoderma* spp. Rhizosphere soil	*Trichoderma* spp. Non-rhizosphere soil	*Fusarium* spp. Rhizosphere soil	*Fusarium* spp. Non-rhizosphere soil
None	4.2 c[5]	1.5 d	1900.0 a	920.0 a
Seed coating[2]	480.0 b	9.0 c	35.0 b	750.0 a
Conidial suspension[3]	51000.0 a	20000.0 b	41.0 b	30.0 b
Preparation[4]	55000.0 a	55000.0 a	43.0 b	35.0 b

(1) The soil was inoculated with $2x10^8$ microconidia/g soil.

(2) $7.5x10^4$ conidia of T-35 per seed.

(3) $5x10^6$ conidia of T-35 per g soil.

(4) Wheat bran-peat (1:1; v/v) preparation of T-35 consisting of $5x10^6$ CFU/g (Dry wt.) was introduced into soil at a rate of 5 g/Kg soil.

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

LEGEND TO FIGURES

Fig. 1. The effect of T. harzianum (T-35) conidial concentration applied to soil, on chlamydospore germination rate of F. oxysporum f. sp. melonis, after 22 h of incubation in soil enriched with chlamydospore 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 (O). Values marked with an asterik are not significantly different from the control (P=0.05).

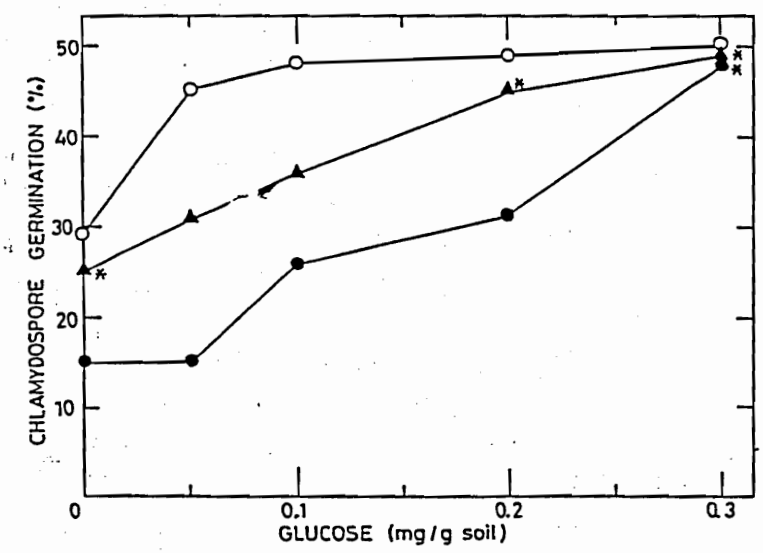
Fig. 2. Chlamydospore germination of F. oxsysporum f. sp. vasinfectum (A) and F. oxysporum f. sp. melonis (B) in cotton and melon rhizosphere soil (□), respectively, as compared with the germination rate in non-rhizosphere soil (O).

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

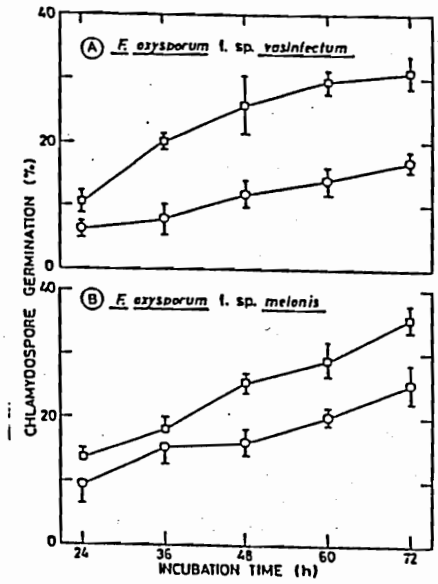
Fig. 4. The effect of cotton seed coating with T. harzianum (T-35) on rhizosphere soil population of T-35 (A) and Fusarium spp. (B) after 8 days of incubation in soil inoculated with microconidia of F. oxysporum f. sp. vasinfectum (10^3/g soil).

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

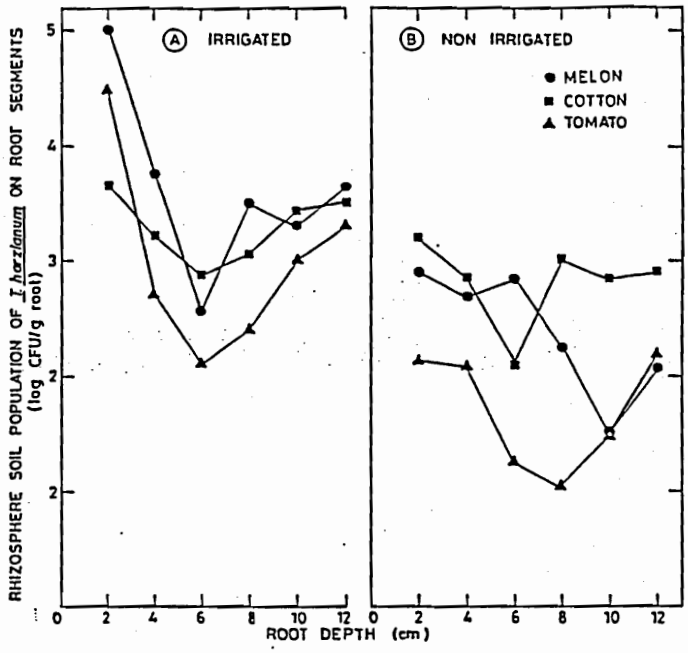
Fig. 6. The effect of T. harzianum (T-35) conidial suspension concentration on colonization of melon rhizosphere by F. oxysporum f. sp. melonis.


CHLAMYDOSPORE GERMINATION (%)
GLUCOSE (mg/g soil)

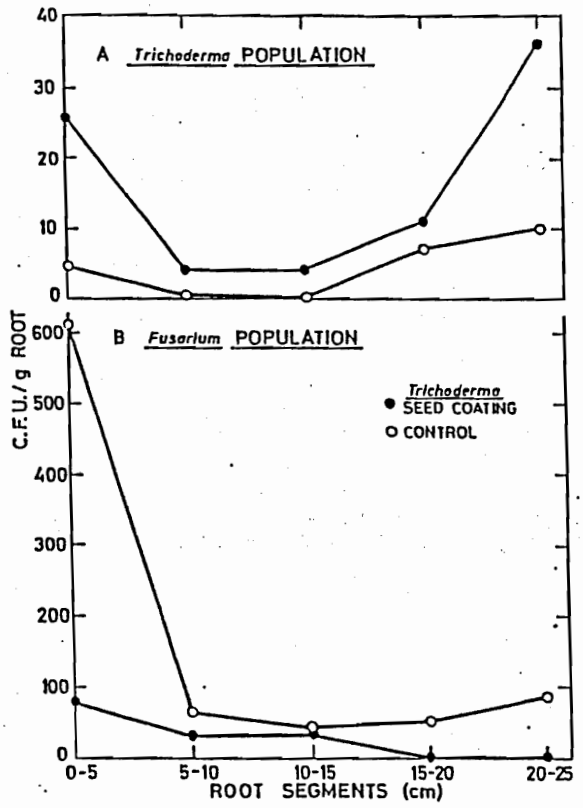
1


A F. oxysporum f. sp. vasinfectum
B F. oxysporum f. sp. melonis
CHLAMYDOSPORE GERMINATION (%)
INCUBATION TIME (h)

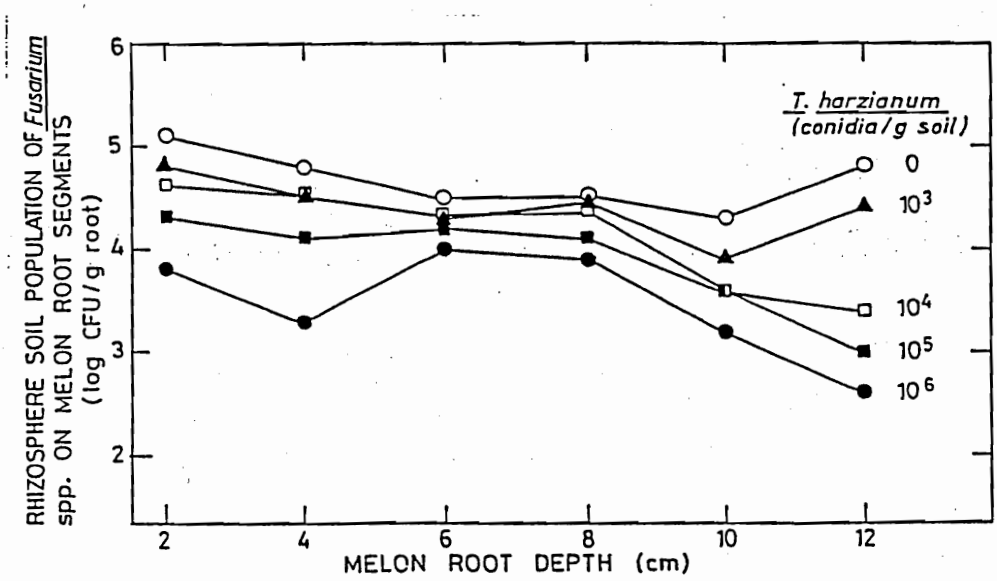
2


A IRRIGATED
B NON IRRIGATED
MELON
COTTON
TOMATO
RHIZOSPHERE SOIL POPULATION OF T. harzianum ON ROOT SEGMENTS (log CFU/g root)
ROOT DEPTH (cm)

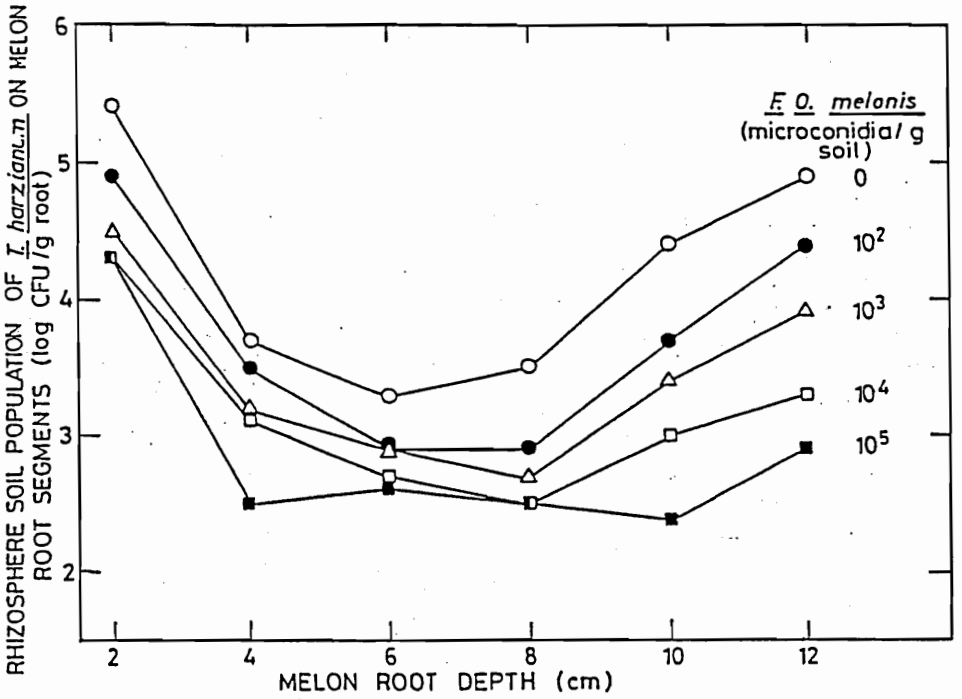
3


A Trichoderma POPULATION
B Fusarium POPULATION
C.F.U./g ROOT
Trichoderma SEED COATING
CONTROL
0-5
5-10
10-15
15-20
20-25
ROOT SEGMENTS (cm)

4


RHIZOSPHERE SOIL POPULATION OF Fusarium spp. ON MELON ROOT SEGMENTS (log CFU/g root)
T. harzianum (conidia/g soil)
0
10³
10⁴
10⁵
10⁶
MELON ROOT DEPTH (cm)

5


RHIZOSPHERE SOIL POPULATION OF T. harzianum ON MELON ROOT SEGMENTS (log CFU/g root)
6
5
4
3
2
F. O. melonis (microconidia/ g soil)
0
10²
10³
10⁴
10⁵
2
4
6
8
10
12
MELON ROOT DEPTH (cm)

6