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**Application of Antagonists to Soil for
Disease Control and Induction of Increased
Growth**

R. Baker, I. Chet

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Title of Research Project:

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Names of Investigators:

Principal Investigator: Ralph Baker

Cooperating Investigator(s) Ilan Chet

Name and Address of Affiliated Institutions:

Principal Institution: Plant Pathology and Weed Science, Colorado State University, Fort Collins, Colorado 80523

Cooperating Institution(s): Hebrew University of Jerusalem, Faculty of Agriculture, Rehovot, 76100 ISRAEL

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Institution's Authorizing Official

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c. Abstract

Four biocontrol agents were effective in control of various plant diseases and their activity was enhanced by various strategies. Trichoderma spp. were particularly enhanced in biocontrol activity and promotion of an increased growth response by mutation to rhizosphere competence. A strain was obtained that was effective against Fusarium wilt diseases. Fluorescent pseudomonad activity against the same diseases was enhanced by the presence of an appropriate iron chelator. A new species, Pythium nunn, was a mycoparasite of Pythium and Phytophthora pathogens and effectively suppressed colonization of organic substrates by Pythium ultimum. Nonpathogenic fusaria induced suppressiveness to Fusarium wilt diseases in raw soil at population densities typical of those found in the field. Trichoderma spp. induced increased growth responses attributable to control of minor pathogens and the production of a diffusible, extractible factor which had biological and chemical properties different from any known plant hormone.

d. Objectives of the original proposal

- i) To use our isolates and to select biocontrol agents that have attributes which can efficiently promote antagonism by mechanisms which were demonstrated to have the most impact, namely competition, hyperparasitism and lysis. It is also desirable that such antagonists have rhizosphere-competence.
- ii) To so manipulate the soil environment that the activity of the biocontrol agents is enhanced to the detriment of the pathogen, and to study the best technique for application.
- iii) To exploit and determine mechanisms associated with the increased growth response induced by antagonists.
- iv) To integrate different biological agents as well as combinations of biological and chemical controls.

e. Body of the report written in the form of a scientific paper

Mechanisms and strategies for enhancement of activity of biological control agents were reviewed by Baker (1983, 1984a,b, 1986) and Baker and Scher (1987). More specific reviews involving mechanisms, enhancement and application of fluorescent pseudomonads were published by Baker et al. (1986) and Baker (1987a). Most of the conclusions reached in these articles resulted from work supported by BARD.

Induction of suppressiveness to Fusarium wilt disease
by fluorescent pseudomonads

Pseudomonas putida was isolated from soils suppressive to Fusarium wilt disease in the Salinas Valley, California. Addition of the bacterium to conducive soil induced suppressiveness (Scher and Baker 1980). This study has already been reported in the previous BARD final report three years ago. Under the present BARD grant, mechanisms were explored and identified, for the most part, as competition for iron (Baker et al. 1986).

In a narrow sense, competition is "active demand in excess of immediate supply of material or condition on the part of two or more organisms" (Clark 1963). In soil, microorganisms compete exclusively for substrate and/or essential elements (Baker 1981). The basic mechanism involved in biological control by competition is that the pathogen must be deprived of a nutrient essential for pathogenesis. Factors found to be potentially essential are nitrogen, carbon (Griffin 1981) and Fe (Scher and Baker 1982). Thus by proper manipulation of one or more of these factors in soil, they could be limiting for pathogenesis. The mechanism by which fluorescent pseudomonads appear to function in biocontrol is by competition for Fe.

The Fe available in soil for plants and microorganisms is defined by the equation:

The principal factor mediating the equilibrium of this equation is the pH of the soil. Thus, the influence of pH may be considerable in determining whether Fe^{3+} is available to the pathogen, and ultimately, whether a soil is suppressive or conducive since Fe^{3+} is necessary for germ-tube elongation from spores or formae specialis of Fusarium oxysporum (Scher and Baker 1982). Indeed, when pH of the suppressive Metz soil (native pH ca 8.0) was reduced to pH 7.0, about half of the suppressiveness was lost. At pH 6.0 the soil was conducive (Scher and Baker 1980).

When Pseudomonas putida, ethylenediaminedi-O-hydroxyphenyl-acetic acid (EDDHA) or the ferrated form (FeEDDHA) was added to conducive soil, the soil became suppressive to Fusarium wilt of flax, cucumber, or radish (Dupler and Baker 1984, Elad and Baker, 1985, Scher and Baker 1980, Scher and Baker 1982). Suppressiveness was not induced by Fe-

ethylenediaminetetra-acetic acid (FeEDTA), or Fe-diethylenediaminetetra-acetic acid (FeDTPA). These results may be explained by the mechanism of competition or Fe illustrated in Fig. 1.

The three ligands bind Fe^{3+} at various stability constants: EDDHA, $\log_{10} K = 33.9$ > DTPA, $\log_{10} K = 27.3$ > EDTA, $\log_{10} K = 25$ (Lindsey 1974). Fe-binding siderophores were detected for most microorganisms examined (Neillands 1984), and the causal agents of Fusarium wilt produced siderophores of the hydroxamate class under Fe-deficient conditions. These have stability constants in the proximity of $\log_{10} K = 29$. This constant is many powers of ten less than most bacterial catechol siderophores. *P. putida* produces a mixed hydroxamate catechol siderophore (pseudobactin as reported by Teintze et al. 1980).

Fig. 1. Mechanisms and pathways involved in soil suppressive to Fusarium wilt pathogens through competition for iron (Fe).

By managing iron availability in such a system, control could be synergistic. Given an alkaline soil in which iron availability is low, pseudomonads could be favored in growth and activity by adding a chelate that binds iron so that it is capable of being produced by the siderophores of the antagonist but not by those of many other microorganisms, including fungal pathogens. Thus Scher et al. (1984a) demonstrated that the addition of FeEDDHA stimulated fluorescent pseudomonad growth in the rhizosphere of cucumber grown in monoculture. This is a particularly attractive strategy because the plant can utilize the iron in the FeEDDHA; consequently, the ligand, deprived of this element, specifically bind more iron from the Fe^{3+} pool (Scher and Baker 1982). In such a system, therefore, the plant itself participates in depriving the pathogen of iron.

Theory expansion suggests that adequate Fe^{3+} is available to Fusarium wilt pathogens for germination and penetration through root tips of the host in conducive soil. Since many pseudomonads have high rhizosphere-competence, there should be intense competition for Fe^{3+} at the rhizoplane (infection court) when *P. putida* produces siderophores, especially in alkaline soils. Therefore, Fe^{3+} is bound in such a way that it is unavailable to *F. oxysporum*.

The ligand EDDHA has a higher stability constant than the siderophore produced by the pathogen. Fe^{3+} complexed by this ligand in the rhizosphere would be unavailable to the pathogen; however, EDDHA-bound Fe can be utilized by the host. This frees EDDHA to bind more Fe^{3+} at the root surface (Lindsey 1974) and the soil is suppressive. This is

a dynamic system in which the siderophores produced by pseudomonads, the EDDHA, and the root combine to render Fe^{3+} limiting for pathogenesis by *Fusarium* wilt pathogens.

Sneh et al. (1984) isolated over 700 bacteria from the suppressive Metz soil. The isolates were capable of producing fungal cell-wall degrading enzymes and/or lysing living or dead germ tubes of chlamydospores and/or producing fluorescent compounds (siderophores) in Fe-deficient nutrient medium. Representative groups with or without these attributes were added to conducive soil and evaluated for their influence on chlamydospore germination of *F. oxysporum* f. sp. *cucumerinum* in rhizosphere and non-rhizosphere soil, and for their ability to induce suppressiveness to *Fusarium* wilt of cucumber. With only one exception (*Enterobacter cloacae*), non-fluorescent bacteria induced little or no inhibition of chlamydospore germination or disease suppression. There was, however, a direct correlation ($r = 0.99$) between the amount of siderophore production (*in vitro*) by various fluorescent pseudomonads and their inhibition of chlamydospore germination in soil. There was also a direct correlation between siderophore production by the various strains and suppression of *Fusarium* wilt of cucumber ($r = 0.747$ [Sneh et al. 1984]), radish ($r = 0.825$) or pea ($r = 0.872$ [Elad and Baker 1985b]).

Other possible mechanism for suppression were tested. As noted above, ability of potential antagonists to induce lysis and/or enzymatic disintegration of germ tubes was not related to suppressiveness (Sneh et al. 1984). An alternative mechanism could be cross protection. In unpublished research, carnation cuttings took up (through the wounded tissue) a suspension of the Metz soil for 2 hr, were rooted, and eventually challenge inoculated with *F. oxysporum* f. sp. *dianthi*. There was no evidence of decrease in incidence of disease by such treatment suggesting that cross protection was not involved in suppressiveness.

Antibiosis was considered as another candidate mechanism for suppression by pseudomonads. Wong and Baker (1984) found, however, no correlation between *in vitro* antibiosis in nutrient agar and suppression of *Gaeumannomyces graminis* var. *tritici* or var. *avenae* although disease suppression was induced by siderophore producing strains.

Chlamydospore germination in rhizosphere soil was inhibited slightly by addition of a non-siderophore-reducing mutant (Elad and Baker 1985b). Available Fe added to the system did not influence inhibition suggesting that another essential element, probably carbon (C), was limiting. This inhibition was not substantial enough, however, to account for suppressiveness. Indeed, such studies provided no evidence for the induction of suppressiveness by increases in biomass that should limit C availability. Growth rates and, thus, biomass of pseudomonads added initially in similar concentrations to soil were not significantly different. Yet, their ability to inhibit chlamydospore germination in soil (Sneh et al. 1984), in the rhizosphere or to decrease disease (Elad and Baker 1985) differed. Such responses were correlated directly with production of siderophores.

Thus, no evidence for participation of pseudomonads in exploitation, antibiosis, cross protection or competition for C in the induction of suppressiveness was obtained. So far, the only mechanism clearly identified that induces substantial suppressiveness in soil is competition for Fe.

The profound influence of soil reaction on suppressiveness and Fe availability (Scher and Baker 1982) was reviewed above. It is well known that siderophore production also occurs only in low Fe environments (Neilands et al. 1984).

This phenomenon was illustrated when fluorescent pseudomonads or partially purified siderophore preparations were applied to soils with pH 5.0 to 7.0 and germination of chlamydospores observed (Elad and Baker 1985b). Predictably, more inhibition of

chlamydospore germination occurred in soil at pH 6.0 to 7.0 than at 5.0 to 5.5 by either treatment. However, the applied siderophore preparation reduced chlamydospore germination significantly at pH 5 to 5.5, whereas the bacteria did not, even though present at population levels of 5.7×10^8 cfu (colony forming units)/g soil.

Baker (1968) proposed that competition for a particular element can be determined by adding the candidate limiting factor to the system and observing whether biological control was nullified. Thus, Sneh et al. (1984) added available Fe to soil and observed that it counteracted the inhibitory effect of fluorescent pseudomonads on chlamydospore germination. Counteraction also was observed, however, when soil was amended with Zn^{2+} , Co^{2+} , Mn^{2+} , and MoO_4^{2-} . In their investigations, Elad and Baker (1985a) suggested two lines of evidence to explain these phenomena: i) added micronutrients delayed multiplication of pseudomonads in soil thereby extending the lag phase. Thus, it appears that high population densities and/or activity of the biocontrol agent are necessary for inducing inhibition in the initial stages of chlamydospore germination. ii) Siderophores produced by pseudomonads apparently lack specificity for binding Fe^{3+} and may incorporate or substitute other cations on binding sites. However, when Fe^{3+} was added to siderophore preparations originally provided with these other cations, Fe gradually replaced these previously bound microelements. This suggests that the affinity of the siderophore-binding-site for Fe^{3+} is higher than the other cations tested.

The ligand, EDDHA, has a higher stability constant than hydroxamate siderophores produced by *Fusarium* pathogens and, indeed, induced suppressiveness when added to conducive soil (Scher and Baker 1982). However, quantities of Fe limiting to germination of chlamydospores should only occur on the host rhizoplane where there is intense competition for this element by both the root and rhizobacteria, like fluorescent pseudomonads, which utilize Fe in FeEDDHA (Scher et al. 1984). Once stripped of its Fe, the ligand binds more Fe from the available Fe^{3+} pool (Lindsey 1974). Since there are no roots or active rhizosphere bacteria to compete for this trace element in soil distant from the root, the amount of Fe^{3+} would be determined by soil pH regardless of the addition of FeEDDHA. Thus, the FeEDDHA was only effective in reducing chlamydospore germination in the rhizosphere but not in soil (Elad and Baker 1985b, Scher and Baker 1983). These phenomena suggest that there is an increment of control in suppressive soils attributable to the host.

There is probably more to this interaction than a simple qualitative relationship. Elad and Baker (1985) observed that siderophore production was reduced proportionally more than rate of bacterial cell division when fluorescent pseudomonads were grown in dilution series of synthetic medium containing low Fe. Therefore, in plants producing lower amounts of exudates in the rhizosphere, pseudomonads should produce lower amounts of siderophores. Evidence for this hypothesis was obtained by observing the level of chlamydospore germination of many isolates of *Fusarium* spp. germinating in rhizospheres of their respective hosts in soil infested or not infested with a siderophore-producing strain of *P. putida*. There was a significant, direct correlation between the germination of chlamydospores in soil to which the pseudomonad was not added and the degree inhibition induced when the strain was introduced. This suggests that the more nutrients exuded in the rhizosphere (as reflected by higher levels of chlamydospore germination) the more inhibition induced by the pseudomonad (Fig. 2). Therefore, increased inhibition results in the rhizosphere when more nutrients (except Fe) are available for the production of siderophores.

CHLAMYDOSPORE
GERMINATION
IN RHIZOSPHERE
(—)

CHLAMYDOSPORE
GERMINATION OF 12
ISOLATES OF FUSARIUM SPP.
UPON ADDITION OF
PSEUDOMONAS SPP. TO SOIL
(---)

PLANT SPECIES
(CUCUMBER, CORN, TOMATO, RADISH,
FLAX, SUGAR BEET, BEAN, WHEAT, PEA)

Fig. 2. Diagrammatic presentation of the evidence accumulated by Elad and Baker (1985b) suggesting that the more nutrients supplied by root exudates of various plants (as reflected by higher levels of chlamydospore germination of many Fusarium spp.) the more inhibition induced by siderophore-producing pseudomonads.

In the past, experimenters have applied one application of organic nitrogen and/or carbon sources to induce chlamydospore germination of Fusarium spp. in soil (e.g. Smith 1977, Smith and Snyder 1972). This, of course, does not reflect the situation found in soils where root exudates triggering germination are continuously introduced into certain areas of the rhizosphere. For a closer simulation of this situation, nutrients were supplied in pulses and striking differences were observed in germination inhibition in soil induced by fluorescent pseudomonads between this procedure and a one-time application of nutrients at the beginning of the experiment (Elad and Baker 1985b). Except at extremely low or high concentrations (where no differences were observed), inhibition of germination was less when nutrients were pulsed than when applied in total at one time. This provides at least a partial explanation of the results of Sneh et al. (1984) who observed less inhibition induced by fluorescent pseudomonads in the rhizosphere as compared to a test in which soils were supplied with one application of nutrients.

Soil was suppressive in the Salinas Valley to formae specialis of F. oxysporum but not to other Fusarium spp. (Smith 1977, Smith and Snyder 1972). A hypothesis explaining this phenomenon was advanced by Elad and Baker (1985b).

Low levels of inhibition of chlamydospore germination in the rhizospheres induced by addition of fluorescent pseudomonads was noticed in isolates of F. solani attributable to C but not Fe. Also, addition of the ligand, EDDHA (that binds Fe in soil so that it is not available to the hydroxamate siderophores of Fusarium spp. [Neilands et al. 1984]), suppressed germination of isolates of F. oxysporum but not F. solani or F. graminearum. What is the explanation for these phenomena? In general, chlamydospores of F. oxysporum are smaller than in other species. Indeed, when chlamydospores of F. solani f. sp. phaseoli and F. oxysporum f. sp. cucumerinum were compared, the ratios of volume, weight and Fe-content were three to five times greater for F. solani (Fig. 3); the larger chlamydospores of F. solani contained more Fe per propagule than F. oxysporum. Therefore, they did not require an exogenous supply of this element even in low Fe environments.

CHLAMYDOSPORE CHARACTERISTICS	FUSARIUM OXYSPORUM F. SP. CUCUMERINUM (SOIL IS SUPPRESSIVE)	FUSARIUM SOLANI F. SP. PHASEOLI (SOIL IS NOT SUPPRESSIVE)
SIZE	○	○
VOLUME	1	4.99
WEIGHT	1	4.60
IRON CONTENT	1	3.00

Fig. 3. Ratios of some properties of chlamydospores of Fusarium oxysporum f. sp. cucumerinum and F. solani f. sp. phaseoli (Elad and Baker 1985b).

Sneh et al. (1984) added available Fe to soil and found that it counteracted the inhibitory of fluorescent pseudomonads on chlamydospore germination. Misaghi et al. (1982) reported that Fe counteracted inhibition of fungal growth by a partially purified siderophore in vitro, but other trace elements had no effect; however, partial counteraction of inhibition by siderophore-producing pseudomonads was observed by Sneh et al. (1984) when soil was amended with Zn^{2+} , Mn^{2+} , and MoO_4^{2-} . Two lines of evidence may explain these phenomena (Elad and Baker 1985a). First, added micronutrients delayed multiplication of Pseudomonas spp. in soil, thereby extending the lag phase. Indeed, when application of pseudomonads was delayed by 1-3 hr, the bacteria did not induce inhibition of chlamydospore germination, again suggesting that this was due to delay in multiplication of added bacteria. Thus, it appears that high population densities (or activity) of the biological control agents are necessary to be effective in the initial stages of chlamydospore germination. This may not explain, however, why partial nullification of germination was observed at lower concentrations of 10^{-4} mM Fe^{3+} , 10^{-5} mM Cu^{2+} , or Zn^{2+} /g of soil.

Second, siderophores produced by pseudomonads apparently lack specificity for binding Fe^{3+} . Unlike chelators such as EDDHA, which specifically bind Fe^{3+} over a wide range of hydrogen ion concentrations (Lindsey 1974), siderophores may incorporate or substitute other cations on binding sites. However, when Fe^{3+} was added to siderophore preparations originally provided with these cations, Fe gradually replaced the microelements as indicated by a significant increase in optical density of the solution at 410 nm. This suggests that the affinity of Fe^{3+} to the siderophore-binding site is higher than that of the other cations tested.

Fe^{3+} applied in pulses was more effective than when applied (in total) at the beginning of the experiment in counteracting inhibition of germination of chlamydospores by pseudomonads. Fe resupplied over time maintains an available pool of Fe^{3+} . Thus, a relatively large amount of Fe added at the beginning of the experiments may shift the equilibrium to the left and immobilize Fe. Fe supplied in pulses renews the pool of Fe^{3+} each time it is applied.

Obvious inhibition of germination of chlamydospores by Pseudomonas sp. strain 346 added to soil was observed at high pH levels but inhibition was at low levels at pH

values lower than 6.0. In acid conditions, populations levels of pseudomonads were lower than in alkaline conditions. According to theory, available Fe was in good supply (Lindsey 1974, 1979); therefore, siderophores were not produced (Neillands 1973). This provides evidence for the hypothesis outlined above. Further support was obtained when concentrated siderophore preparations were added to soil. These induced suppressiveness to chlamydospore germination in soil even at relatively low pH values.

The catechol hydroximate siderophores of fluorescent pseudomonads have a higher stability constant than the hydroximate siderophores of *F. oxysporum* and, thus, are able to compete successfully for available Fe (Scher and Baker 1982). The ligand EDDHA also has a higher stability constant than hydroximate siderophores and induced suppressiveness when added to soil. However, low quantities of Fe limiting germination of chlamydospores would only occur on the host rhizoplane where there is intense competition for this element by the root and rhizosphere bacteria capable of utilizing Fe in FeEDDHA--once stripped of its Fe the ligand binds more Fe from the available Fe^{3+} pool. In contrast, soil distant from root surfaces should maintain a level of Fe^{3+} determined by soil pH regardless of the addition of FeEDDHA since there are no root or active rhizosphere bacteria to compete for the trace element. Thus, FeEDDHA was only effective in reducing chlamydospore germination in the rhizosphere (Sneh et al. 1984) but not in soil (Scher et al. 1983, Elad and Baker 1985b). The effect of the ligand (EDDHA) alone, however, was similar to the effect of purified siderophores in that germination of chlamydospores in soil was inhibited in the presence of Fe in a lower molarity than that of the chelator.

These results expand and confirm developing theory regarding the mechanisms involved in the induction of suppressiveness to *Fusarium* in soil. Also, it is apparent that the presence of excessive amounts of some essential cations results in competition for binding site on siderophores and inhibits multiplication of pseudomonad biocontrol agents. This diminishes competition for Fe and reduces the efficacy of biological control.

A rifampin-resistant mutant was used to monitor population densities to determine survival characteristics of *P. putida* in soil (Dupler and Baker 1984). Recovery was greatest immediately after addition at high matric potential but when added at -100 bars, no *P. putida* was detected. When soils were dried slowly to -100 bars, the bacterium survived. *P. putida* survived through winter in two Colorado soils and population densities increased after January. Densities also remained high in rhizospheres of cucumber and radish plants in field trials.

For eventual genetic manipulation to increase efficiencies, factors involved in selection of traits for enhancement of biocontrol agents must be identified. Various strains of fluorescent pseudomonads applied to seed of cucumbers demonstrated different efficiencies in biocontrol of *Fusarium* wilt (Park et al. 1988). The reason for higher efficiency was not apparent in tests for *in vitro* antagonism, production of siderophores in iron-deficient medium, or by observation of inhibition of chlamydospores by the strains in rhizospheres of cucumbers. A more efficient agent in biocontrol (NIR) had higher rhizosphere-competence and produced siderophores at higher iron levels than an inferior strain (A-12).

The activity of Fe^{3+} was controlled in the absence of soil by amorphous Fe hydroxide or added chelating agents. The activity of Fe^{3+} could then be calculated through known equilibrium constants.

Through careful manipulation of Fe^{3+} activity in nutrient solution, the determination of the critical Fe level necessary for suppression of germination of spores of *F. o. f. sp. cucumerinum* by *P. putida* A-12 in this system was achieved (Simeoni et al. 1987). When Fe^{3+} activity was high, there was enough available Fe for

both the fungal pathogen and the bacterial antagonist. As Fe^{3+} activity dropped between 10^{-22} and 10^{-27} M, Fe competition between the two organisms was enhanced and germination of *F. o. f. sp. cucumerinum* was significantly decreased (Fig. 4). The critical Fe^{3+} below which chlamydospore germination was suppressed was between 10^{-19} and 10^{-22} M.

As Fe^{3+} activity was decreased below 10^{-27} M, germination suppression of *F. o. f. sp. cucumerinum* by the pseudomonad isolate also decreased (Fig. 1). One explanation for this behavior could be that the smaller bacterial cells do not contain as large an internal Fe supply as the larger fungal chlamydospores. Siderophores are secondary metabolites (Raymond et al., 1984), and their production depends on overall metabolic activity. Under extreme Fe stress, *F. o. f. sp. cucumerinum*, which contains 0.06 ng of Fe per chlamydospore (Elad and Baker 1985), would have a competitive advantage over the pseudomonad isolates, which face a reduction in metabolic rate and in siderophore production. The significant reduction in both overall respiration and siderophore production by *P. putida* A-12 as Fe was decreased to extremely low levels (Fig. 5) supports this explanation. An Fe^{3+} activity between 10^{-22} and 10^{-27} M is, thus, the optimal range for competition between *P. putida* A-12 and *F. o. f. sp. cucumerinum*.

In a well-aerated soil, the solubility of Fe is generally controlled by a ferric hydroxide, which is referred to as soil-Fe (Lindsey 1979). In a soil at pH 7.6 in equilibrium with soil-Fe, the calculated Fe^{3+} activity is 10^{-20} M. When all inorganic ferric hydrolysis species are considered, this corresponds to a total solution Fe concentration of 10^{-10} M. Thus, the critical Fe^{3+} level, between 10^{-19} and 10^{-22} M, associated with germination suppression of *F. o. f. sp. cucumerinum* by *P. putida* A-12 could occur in a natural soil solution. The Fe^{3+} activity level for optimal suppression, between 10^{-22} and 10^{-27} M, could occur in the rhizosphere when plant uptake and microbial utilization are considered.

Additional analysis of the system under extreme Fe stress permits quantification of the Fe requirement for chlamydospore germination of *F. o. f. sp. cucumerinum* in the presence of *P. putida* A-12. During experimental procedures and the 12-hr incubation, death of some organisms is expected. This microbial turnover would lead to a release of Fe to the system. When no Fe or EDDHA was added to the nutrient solution, some of the microbially released Fe became available to *F. o. f. sp. cucumerinum*, and the chlamydospore germination was comparable to that obtained under the maximum Fe^{3+} activity of 10^{-19} M (Figs. 1 and 6). Iron concentration in this nutrient solution before and after the 12-hr incubation was insignificant (Fig. 6), thus, any Fe released was used by the organisms on the membrane and did not remain in solution.

In contrast, Fe from microbial turnover was not as available to *F. o. f. sp. cucumerinum* when EDDHA was in the solution. Released Fe was held in solution by the chelate, which has a much stronger affinity for Fe than the Fusarium siderophore (Scher and Baker 1982). The 6 μg of Fe per kilogram increase after incubation (Fig. 6) was a measure of the Fe necessary to increase chlamydospore germination of *F. o. f. sp. cucumerinum* by 21%. The 6 μg of Fe per kilogram of nutrient solution that was measured before incubation is probably the result of EDDHA scavenging Fe from the glucose source and keeping this Fe in solution.

Fig. 4. Effect of decreasing Fe^{3+} activity on chlamyospore germination of *Fusarium oxysporum* f. sp. *cucumerinum*. A, Comparison of fungal spores alone and in the presence of *Pseudomonas putida* A-12. B, Comparison of fungal spores alone and in the presence of *Pseudomonas putida* A1/UV/AB-6, a non-siderophore-producing mutant. Ninety-five percent confidence intervals of the regression lines are shown.

Fig. 5. Influence of different Fe^{3+} activities and the presence of *Pseudomonas putida* A-12 on microbial activity (CO₂ evolved) and siderophore production (absorption at 410 nm). Columns headed with different letters for a given variable differ statistically at $P = 0.05$.

Fig. 6. Effect of added ethylenediamine di-o-hydroxyphenylacetic acid on chlamyospore germination of *Fusarium oxysporum* f. sp. *cucumerinum* in the presence of *Pseudomonas putida* A-12 in solutions containing no added Fe. Changes in nutrient solution Fe concentration are also shown. Columns headed with different letters for a given variable differ statistically at $P = 0.05$.

Toxicity of the nutrient solution to the pseudomonad isolates when EDDHA is absent or present in low concentrations (Fig. 7) indicated that other elements may have affected the competitive mechanism. This toxicity also helps explain the reduction in respiration and siderophore production of the pseudomonad isolates under extreme Fe stress, as the solutions used did not contain EDDHA. The toxic effects were partly alleviated by the addition of Fe to the solution (Fig. 5), but not enough to affect chlamydospore germination of *F. g. f. sp. cucumerinum* significantly.

The detrimental effect of the trace element addition to a simple phosphate buffer on the population density of *P. putida* A-12 (Fig. 8) suggests that these were the elements responsible for the observed toxicity. Both Cu^{2+} and Zn^{2+} are strongly bound by EDDHA in the absence of Fe^{3+} (Lindsey 1979). Because Cu^{2+} is present in the solution at $10^{-6.5}$ M and Zn^{2+} at $10^{-5.8}$ M, the addition of at least 10^{-5} M EDDHA should be enough to bind these elements, render them unavailable to the bacteria, and relieve their toxic effects.

The Zn/Cu ratio in a soil solution at pH 7 in equilibrium with reported soil-Zn and soil-Cu is around 1,000/1 (Lindsey 1979). The Zn/Cu ratio in the two-fifths Hoagland solution, however, is 5/1. This relatively large increase in Cu could account for the toxic effects of the nutrient solution to a bacterium whose natural environment more closely resembles that of soil solution. Ecker et al. (1982) have noted a decrease in fungal siderophore synthesis brought on by the presence of $10^{-7.1}$ M Cu in a growth medium. Clearly, the interactions of trace elements on the competition between fungal and bacterial siderophores are complex and need further study.

The mechanism of biological control of Fusarium wilt by Fe competition induced by a fluorescent pseudomonad is a complicated interaction, which is affected by soil minerals, pH and Fe levels, siderophore production, and plant roots. By simplifying the system, quantification of the competitive response is possible. A knowledge of the Fe^{3+} activity necessary for optimal biological suppression could have many applications in the future. Maintenance of the proper Fe level during selection of potential bacterial antagonists is one such application. Another is the use of the value as a predictor for whether biological suppression is possible when other factors affecting the mechanism are known.

Trichoderma spp. -- increased growth response, mutation and mechanisms

Baker et al. (1984) first reported promotion of plant growth in raw soil by application of Trichoderma harzianum either in the form of conidia or in a peat-bran culture formulation (Sivan et al. 1984). Radish dry weight was increased after 6 wk growth by 150% to over 250% by the addition of 10^5 cfu/g of raw soil. These preliminary observations suggested that increased growth could be induced in raw soil as well as under gnotobiotic conditions (Lindsey and Baker 1967).

Chang et al. (1986) noticed growth responses induced by Trichoderma spp. in various floricultural and horticultural crops. Steamed or raw soil infested with the fungus hastened flowering of periwinkle, increased the number of blooms on chrysanthemums and petunias, and increased dry weights of these and other plants like tomato, pepper and cucumber. Pepper seed germinated 2 days earlier than untreated controls.

Most of the responses cited above were noticed under laboratory and university greenhouse conditions. To determine whether phenomena could occur under commercial conditions, bedding plant growers were supplied with the peat-bran formulation containing approximately 10^8 cfu/g of product of strains of T. harzianum and instructed to apply the formulation 1% by volume to soil mixes. Obvious increases in growth and

hastening of flowering were observed in allyssum, marigold, pepper, periwinkle, and petunia under these conditions (Baker et al. 1986).

Fig. 7. Effect of increasing amounts of ethylenediamine di-o-hydroxyphenylacetic acid in nutrient solution on the population density of *Pseudomonas putida* strains after a 12-hr incubation period. Initial population density added to 50 ml of solution was $10^{7.6}$. Ninety-five percent confidence intervals of individual means are shown.

Fig. 8. Effect of low and high amounts of ethylenediamine di-o-hydroxyphenylacetic acid in three different solutions on the population density of *Pseudomonas putida* A-12 after 12-hr incubation. Initial population added to 50 ml of solution was $10^{7.6}$. Trace elements include Cu, Zn, Mn, B, and Mo. Columns headed with different letters differ statistically at $P = 0.05$.

A problem involving timing for flowering, which was solved by use of a strain of *T. harzianum*, illustrates the potential for use of such growth-regulating agents. Home gardeners prefer to purchase bedding plants in bloom--in Colorado transplanting in gardens can occur in May. Yet periwinkle does not produce blooms by that time even if seeded in January or February. In an experiment performed in a commercial greenhouse (Baker et al. 1986), periwinkle treated by the grower with the peat-bran formulation of *T. harzianum* flowered by May 2 and was three times taller than the control, which was not in full flower until another month had elapsed. In cold winter and spring climates (as in Colorado), there is also an additional advantage from treatments inducing speedy flowering. A month or more of greenhouse heating bills may be saved.

Cuttings may root more rapidly when treated with *Trichoderma* spp. This response is over and above that observed with application of recommended rooting hormone. Fig. 9 illustrates responses of carnation. In this case, a mutant tolerant of benomyl (a fungicide commonly used in the hormone solution) was employed. Chrysanthemums also root faster (Paulitz et al. 1985). In such tests, cuttings usually root at least 2 days earlier in the presence of *Trichoderma* spp.

Fig. 9. Increased rooting of carnation cuttings induced by *Trichoderma harzianum*.

When chrysanthemum cuttings were treated in the propagative bed with the peat-bran formulation, there was not only increased rooting, but the transplants subsequently had significantly greater heights, dry and wet weights, and numbers of flower buds (Chang et al. 1986). Currently, tests are underway to determine effects on subsequent carnation flower production by treatment in propagative beds with the peat-bran formulation. This exploits the exciting possibility that rooted cuttings may carry in the root ball an increased growth response factor evident later during flower production.

Extensive experimentation involving application of *Trichoderma* spp. to higher plants has been done over a wide range of environmental conditions in growth chambers or university and commercial greenhouses. Occasionally, little or no increased growth responses were observed from treatment with *Trichoderma* spp.

Trichoderma - plant interactions and its effect
on increased growth response

Germination of seedlings. Seeds of five kinds of vegetables were sown in soil. Trichoderma harzianum (T-203) was applied to the soil as: a peat-bran preparation (5×10^6 g/soil), conidial suspension (5×10^6 g/soil) or as seed coating ($10^4 - 10^5$ per seed).

It was found (Table A) that the peat-bran preparation in soil induced significant increases of all the vegetable seedlings and the conidial suspension induced significant increases only in bean, radish and cucumber at final emergence. However, seed coating induced significant increase only in tomato. The polysaccharide 'Pelgel' which was used for coating had no effect at all. Responses were observed when T-203 was applied to petri-dishes, wet filter paper, as seed coating (10^5 conidia/seed), 1 ml conidia suspension (10^6 conidia/ml), or seed coating and conidia suspension together (Fig. A). The effect was observed on the germination development percentage and on the final germination rate.

TABLE A. Germination of vegetable seedlings when Trichoderma harzianum (T-203) was added to soil as peat-bran preparation, conidial suspension or seed coating.

Plant	Control	Germination of seedlings (%)		
		Peat bran preparation	Conidial suspension	Seed coating
Bean	74	100 ^a	100 ^a	77
Radish	65	100 ^a	100 ^a	58 ^a
Tomato	85	100 ^a	70 ^a	100 ^a
Pepper	85	95 ^a	90	90
Cucumber	90	100 ^a	100 ^a	90

a Differs significantly from the control (LSD = P = 0.05). Means of 6 replicates.

b Germination of bean, radish, tomato and cucumber, 5 days after planting. (Pepper, 12 days after planting.

Fig. A. Germination of pepper seeds in petri-dishes. *Trichoderma harzianum* T-203 were added to seeds. ○ - control, ● - conidial suspension, Δ - seed coating, ◻ - seed coating + conidial suspension. Values in each day followed by the same letter do not differ significantly ($P = 0.05$).

Effect of the preparation components

In order to separate the effect of the *Trichoderma* from the secondary effect of the peat-bran, we added to soil: autoclaved peat-bran, peat bran preparation and conidial suspension (Fig. B).

The peat bran preparation and conidial suspension were found to be the best treatments, showing a significant increase in germination, seed length and leaf area of pepper. Peat bran and the autoclaved preparation also induced increased growth response in pepper (Fig. B).

Growth response in different plant growth substrates.

A water suspension of conidia (5×10^6 ml) of isolate T-203 was applied to 5 different plant growth substrates; sandy loam soil, autoclaved soil, vermiculite, peat-vermiculite (1:1, v/v) and peat. There was a significant increase in dry weight of cucumber, 15 days after sowing, in all the treated media (Table B). The growth response was most prominent in natural sandy loam soil (43% increase) while the increase of dry weight in autoclaved soil was only 26%. Conidia applied to vermiculite had little effect on dry weight (17%).

Fig. B: Germination, length and leaf area of pepper 30 days after planting and treated with *Trichoderma* preparation (T-203), and the components of the preparation.
 □ - control, ▨ - peat bran preparation, ▤ - conidial suspension, ▩ - autoclaved peat-bran, ▪ - autoclaved preparation.
 Values followed by the same letter do not differ significantly at $P = 0.05$.

Table B: Dry weights of cucumber plants 15 days after planting and treated with conidia of *Trichoderma harzianum* T-203 at 5×10^6 /ml of media

Plant growth substrate	Dry weight (g/plant)		% increase
	Control	Treated	
Sandy loam soil	A 0.14 a	B 0.20 b	42.8
Autoclaved soil	A 0.19 a	B 0.24 c	26.3
Vermiculite	A 0.12 a	B 0.14 a	16.7
Peat	A 0.13 a	B 0.16 a	23.1
Vermiculite/peat (1:1, v/v)	A 0.20 b	B 0.27 d	35.0

Numbers followed by the same letter do not differ significantly ($P = 0.05$). Capital letters between the columns and small letters within a column.

Cucumber and tomato plants which were grown in Hogland solution responded to an application of T-203 to roots. The effect was not consistent but there was an increase of 20% in length and 10% in dry weight of seedlings, 21 days after planting.

Presence of Trichoderma in roots

Trichoderma harzianum was found in roots of plants growing in soil previously treated with the fungus. After washing and disinfecting the roots it is possible to see the hyphae of the fungus under direct microscopy (Fig. C) and indirect observation after incubation of root segments on selective medium (Fig. D).

Until 20 days after planting, the population of roots infected by Trichoderma varied from 40-90% between the different test plants (Fig. E).

Fig. C: Trichoderma hyphae in pepper root treated with conidial suspension (x 400).

T. harzianum isolate T-203 increased growth response in vegetables from the time of germination until 30 days after planting (Table A). These results were similar to previous observations. Responses were determined by measuring the length and leaf area of pepper and dry weight of cucumber plants.

The most prominent effect was observed in the peat-bran preparation treatment while seed coating was the least effective. This can be explained by the small amount of fungus applied to soil by this treatment (10^4 - 10^5 cfu/seed) resulting in a soil population of only 10^3 cfu/gr. The population of the fungus in all the other treatments was 10^6 cfu/gr according to previous works.

Fig. D: Trichoderma hyphae grow from pepper roots treated with conidial suspension (up)

The effects of the peat-bran preparation are apparently due to the effects of the fungus, metabolites of the fungus or degradation productions of peat-bran after fungal growth (Fig. B).

The increased growth response of plants caused by Trichoderma depends on the ability of the fungus to survive and develop in the rhizosphere. The peat-bran is a food base reservoir for the fungus and this gives the Trichoderma advantage over Trichoderma applied to soil as a conidial suspension. The ability of the Trichoderma to induce growth response in plants varied with different substrates (Table B).

The increase in dry weight of cucumber grown in sandy loam soil after autoclaving and addition of a conidial suspension of 10^6 cfu/gr was 26.3% as compared to 42.8% without autoclaving. This effect can be explained by the ability of the Trichoderma to inhibit minor pathogens in the rhizosphere.

Trichoderma is a known biocontrol agent. When it is introduced to the soil it is possible that Trichoderma inhibits pathogenic but not non-parasitic microorganisms in the rhizosphere, or it may destroy the phytotoxins produced by these microorganisms.

The increase growth response caused by Trichoderma is not only a result of the protection against minor pathogens, but these effects are also observed in semi-sterile plants which were grown in Hogland solution. Trichoderma had a direct effect on plants grown without soil.

The effects of the fungus were not consistent because of difficulties in growing the fungus in this media, however there was an increase of 20% in the length and 10% in dry weight of cucumber and tomato plants, respectively.

Mycorrhizae fungi that form symbiotic associations with plant roots enhance the growth of plants. A fungal sheath or mantle forms over the roots and hyphae penetrate between cells of the outer cortex.

In our study it was observed for the first time that the Trichoderma like mycorrhizae fungi penetrate and live in plant roots (Figs. C, D.).

It is clear to us that upon interaction between Trichoderma and plants, the effect on plants involves more than one mechanism. Understanding this mechanism may improve the use of the fungus in inducing increased growth response of various crops besides its use as a biocontrol agent.

Fundamentally, Trichoderma spp. suffer from the defects common to all exploited biological entities, namely, the deleterious influence of unfavourable environment on their activity (Baker 1986). They require appropriate substrates (Baker et al. 1984), must colonize infection courts (Harman et al. 1980) and absorptive tissues of roots.

(Ahmad and Baker 1986) and should be active in unfavorable environmental conditions-- particularly temperature (Harman et al. 1981), moisture and soil pH (Chet and Baker 1980, 1981, Liu and Baker 1980). Numerous strategies have been proposed to make such agents more efficient and to increase their activities in unfavorable environments (Baker 1986, Baker and Scher 1986); however, one obvious solution to the problem, as it is related to the increased growth response, is to apply Trichoderma spp. where plant growth environments correspond to the optimum conditions for activity of these fungi. Many of these conditions are met in the ornamental industry which utilizes environmentally controlled, covered structures. Therefore, most attempts at commercialization of the product have been done in the greenhouse industry. Here, temperatures are controlled. Soil mixes provide favorable soil moisture potential. Acid peat is usually a component of soil mixes, and Trichoderma spp. usually function best at such soil reactions (Chet and Baker 1980).

Even so, variable degrees of responses were observed in commercial tests. A candidate source of such variability was the different media used to support plant growth by growers. Almost all of the media contain peat and vermiculite mixed in various proportions by growers or commercial distributors. The influences of mixes on the increased growth response were measured by mixing peat and vermiculite in various ratios to form 0% to 100% peat mixes (Paulitz et al. 1986). Greatest increases were observed when T. harzianum was added to mixes containing 20% or 80% peat. Smallest increases occurred in 0% or 100% peat mixes.

Other factors also influence responses (Windham et al. 1985a,b). Responses attributed to T. harzianum were observed in tomatoes at soil pH of 5.5 or 6.2 but not at 7.8. Radishes treated with the fungus, however, had significantly greater growth over this entire range of soil reactions. Predictably, some Trichoderma spp. and strains stimulate plant growth better than others. Increases in dosage of the agent provided corresponding increases in plant growth (Baker et al. 1984); however, the peat-bran formulation applied to provide 10^8 cfu/g soil induced stunting of eggplants (Baker 1988).

Hypotheses have been advanced to explain why microorganisms may promote growth of plants. Effects could result from inhibition and alteration of normal root microflora. Direct effects of growth-stimulating substances (hormones, growth factors), nutrient availability or stimulation of nutrient uptake could be involved. There is also the possibility that low levels of substances in soil inhibitory to plant growth could be decreased in concentration.

Evidence for the hypothesis that growth-stimulating soil microflora antagonize minor pathogens (Salt 1978) whose activities are insidious (i.e., they parasitize root tips and cortical cells, thereby inducing no obvious symptoms except to reduce growth and yield) is substantial. Over two decades ago, Wilhelm (1965) identified Pythium ultimum as a major component of minor pathogen activity in strawberries. Soil fumigation reduced activity of Pythium spp. and increased growth in comparison with soils not fumigated resulted.

Many root-colonizing bacteria are known to produce auxins, gibberellins, and cytokinins. Such hormones are not involved in the action of plant growth-promoting rhizobacteria like the fluorescent pseudomonads, however. In this case, growth promotion was not observed in gnotobiotic plant systems in which only the bacteria were introduced (Kloepper and Schroth 1981). Increased growth was only observed when small volumes of field soil (presumably containing minor pathogens) was reintroduced in the presence of the bacteria. Indeed inhibition of plant growth was correlated with production of indole acetic acid in culture by bacterial strains deleterious to root elongation of sugar beet (Loper and Schroth 1986). Thus, the mechanisms of action of

fluorescent growth promoting pseudomonads appears to be involved with antagonism to minor pathogens.

In contrast, growth responses induced by Trichoderma spp. appear to be due to both the control of minor pathogens and production of a growth-regulating factor. The impact of the component of control of minor pathogens is currently being explored and appears to be substantial (Ahmad unpublished). The inhibition of "root nibblers" like Pythium spp. could be caused by an antibiotic produced by Trichoderma spp. detectable only at the microscopic level (Lifshitz et al. 1986). Since the initial reaction of P. ultimum is a microsecond retreat of the protoplasm from the hyphal tip, the phenomenon was named the routing factor. This system counterpoises factors that operate against significant production of antibiotics in soil (Baker 1985). The food base for activity of Trichoderma spp. could be substantial enough for production of the routing factor. Indeed, rhizosphere-competent mutants were found to population densities approaching 10^6 cfu/g of rhizosphere soil at root tips of plants (Ahmad and Baker 1987b). Again, interactions occurring between Pythium spp. and the antagonist would occur on the rhizoplane of the root tip where there is some isolation from charges on colloidal material that could adsorb antibiotics.

There is direct evidence for a growth-promoting factor (Windham et al. 1986). Trichoderma spp. added to previously germ-free radish plants under gnotobiotic conditions induced production of higher dry weights than germ-free controls. Rate of seed germination in maize, tomato and tobacco was increased by 1-2 days compared with controls where Trichoderma spp. were not present when seeds were separated from the fungi by a cellophane barrier. The growth-stimulating factor has been extracted from Trichoderma spp. and is currently in the process of being chemically identified.

The problem of delivery of biocontrol agents to plant material is often the principal deterrent factor in commercial exploitation of the product. Most Trichoderma spp. grow well in organic media, however, and a large biomass can be produced in 7-10 days. This suggests that the product can be packaged with the medium previously used for culturing and this applied in small proportions to planting media. Thus, the agents are added to soil together with organic material which insures greater activity in inducing growth enhancement and/or biocontrol. We have used a peat-bran/water mixture (1:1:0.7 v/v/v) developed in Israel (Hadar et al. 1979). Peat is used to maintain an acid pH, but it evidently contains fungi that may be thermophilic since repeated autoclaving of the mixture is necessary to eventually produce sterile medium to support axenic cultures of Trichoderma spp. In approximately 7 days, the culture may be air-dried, pulverized, passed through a 2-mm diameter screen, and stored. Sivan et al. (1984) stored peat-bran formulations for 1 yr and observed an approximately 91% decrease in population density from 4.9×10^9 to 4.5×10^8 cfu/g of medium. Even after such storage, however, this population density is high enough to achieve biological control (Liu and Baker 1980) or increased growth response (Baker et al. 1984) when applied at the rate of 1% to soil.

Cost estimates as low as \$0.40/lb have been made for commercial production of the peat-bran formulation. This could be sold to growers for \$1.00/lb to provide sufficient profit. Thus, a grower who mixes the formulation at a rate of 1% into his potting mixes would pay \$0.002 to \$0.02/plant unit in a typical bedding plant operation.

Seed treatment (Harman et al. 1980, 1981, Lifshitz et al. 1986) is another hypothetical strategy for delivery of Trichoderma spp. to a developing plant. It should be much more cost-effective than broadcast application of the peat-bran, germling or alginate pellet formulations. However, wild-types of Trichoderma spp. as well as other fungal biocontrol agents are not rhizosphere-competent; that is, they do not colonize the other surface of developing roots when applied to seed.

Recently, mutation of Trichoderma spp. has been accomplished so that the mutant strains are rhizosphere-competent (Ahmad and Baker 1987b). One reason for the attribute of rhizosphere-competence is the ability of the mutants to produce higher quantities of cellulase than wild-types (Ahmad and Baker 1987a). Such strains utilize the mucigel of the roots which contains the remains of the primary cell walls of the epidermis (Foster et al. 1983). Thus, rhizosphere-competent mutants could be placed on seeds and growth with the roots by utilization of a substrate that is produced by the tissues and sloughed off in the normal course of events. The mutant fungus has been present at almost 10^6 cfu/g rhizosphere soil (Ahmad and Baker 1987b) at the root tip; therefore, it could produce the growth stimulating factor (Windham et al. 1986) at this site as well as protect against minor pathogens. This could be an exceedingly efficient delivery system.

In this report, reference will be made to a rhizosphere-competence (RI) index (Ahmad and Baker 1987a):

$$RC \text{ index} = \frac{\sum_{i=1}^n [\log (p_i + 1) \cdot \ln (d_i + 1)]}{n}$$

where p = population density per milligram of rhizosphere soil, d = root depth, and n = total root length. By use of such an index, data can be subjected to analyses of variance and/or regression analyses more easily and means separated with an FLSD.

Seed treatment with conidia of rhizosphere-competent mutants of T. harzianum reduced the incidence of preemergence damping-off of barley, cucumber, pea, radish, and tomato induced by P. ultimum (Ahmad and Baker 1988c). Wild-type parents of these mutants were less effective in control. When rhizosphere-competent mutants were applied to seed or when a peat-bran preparation was added to soil, the resulting plants produced significantly higher fruit weight and higher dry weights than those treated with rhizosphere-incompetent wild-types and controls (Fig. 10). Seed treatment with mutants increased the incidence of emergence and resultant plant growth was significantly better than when mutant strains were added to soil in peat-bran (Fig. 11). There was, however, no significant difference between the two types of application of the wild-types. When cucumber seeds treated with a T. harzianum rhizosphere-competent mutant (T-95) (Fig. 12) or its parent wild-type (WT) were sown in raw soil kept under constant matric potential with no additional water added, the roots grew 8 cm in 8 days (Fig. 13). Untreated seeds produced roots 7 cm long. Fewer cfu of P. ultimum per mg were isolated from rhizosphere soil of the T-95 treated seedlings than in the untreated controls and those treated with WT. P. ultimum was not detected in the 8th-cm (farthest from seed) root segment of T-95 treated seeds, whereas the last cm of root segment from untreated and WT treated seeds yielded 3000 cfu/g rhizosphere soil.

Germination of sporangia of P. ultimum was reduced considerably in rhizospheres of cucumbers when a rhizosphere-competent strain (T-95) of T. harzianum was applied to cucumber seeds in comparison to seeds treated with the parent wild-type (WT) or non-treated controls (Fig. 14).

These studies (Ahmad and Baker 1988c) suggest that the attribute of rhizosphere-competence in a biocontrol agent potentially contributes to the enhancement of efficiencies, plant growth and increased yield.

Fig. 10. Effect of caryopses or seed treatment and peat-bran application of *Trichoderma harzianum* strains on A, dry weight of 2-wk-old barley shoots, B, dry weight of 8-wk-old cucumber shoots, C, fresh weight of cucumbers per plant, D, average number of cucumbers per plant, E, dry weight of 8-wk-old pea shoots, F, average number of pods per pea plant, G, fresh weight of pods, H, fresh weight of 8-wk-old radish roots, and I, dry weight of 9-wk-old tomato shoots. Bars in each figure labelled by the same letters are not significantly different ($P = 0.05$).

Fig. 11. Effect of caryopses or seed treatment and peat-bran application of *Trichoderma harzianum* strains on emergence of A, barley (4 days after seeding), B, cucumber (4 days), C, pea (6 days), D, radish (4 days), and E, tomato seedlings (5 days). Bars in each figure labelled by the same letters are not significantly different ($P = 0.05$).

Fig. 12. Population density of *Trichoderma harzianum* strains in the rhizosphere soil of root-tips taken from 9-wk-old tomato plants. Bars labelled by the same letters are not significantly different ($P = 0.05$).

Fig. 13. Spatial distribution (A) and population index (B) (Ahmad and Baker 1987b) of *Pythium ultimum* in the rhizosphere soil of 8-day-old cucumber seedlings grown from seeds treated with *Trichoderma harzianum* strains T-95 and WT. Bars labelled by the same letters are not significantly different ($P = 0.05$).

Fig. 14. Percent germination of *Pythium ultimum* sporangia on a membrane placed on A, cucumber seed and B, rhizosphere soil of cucumber seedlings, treated with *Trichoderma harzianum* strains T-95 and WT. Bars labelled by the same letters are not significantly different ($P = 0.05$).

There are at least three factors that may be connected with the development of rhizosphere-competence by mutants of *Trichoderma* spp. The first of these is obvious; they must have a linear growth rate equal to or greater than a developing plant root. The strains of *T. harzianum* have growth rates greater than roots at least at soil temperatures greater than 19 C. Mutants were selected originally for benomyl-resistance which may or may not be associated with rhizosphere-competence. Finally, evidence (Ahmad and Baker 1987a) suggests that a key factor is the increased production of cellulase by the mutants so that utilization of the mucigel on the root is possible.

Two species, *T. viride* and *T. polysporum*, were sent to us from Sweden. These were being used as biocontrol agents and the suppliers wished to enhance their efficiency by mutations to rhizosphere-competence. There was some concern about accomplishing this task since *T. polysporum*, especially, is a slow-growing species. Thus, mutations would not only be necessary for increased cellulase production but for increased linear growth rate as well. *T. koningii* also has a slow growth rate. A report by Ahmad and Baker (1988b) is reviewed here.

One of 13 mutants of *T. harzianum* (WT) and three of 12 mutants of the same species (T-12) were benomyl-resistant but not rhizosphere-competent (Fig. 15). All mutants of *T. koningii* (T-8) were rhizosphere-competent (Fig. 15C) and had significantly higher linear growth rates (12.5 mm/day) than their parent (7.5 mm/day).

From over 3000 mutants, two strains each of *T. polysporum* and *T. viride* were selected for benomyl tolerance and linear growth rates that appeared to be higher than their wild-type parents. When statistical analyses were applied, the mutants of *T. polysporum* had significantly higher growth rates (12.5 mm/day) when compared to the wild type (6 mm/day); however, growth rates of the mutants of *T. viride* were not significantly different from the wild-types (7.5 mm/day). Growth rate of *T. viride* approached that of the cucumber root which is approximately 1 cm/day at 25 C (Ahmad and Baker 1987a). The mutants of *T. polysporum* and *T. viride* were rhizosphere-competent (Fig. 2A,D,E).

Fig. 15. Rhizosphere-competence (RC) index of *Trichoderma* spp. and strains A, benomyl tolerant mutant strains of *T. harzianum* (WT), B, benomyl tolerant mutant strains of *T. harzianum* (T-12), C, benomyl-tolerant mutant strains of *T. koningii* (T-8), D, benomyl tolerant mutant strains of *T. polysporum* (T.P.) and E, benomyl tolerant mutant strains of *T. viride* (T.V) compared with their respective wild-type parents.

These results suggest that benomyl tolerance is not necessarily associated with rhizosphere-competence (Fig. 15A,B). Also, it was possible to mutate species with relatively slow linear growth rates like *T. polysporum* and *T. koningii*, so that they were rhizosphere-competent (Fig. 15C,D,E).

Previously, a direct correlation between cellulase production, biomass from growth on cellulose or competitive saprophytic ability on cellulose substrates in soil with the degree of rhizosphere-competence among strains of *Trichoderma* spp. was reported (Ahmad and Baker 1987a). To determine whether this was also an attribute of the *Trichoderma* spp. and strains tested here, biomass resulting from either glucose (1% w/v) or cellulose (cotton linters, Sigma, 1% w/v) was measured after 6 days in a medium supplemented with minerals at comparable levels to Czapek-Dox medium. All rhizosphere-competent strains of *Trichoderma* spp. produced significantly higher biomass than their wild-type parents or the nonrhizosphere-competent mutant strains when cotton linters were the sole carbon source (Fig. 16A-E). No significant differences in biomass were observed when glucose was the sole carbon source.

This study supports findings (Ahmad and Baker 1988b) that strains of even slow-growing *Trichoderma* spp. can be induced to become rhizosphere-competent by appropriate mutations. Further, it provides evidence for the hypothesis that rhizosphere competence of the mutants can be explained by their increased capacity to utilize cellulose substrates on or near the rhizoplane.

Fig. 16. Dry weight (g) of mycelium of strains of *Trichoderma* spp. A, *T. harzianum* (WT), B, *T. harzianum* (T-12), C, *T. koningii* (T-8), D, *T. polysporum* (T.P.), and E, *T. viride* (T.V.), grown for 6 days in Czapek-Dox broth with 1% cotton linters or glucose as sole carbon source. All rhizosphere-competent mutants (see Fig. 20) produced significantly ($P = 0.05$) higher biomass than the wild-type parents or nonrhizosphere-competent mutants.

The RC index of mutant T-95 on cucumber seedlings from seeds treated with conidia at $\log_{10}$ 1, 2, 3, 4, 5, and 6 per seed is presented in Fig. 17 (Ahmad and Baker 1988c). A linear relationship (significantly higher than zero) between the RC index and population densities on seed was observed. Thus, the more conidia added to a seed, the greater the population density of the antagonist in the rhizosphere.

Fig. 17. Relation of rhizosphere-competence (RC) index of *Trichoderma harzianum* strain T-95 to the population density applied on the seed.

Typical RC indices of repeated experiments with homogeneous variances at various soil matrix potentials are presented in Fig. 18. RC index at -0.03 bars was significantly higher than -0.01, -0.02, and -0.10 bars. However, there were no significant differences among RC indices at soil matrix potentials -0.01, -0.02, and -0.10 bars. Population densities of strain T-95 per milligram rhizosphere soil of cucumber seedlings at each soil matrix potential are presented in Fig. 19.

Fig. 18. Rhizosphere-competence (RC) index (Ahmad and Baker 1988b) of *Trichoderma harzianum* strain T-95 at various soil matrix potentials.

Fig. 19. Population densities per milligram rhizosphere soil of *Trichoderma harzianum* strain T-95 at various soil matrix potentials (denoted by numbers at the top left corner of each figure).

Strain T-95 was rhizosphere-competent at all soil matric potentials. Colonization of roots by seed treatment could result from conidia being moved by gravimetric movement of water (Chao et al. 1986) or by possession of the attribute of the rhizosphere competence (Ahmad and Baker 1987b). In this study, gravimetric movement of water would have been highest at soil matric potential of -0.01 bars and lowest at -0.10 bars; however, the RC index of T-95 at -0.01 bars was not significantly different than at -0.10 bars (Fig. 18). Further, the population density at the root-tip was significantly higher at -0.10 than at -0.01 bars (Fig. 19). This suggests that the downward movement of conidia of T-95 by gravitational movement of water was not necessary for the agent to be found in the rhizosphere.

The highest RC index was observed at a soil matric potential of -0.03 bars (Fig. 18), which suggests that this was near optimum for activity of T-95 in the rhizosphere. The lowest RC index occurred at -0.01 bars, and this was reflected in the significantly reduced population density in the rhizosphere at all other matric potentials (Fig. 19). This may reflect effects of anaerobic conditions and high soil moisture on root physiology at -0.01 bars (Burstrom 1965), or a direct effect on the growth rate of strain T-95.

A knowledge of mechanisms can lead to intelligent approaches to enhancement of biocontrol agents (Baker and Scher 1986). A peculiar anomaly presented an opportunity to explore mechanisms associated with biocontrol of Trichoderma spp. Despite the similarity in disease suppression in preemergence damping-off induced by Rhizoctonia solani and by Pythium spp., we found it difficult to generate suppressiveness in soil to the latter pathogen when Trichoderma spp. were mixed into conducive soil; only seed treatment was successful in control. In contrast, suppressiveness to R. solani was induced by both seed and soil treatment.

Biological control induced by seed treatment with Trichoderma spp. against pathogenic species of Pythium could result from mycoparasitism, competition for nutrients in seed exudates, and/or antibiosis.

Harman et al. (1980) suggested that mycoparasitism was the principal mechanism of Pythium damping-off when seeds were coated with Trichoderma hamatum. Their suggestion was based on evidence that in the presence of Pythium spp., T. hamatum was capable of producing the hydrolytic enzymes β -1,3-glucanase and cellulase and on observation of hyphal parasitism in vitro on Pythium spp. (Chet et al. 1981). Another indication of the involvement of mycoparasitism was provided by Elad et al. (1981), who showed that the ability of isolates of T. harzianum to control damping-off caused by P. aphanidermatum was correlated to the level of hydrolytic enzyme production in soil.

Considerations related to the time sequence of the host-pathogen interaction, inoculum potential relationships, and collocation of the biocontrol agent and pathogen in the infection court suggest that mycoparasitism is not a likely mechanism in the control of Pythium damping-off by seed treatment with at least some isolates of Trichoderma spp. A fundamental observation leading to this conclusion is that seed treatment with isolates of Trichoderma spp. was effective in controlling both Pythium and Rhizoctonia damping-off, whereas mixing these same biocontrol agents in soil suppressed R. solani but not Pythium spp. (Chet and Baker 1981, Harman et al. 1980, Lifshitz and Hancock 1981). How can this phenomenon be explained?

First, inoculum densities of the two pathogens found in nature are exceedingly different. R. solani was isolated from field soils below 1-10 propagules per gram (Henis et al. 1978, Ko and Hora 1971), whereas inoculum densities of Pythium spp. were considerably higher. R. solani compensates for such low inoculum densities by having a relatively high linear growth rate (Baker 1978). Evidence summarized by Baker and Drury (1981) also suggests that propagules of R. solani respond to seed exudates at some

distance from the host infection court. This provides Trichoderma spp. with the opportunity to "intercept" this pathogen as it grows toward the host infection court; relatively few propagules of R. solani touch the seed coat, thereby avoiding antagonism in the volume of the spermosphere. In contrast, propagules of Pythium spp. may have to touch the infection court (Stasz et al. 1980, Baker and Drury 1981) to succeed in infecting the host; relatively high inoculum densities, in this case, ensure that an adequate proportion of the total inoculum in the soil is adjacent to the seed. Therefore, biocontrol agents must interpose a barrier between the propagules of the pathogen and the host, namely, a coating of the agent on the seed.

Trichoderma spp. parasitized hyphae of isolate P. ultimum in vitro; the time restraints, however, are critical for mycoparasitism to occur (Lifshitz et al. 1986). Hyphal coilings, suggesting early stages of mycoparasitism, were observed rarely and occurred only after 24 hr from the time mycelial contact was made. This imposes a time limit for mycoparasitism to occur, since our results confirmed the observations of Stasz et al. (1980) that seed coat colonization by Pythium spp. occurs within 10 hr and substantial embryo infection occurs within 24-48 hr in a susceptible pea cultivar. In addition, the time available for biocontrol agents to parasitize Pythium spp. is mediated by the time required for germination of propagules of Trichoderma spp. Whereas sporangium germination occurred within 90 min after introduction into the infection court, conidia of T. harzianum required 10-14 hr for over 50% germination at 26 C; these isolates still protected seed from damping-off at this temperature, however. It is therefore doubtful that mycoparasitism fully explains the mechanism of antagonism involved in seed treatment with Trichoderma spp. for control of Pythium damping-off.

Competition for nutrients in seed exudates also is a candidate for the mechanism of biological control of Pythium damping-off by seed treatment. The concentration of seed exudates affects successful infection. Our study (Lifshitz et al. 1986), however, suggests that competition for seed exudates is not an important antagonistic mechanism based on the following: i) Addition of nutrients (glucose and asparagine) to the seeds did not affect successful disease control; and ii) The presence of Trichoderma spp. on seed did not reduce germination of sporangia or chlamydospores in the spermosphere. These observations suggest that nutrients required for germination, penetration, and infection by the pathogen were not limited by the activity of Trichoderma spp. on the seed coat.

Fungitoxic action of Trichoderma spp. on pathogenic Pythium spp. was first reported by Allen and Haenseler (1935) and later by Dennis and Webster (1971a,b). Nevertheless, antibiosis was not considered in later reports on biological control of Pythium damping-off by Trichoderma spp. In our study (Lifshitz et al. 1986), toxic metabolites were produced in vitro by seven effective biocontrol isolates of Trichoderma. In addition, stress signs were obvious in hyphae of P. ultimum grown in dual culture 24 hr before a mycoparasitism reaction was observed. This was noted, however, only at a microscopic level and would not have been evident by macrobiotic observations, conventionally used in screening. Because the protoplasm in the hyphal tips of isolate N1 responds to the Trichoderma-produced factor by being disorganized and "thrown into confused light," we suggest it be named the routing factor.

The saprophytic activity of Pythium spp. in soil generally is restricted to early colonization of substrate in advance of other microorganisms. The inability of Pythium spp. to compete as a secondary colonizer was attributed to their great sensitivity to toxic metabolites produced by other microorganisms (Barton 1961, Garrett 1956). Because the interaction between Trichoderma spp. and isolate N1 occurred on dead seed coats, and because such isolates were capable of producing metabolites toxic to the pathogen, we suggest that production of toxic metabolites by Trichoderma spp. on seed coats is the principal mechanism of biological control of Pythium damping-off.

Pythium nunn -- a new biocontrol agent

A potent antagonist was characterized and described as a new species. The agent was discovered by accident in an experiment to test whether weekly addition of bean leaf meal would induce suppressiveness to Pythium spp. due to increased activity of Trichoderma antagonists (Liu and Baker 1980) similar to that observed during radish monoculture for suppression of Rhizoctonia damping-off (Henis et al. 1978). Suppressiveness developed; however, there was no evidence that Trichoderma spp. were involved. Rather an antagonist, Pythium nunn (Lifshitz et al. 1984c), increased in population density whereas P. ultimum was reduced to low levels after five weekly applications of bean meal (Lifshitz et al. 1984b). Rampant mycoparasitism was observed by use of SEM and light microscopy in vitro (Lifshitz et al. 1984a) involving hosts like Pythium spp., Phytophthora spp. and R. solani.

Early lysis of germinating sporangia of P. ultimum exposed to soil containing P. nunn was observed (Lifshitz et al. 1984b). This could be interpreted as a reaction by P. ultimum to antibiosis; however, subsequent studies by Paulitz and Baker (1988a) suggested that competition by ectoparasitism and/or preemption of substrate sites was a more likely explanation.

Clark (1965) concisely stated the principle involved in competition: "basically competition among microorganisms is for substrate in the specific form and under the specific conditions in which that substrate is presented." P. ultimum is a primary colonizer (Garrett 1970) and becomes active (sporangia germinate) in response to the presence of available substrates. The following interpretations of the ecological interactions between P. nunn and P. ultimum are based on principles elaborated by Griffin (1981) from his studies on the influences of soil nutrition on formation of chlamydospores from germinating macroconidia of Fusarium spp.

Fungistatic factors impose dormancy on fungal propagules in soil (Lockwood 1964). Propagules are typically aroused to activity by the presence of available carbon. After this stimulation, the subsequent fate of a soil microorganism, P. ultimum, depends on a number of ecological factors. The presence of P. nunn substantially influences the different possible pathways of development of P. ultimum by interactions as follows (Baker 1988a):

- i) When activity of P. nunn was increased by addition of appropriate substrate, early lysis of germinating sporangia of P. ultimum occurred (Lifshitz et al. 1984b). Such phenomena were considered by Garrett (1965) to be due to autolysis induced by starvation.
- ii) Germ-tubes of sporangia of P. ultimum that had not lysed typically produce new (secondary) sporangia. More aborted small sporangia were formed in soil and were fewer in total number where P. nunn was active than in controls (Lifshitz et al. 1984b). Such reproduction following germination was described by Griffin (1981) and attributed to deficiency in carbon substrates.
- iii) Paulitz and Baker (1988a) amended soil with increasing concentrations of bean leaves, oatmeal, glucose plus L-asparagine, or glucose alone. There was a significant negative correlation between the concentration of these substrates added to soil and the formation of secondary sporangia by P. ultimum. Thus, the concentration of substrates determines whether reproduction (at low carbon concentrations) occurs or whether vegetative growth continues when adequate carbon is available. The impact of the activity of P. nunn was apparent in that the formation of secondary sporangia by P. ultimum in soil amended with the antagonist was significantly greater after substrates were added than when the antagonist was not present. Therefore, vegetative growth and colonization of substrates by P. ultimum was inhibited by the addition of P. nunn.

Based on these phenomena, an explanation is now possible for the suppression of *P. ultimum* by *P. nunn* when carbon substrates are added to soil. When bean leaves were added to soil (at 26 C, pH 7.3) at weekly intervals, inoculum density of *P. ultimum* increased almost tenfold during the first week but thereafter declined to an original or lower level after 6 wk (Lifshitz et al. 1984b). During this same period there was a fourfold increase in the population density of *P. nunn*. Also, organic amendments generally increased frequency of damping-off by *P. ultimum*, but no increase in disease incidence occurred when *P. nunn* was added with these (Paulitz and Baker 1987a). Such observations suggested that *P. nunn* depresses the competitive saprophytic activity of *P. ultimum* by competition and/or ectoparasitic activity which initially decreased its inoculum potential without (necessarily) decreasing inoculum density (Baker 1977) since secondary propagules may be produced (Paulitz and Baker 1988a). Subsequently, however, inoculum densities over time may decline due to mycoparasitic activity of *P. nunn*.

The scenario involving *Trichoderma* spp. contrasts sharply with that existing for *P. nunn*. In the former case, organic amendments apparently are not necessary for increases in activity and thallus production. Repeated replants of radish induced suppressiveness to damping-off (Henis et al. 1978) and decreases inoculum density of *R. solani* (Liu and Baker 1980). There was no change in conduciveness, however, when only radish was planted or when the pathogen was added alone (Henis et al. 1979). This suggested that the radish was necessary for *R. solani* to convert from dormant sclerotia resistant to mycoparasitism to vegetative thallus production susceptible to parasitism by *Trichoderma* spp. (Baker 1982). Indeed, during radish monoculture in the presence of *R. solani*, the population density of *T. harzianum* increased from nondetectable levels to almost 10^6 cfu/g of soil (Liu and Baker 1980), and suppressiveness developed whether or not organic matter radish residues were screened out between successive crops (Wijetunga and Baker 1979). Another evidence for the ability of *Trichoderma* spp. to increase their biomass principally by mycoparasitism was provided by Clevenstein (1986). Increases in population density of the antagonists was observed when an avirulent strain of *R. solani* was added to soil supporting radish growth but no increase occurred where it was not added.

All this suggests that *P. nunn* can compete saprophytically with a plant pathogen like *P. ultimum*. Recent studies (Paulitz and Baker 1988b), however, provide evidence that *P. nunn* is more than just a primary colonizer gaining possession of a substrate before *P. ultimum*.

P. nunn occupied bean leaves previously colonized by *P. ultimum*. For example, after 6 hr in a naturally-infested soil, 20% of the bean leaves were colonized by *P. ultimum*, and none by *P. nunn*. This is predictable, because sporangia of *P. ultimum* germinate in 1-3 hr (Stanghellini and Hancock 1971), while oospores of *P. nunn* germinate in 9 hr (Paulitz, unpublished). Also, the linear growth rate of *P. ultimum* is almost twice that of *P. nunn* at 27 C. Therefore *P. ultimum* colonized the substrate first. However, after 18 hr, 60% of the bean leaf fragments were co-colonized by both *P. nunn* and *P. ultimum*, even though the inoculum density of *P. nunn* was less than 10 cfu/g. Again, bean leaves pre-colonized by *P. ultimum* for 24 hr were still capable of being colonized by *P. nunn* at a high frequency. This ability of *P. nunn* to colonize substrates previously colonized by *P. ultimum* is unlike the *P. ultimum*-*P. oligandrum* system. Martin and Hancock (1986) reported a low frequency of co-colonization. They suggested that in the suppressive soils studied, *P. oligandrum* had higher relative propagule densities, and that organic substrates were first colonized by *P. oligandrum*, thus preventing the subsequent establishment of *P. ultimum*. Our work suggests that *P. nunn* can function as both a primary and secondary colonizer of substrates already occupied.

P. nunn displaced *P. ultimum* from the substrate 12-24 hr after colonization. This effect was more pronounced in soils artificially infested with *P. nunn*, but was evident

in naturally-infested soil after 48 hr. This displacement of P. ultimum from the substrate by P. nunn might explain the results of Lifshitz et al. (1984b), in which the population densities of P. ultimum declined with the repeated additions of bean leaves to the soil. This phenomenon could be due to competition between the two fungi on the substrate, which reduces the activity and leads to reduction in the formation of sporangia by P. ultimum. This reduction in the frequency of colonization could also be due to mycoparasitism. Since P. nunn is an aggressive mycoparasite in vitro (Lifshitz et al. 1984a), reduction in population density of P. ultimum in the presence of P. nunn could also be due to this phenomenon. In the Trichoderma-Rhizoctonia system, the mycoparasite (Trichoderma) utilized the host (Rhizoctonia) as a food base, and increased its inoculum density at the expense of the pathogen (Liu and Baker 1980). But in the P. nunn system, the population density of P. nunn was not affected by the population density of P. ultimum in an aerated steamed soil, and interactions between the two fungi were only observed in the presence of organic substrate (Paulitz and Baker 1987a). Clearly, P. nunn requires an organic substrate for antagonistic activity (Baker 1988a). Mycoparasitism might therefore play a significant role in substrate capture.

Development of another biocontrol system involving suppression of Fusarium wilt diseases

Protection against Fusarium wilt diseases by application of nonpathogenic strains of F. oxysporum or formae speciales not pathogenic to the challenged host is a well-studied phenomenon. In most of these experiments, cut, wounded, or bare roots were dipped or sprayed with suspensions containing high concentrations of propagules of the potential biocontrol agent. Application of agents by use of such artificial methods could bypass resistance mechanisms that might function in intact, non-injured roots and stems. Microconidia, the type of propagule most frequently used in such experiments, are usually not the propagules involved in survival under field conditions. Finally, in most of the experiments, plants were grown under axenic conditions or in steamed greenhouse mixes, which eliminates the typical microbial interactions found in raw soil.

Scher and Baker (1980) and Alabouvette et al. (1980) transferred Fusarium suppressiveness to conducive steamed soils by incorporation of smaller amounts of suppressive soil in greenhouse mixes. The latter workers also reinfested steamed soil with nonpathogenic Fusarium spp. and observed suppressiveness. However, it is not certain whether this actually reflected the reestablishment of the biological buffering characteristic of soils infested with resident microflora. Schneider (1984), however, observed suppression of Fusarium wilt of celery by addition of nonpathogenic strains of F. oxysporum to raw conducive soils. In our experiments reported here, the infection rate of cucumbers growing in raw soil was significantly reduced by incorporating chlamydospores of selected nonpathogenic isolates of F. oxysporum.

Isolate C5 at 10,000 cfu/g was effective in suppressing Fusarium wilt of cucumbers in conducive soil. In cultivated soils, total population densities of Fusarium spp. ranged from 11,000 to 27,000 cfu/g and F. oxysporum was a major component (Nash and Snyder 1965, Windels and Kommendahl 1974). Therefore, population densities of 10,000 cfu/g of nonpathogenic isolates (like C5), which could induce some increment of suppressiveness, are not uncharacteristic in field soils.

Nonpathogenic isolates of F. oxysporum, like pathogenic F. oxysporum, are highly competitive saprophytes, can successfully colonize the cortical tissue of roots, and are well adapted for survival in soil. Non-pathogens may share some of the same niches as pathogens. Therefore, there is probability that effective competition and biological control are greater than if the competitor did not occupy the same niche. Many biological control agents work well in steamed or sterilized soil, but fail in the field because they are not adapted to the same biological and physical environment in which the pathogen operates. The use of the biological control agents that occupies the same

niche as the pathogen could overcome this obstacle, and offer the potential for suppression of a soilborne disease that is difficult to suppress by other control measures.

Integration of biocontrol with chemicals or other agents

An integrated approach could provide some solution of problems of inconsistency and/or inefficiency of biocontrol. Pathogen-specific fungicides could be used to decrease detrimental effects on biocontrol agents and/or beneficial microorganisms already present in soil.

Lifshitz et al. (1985) integrated biological control of damping-off (induced by Rhizoctonia solani) with chemical control by use of an experimental fungicide benodanil. Obviously, the two systems should be compatible and benodanil was not fungitoxic to Trichoderma harzianum at in vitro rates far below those inhibitory to R. solani. The relatively low rate of benodanil inhibitory to radial growth of R. solani in vitro ($ED_{50} = 0.28 \mu\text{g a.i./g}$ of medium) was reflected in soil as measured by DI ($ED_{50} = 1.3 \mu\text{g a.i./g}$ of soil). Thus the two systems are potentially compatible.

In past research, it was difficult to induce suppressiveness to R. solani in alkaline soils by amendments of conidia of T. harzianum (Chet and Baker 1980, Liu and Baker 1980). This was especially true if large propagules of the pathogen were used as inoculum. These propagules are more typical of those found in field soil. Two strategies were used to solve these difficulties. The first was to apply the biological control agent to seed coats. This provided a barrier against the pathogen and resulted in significant control of damping-off compared with inoculated controls. The second strategy involved implementation of monoculture in which repeated weekly plantings of radish induced suppressiveness in soil containing high population densities of T. harzianum. During these experiments, there was little change in the population density of Trichoderma spp. in the alkaline soil. This reflected previous results and suggested that propagules of R. solani were inactivated during monoculture (Liu and Baker 1980).

There was no evidence that benodanil enhanced the activity of Trichoderma spp. during monoculture; however, a possible interaction between seed treatment with T. harzianum and benodanil in soil was tested statistically by factorial design. Analysis of variance indicated a nonsignificant T. harzianum x benodanil interaction, which demonstrated that disease control by T. harzianum and benodanil was additive but not synergistic.

Integrations of biological with compatible chemical controls, as reported here, can lead to enhanced efficiency in integrated pest management. These studies emphasize, however, the importance of strategic application of biocontrol agents (seed treatment in this case) to compensate for environmental conditions (e.g., high soil pH) and/or inoculum status (e.g., large propagules) unfavorable for efficient activity of such agents.

Even if the biocontrol agent is sensitive to a fungicide, mutations can be induced for resistance (Chang et al. 1986). Conidia of a benomyl-resistant isolate of T. harzianum applied in rooting hormone to carnation cuttings reduced the incidence of Rhizoctonia rot 50% or more during propagation (Fig. 20). A similar amount of control was obtained by adding benomyl to the hormone solution. When both were added, 100% control was achieved (Baker and Scher 1987).

Fig. 20. Integrated chemical and biological control of *Rhizoctonia* stem rot during propagation of carnation cuttings. The T-95 is an isolate of *Trichoderma harzianum* mutated so that it was tolerant to benomyl. Benomyl (50% a.i.) was applied at 5% suspension in the rooting hormone.

Frequently, suggestions are made that combinations of biocontrol agents may be better than any component agent alone. This is not necessarily the case, however. When *T. harzianum* was added to soil containing high *Pseudomonas* spp. population density and low iron availability, the fungus was sensitive to iron deprivation induced by the fluorescent pseudomonads and was ineffective as a biocontrol agent (Hubbard et al. 1983). However, in an unpublished experiment when a rhizosphere competent mutant of *T. harzianum* (Ahmad and Baker 1987a) was applied in various combinations with a fluorescent pseudomonad, there was no interference between them as reflected in their respective population densities in the rhizosphere of cucumbers (Fig. 21). This has intriguing potential for enhancing these biocontrol agents.

As reviewed above, some nonpathogenic isolates of *Fusarium oxysporum* induced suppressiveness to *Fusarium* wilt of cucumber whereas others did not. The addition of a fluorescent pseudomonad strain N1R of *Pseudomonas putida* induced suppressiveness at soil pH 6.7 but strain A-12 did not. When three isolates of *F. oxysporum* (C14, Sal 15, Fo47), ineffective in inducing suppressiveness themselves, were added together with A-12, significant suppression of *Fusarium* wilt resulted (Fig. 22). Thus, combinations of bacteria and *F. oxysporum*, ineffective by themselves, induced suppressiveness (Park et al. 1988).

Fig. 21. Population densities of *Pseudomonas putida* applied to seed (10^6 cells per seed) or soil (10^8 cells per g) or *Trichoderma harzianum* T-95 applied to seed (10^6 conidia per seed) or soil (10^6 conidia per gram) in rhizospheres of cucumbers after 8 days of incubation in Nunn sandy loam. Population densities of *P. putida* when applied to soil, A, or seed, B with (o) or without (•) *T. harzianum* applied to seed, A, or soil, B. Population densities of *T. harzianum* when it was applied to seed, C, or to soil, D, with (•) or without (o) *P. putida* applied to soil, C, or to seed, D.

Fig. 22. Biological control of Fusarium wilt of cucumber with non-pathogenic isolates of *Fusarium oxysporum* alone and in combination with *Pseudomonas putida* strains A-12 or N1R in soil of pH 6.7. *F. oxysporum* f. sp. *cucumerinum* added at 1000 cfu/g soil and nonpathogenic isolates of *F. oxysporum* added at 10,000 cfu/g soil. Bacteria added at 10^8 cfu/g soil. All treatments significantly different from infested control ($P = 0.05$) except for those marked with *.

The profound effects of soil pH on the ability of fluorescent pseudomonads to induce suppressiveness to Fusarium wilt diseases was reviewed above. Thus, in this study, no significant biocontrol occurred when either strain N1R or A-12 was added to soil of pH 5.5 (Fig. 23). At pH 6.7, strain N1R significantly delayed the onset of symptoms of Fusarium wilt of cucumber but there was no difference in disease incidence when compared with the infested control after 30 days. Neither *P. putida* strain A-12 nor nonpathogenic isolate Sal 15 added alone induced suppressiveness, but when added together these isolates effectively reduced disease incidence. At pH 8.1, *P. putida* strain N1R and A-12 + Sal 15 induced suppressiveness. Therefore, as the pH of soils was increased, suppressiveness induced by *P. putida* strain N1R or strain A-12 with Sal 15 became more pronounced. Such biocontrol phenomena associated with soil pH have been explained by the mechanism of iron competition as reviewed previously.

The reason(s) why two relatively inefficient biocontrol organisms (*P. putida* A-12 and *F. oxysporum* Sal 15) show enhanced biocontrol activity when combined is not completely understood. However, several possibilities exist.

Fig. 23. Effect of soil pH on biological control of *Fusarium* wilt of cucumber with two strains of *Pseudomonas putida* (A-12 and N1R) and a nonpathogenic isolate of *Fusarium oxysporum* (Sal 15) A - pH 5.5, B - pH 6.7, C - pH 8.1. *F. oxysporum* f. sp. *cucumerinum* added at 3,000 and Sal 15 added at 10,000 cfu/g soil. Bacteria added to soil at 10^8 /g. All *Fusarium* spp. and bacteria added at the beginning of the experiment. Treatments marked with * are significantly different from inoculated control, $P = 0.05$.

P. putida strains N1R and A-12 reduced germination of chlamydospores of either pathogenic or nonpathogenic isolates of *F. oxysporum* in rhizospheres of cucumber (Table 1). Such reductions in chlamydospore germination have been directly correlated with the level of nutrients in root exudates of various plants and, in turn, related to enhanced production of siderophores by pseudomonads at higher carbon levels (Elad and Baker 1985b). Thus, one hypothesis to explain the phenomena is that the addition of nonpathogenic strains of *F. oxysporum* increased root exudation thereby enhancing the

Table 1. Germination of chlamydospores of isolates of Fusarium oxysporum in rhizospheres of cucumber infested with strains of Pseudomonas putida.

Strains of <u>P. putida</u>	Germination of chlamydospores (%) ^a						
	C1	C5	C10	C14	Isolates Sal 15	Fo47	<u>F. o. cucumerinum</u> ^b
Control	66a	28a	55a	13a	52a	54a	75a
A-12	22b	12b	16b	8a	10b	16b	10b
N1R	36ab	8b	10b	2a	15b	12b	13b

^aNumbers within each column followed by the same letter are not significantly different ($P = 0.05$).

^bF. o. cucumerinum = Fusarium oxysporum f. sp. cucumerinum.

activity of added fluorescent pseudomonads in the rhizosphere. This may explain why nonpathogenic isolates of F. oxysporum added to alkaline soil at a relatively high population density (10,000 cfu/g soil) induced significantly higher population densities of either strain A-12 or N1R in the rhizosphere than when the fungus was not added (Fig. 24).

"The impact of interaction of microbial populations in the root-soil interface on pre-penetration activity, infection and resulting disease is a critical area that has received relatively little attention . . . [and] the interactions that occur are of major significance" (Mitchell 1976). Appropriate systems to study such interactions are difficult to identify. Further, technical difficulties connected with methodology complicate quantitative measurements. The biocontrol system described in this paper may be an appropriate tool for definitive description of such interactions in microbial populations.

There is an intriguing possibility that two or more biocontrol systems could be to produce additive or even synergistic effects at different microsites. For example, rhizosphere-competent Trichoderma spp. applied in seed treatment could be used to protect infection courts (seed, root tips) against pathogenic activity of Pythium spp. P. nunn could be used to eradicate inoculum of the same pathogens present in sites distant from infection courts.

Fig. 24. Effect of soil pH and isolates of *Fusarium oxysporum* on population density of *Pseudomonas putida* strain A-12, A, or strain N1R, B, in rhizosphere of root tips of cucumbers. Three treatments were used: raw soil, raw soil infested with *F. oxysporum* f. sp. *cucumerinum* (Foc) (3,000 cfu/g), and soil infested with Foc plus a nonpathogenic isolate of *F. oxysporum* (Sal 15) added at 10,000 cfu/g. *P. putida* strains A-12 or N1R were added to the soil at 10^8 cells/g at the beginning of the experiment. Treatments marked with * are significantly different from noninoculated control ($P = 0.05$).

As this is being written, a preliminary experiment was completed testing the hypothesis elaborated above. Cucumbers were sown in aerated steamed (60 C) or raw soil, amended or not amended with bean leaf meal and seeds were treated with *T. harzianum* (T-95) and/or soil was infested with *P. nunn*. The raw soil contained indigenous strains of *P. ultimum* and *P. nunn*. The results (Fig. 25) suggest:

- i) Seed treatment with T-95 was effective in control of damping-off.
- ii) *P. nunn* controlled damping-off in either steamed or raw non-amended soil but at less efficiency than T-95.
- iii) When bean meal was added, *P. nunn* was more effective in control. Instead in raw amended soil, the two biocontrol agents induced better than additive control at the first planting (Fig. 25G).

Fig. 25. Damping-off of cucumbers repeated by planted at weekly intervals in raw or aerated-steamed soil (60 C) in seed treatments with *Trichoderma harzianum* (T-95) and/or infestation with *Pythium nunn* (300 cfu/g soil). Bean leaf was added (C, D, G, H) at 0.3% w/w in some treatments. *P. nunn* and *P. ultimum* was present in the raw soil at low levels.

Fusarium spp. and Macrophomina phaseolina - a new target for Trichoderma control under field conditions

A. Biological control of Macrophomina phaseolina (Tassi) Gold by Trichoderma harzianum

Four isolates of Trichoderma harzianum Rifai inhibited linear growth and microsclerotia production in Macrophomina phaseolina. The antagonist proliferated in dual liquid culture with M. phaseolina and significantly decreased the number of its viable propagules. Peat bran preparation of T. harzianum reduced disease incidence, in greenhouse-planted beans, by 37-74%. Coating melon seeds with T. harzianum conidia reduced disease incidence by 37.5 - 46.3%. T. harzianum growth was affected less than that of the pathogen, by Pentachloronitrobenzene or Dazomet. Though the biocontrol agent was as effective as the chemicals, however disease control was enhanced by combining both, under greenhouse conditions. In the field, "charcoal root rot" of melons and corn was reduced by 22 and 28%, respectively. Moreover, treatments with T. harzianum in melons, resulted in earlier fruit yields.

Macrophomina phaseolina (Tassi) Gold) is a sclerotium-producing fungus, which causes "charcoal rot" in corn, melon, bean and other economically important crops in arid and semi-arid areas. Although the pathogen penetrates its host at a young age, the symptoms appear only later during the growth season. M. phaseolina populates roots and stems of young plants, producing, in the collapsed plants, many sclerotia whose (unusually) high survivability defeats common control practices. Though eradicants can control "charcoal rot", no effective and economically feasible means are available for large field crops.

There are a few reports on biological antagonism against M. phaseolina by Aspergillus sp., Bacillus spp., Streptomyces and growth filtrate of Trichoderma viride. We have studied the potential of T. harzianum in controlling M. phaseolina under both greenhouse and field conditions.

Antagonistic activity

Fungi were isolated from field soil naturally infested with Macrophomina phaseolina. Of the 96 Trichoderma spp., isolate 30 inhibited growth of the plant pathogen when grown towards it on SM, however, T. harzianum isolates T-203, T-243, T-134 and T-315 were the most effective in inhibiting and growing into the colony of the host fungus. In order to test the production of inhibitory substances by T. harzianum, the antagonistic isolates and M. phaseolina (control) were grown on top of a cellophane membrane placed on SM. M. phaseolina was inoculated following removal of the membranes. The linear growth of M. phaseolina 21, 25 mm, after four days of incubation on a medium previously supporting growth of the same fungus, was significantly inhibited, by 74.5, 83.0, 62.8 and 37.4% by replacing this growth medium with others previously supporting growth of T. harzianum isolates T-203, T-243, T-134 and T-315, respectively. Moreover, when grown on media previously supporting growth of T.

harzianum, M. phaseolina mycelium was more scattered and produced fewer microsclerotia, compared with its growth on a medium previously supporting growth of M. phaseolina. No inhibitory volatiles were detected. Growth rates of T. harzianum isolates T-203 and T-134, on media previously supporting growth of M. phaseolina, increased by 32.2 and 122.2%, respectively, compared with their growth on media previously supporting growth of the same antagonists.

Isolate T-134 was grown on carbonless SM (control) or SM containing either glucose, laminarin, chitin or purified cell walls of M. phaseolina. Dry weight of T. harzianum mycelium after inoculation of 5 days with the carbonless medium, was 17 mg per plate. The above mentioned carbon sources increased the dry weight of the fungus by 2.9, 3.1, 2.8 and 4.5 times, respectively, compared with the control M. phaseolina cell wall components also increased conidia production by T. harzianum. Conidia of T. harzianum 134 were inoculated into submerged cultures of M. phaseolina (Fig. 26). The number of viable T. harzianum propagules increased, during 8 days of incubation, from 100 to 3000 and to 2×10^6 per ml in viable and heat killed cultures of the plant pathogen. At the same time, viable propagules of M. phaseolina in the dual culture decreased from 3400 to 100 per ml.

Control of charcoal root rot under greenhouse conditions

Four isolates of T. harzianum were applied, as conidial suspension or peat:bran preparation, to raw soil infested with M. phaseolina before planting with beans. Thirty one days later, disease severity was reduced by 37-74%, but no significant difference was found between the two methods of application (Table 2).

Table 2: Effect of inoculum type of various Trichoderma harzianum isolates on biological control of bean disease caused by Macrophomina phaseolina*

<u>T. harzianum</u> isolate	Diseased plants (%)	
	Peat:bran culture**	Conidia**
-	66.4 a ⁺	66.4 a
T-315	41.7 b	35.9 bc
T-203	27.9 bcd	23.1 cd
T-243	22.8 cd	22.0 cd
T-134	17.2 d	20.0 cd

* Percentage of diseased plants after 31 days of incubation.

** Peat:bran culture or conidial suspensions of the various isolates were mixed with infested soil before planting to give 1×10^6 colony forming units/g.

+ Numbers followed by a common letter are not significantly different ($p = 0.05$).

Mixing either a conidial suspension of peat:bran culture of isolate T-134 with autoclaved soil, previously infested with M. phaseolina, significantly reduced disease incidence by 32.7 - 42.4%, respectively, from the 51% of diseased beans in the untreated control.

Melon disease, induced by M. phaseolina, gradually reached 33% during 46 days of incubation. Seed application of T. harzianum isolate 243, reduced this incidence by 46.3, 37.5 and 41.2% - 34, 38 and 46 days after planting, respectively (Fig. 27).

Integrated chemical-biological control of M. phaseolina

SM supplemented with 50, 100 and 200 ug/ml Bazamid inhibited the linear growth of M. phaseolina by 41, 76 and 80%, respectively and T. harzianum 134 by 5, 18 and 22%, respectively. Adding 5, 10 and 50 ug/g PCNB to the SM inhibited M. phaseolina growth by 27, 57 and 84%, respectively, but T. harzianum only by 0, 2 and 10%, respectively.

Conidia of T. harzianum 134, at a rate of 10^6 propagules/g soil, and PCNB, at a rate of 25 ug/g soil, or a combination of both, were applied to soil infested with M. phaseolina. Disease incidence in beans, recorded after 42 days of incubation in the greenhouse, reached 75.4% in the untreated control. T. harzianum and PCNB significantly decreased disease incidence by 57 and 50% respectively, whereas a combination of both treatments reduced disease incidence by 64%. Combining Trichoderma with Bazamid did not enhance the control of "charcoal rot" in beans.

Field experiments

In the first field experiment, T. harzianum (203) and PCNB were tested for their ability to control "charcoal rot" of melons in a commercial field, naturally infested with M. phaseolina. Prior to planting, treatments consisted of: broadcast or furrow application of peat:bran Trichoderma preparation; Application of Trichoderma to the seeds; Furrow application of PCNB; and furrow application of a combination of PCNB and peat: bran Trichoderma preparation. Though disease symptoms were not observed during the growth season, up to 30% of the surface-sterilized plant samples, plated on a medium selective for M. phaseolina, yielded the plant pathogen. Root colonization by the pathogen was observed in 21% of the samples taken from Trichoderma-treated plots, whereas between 23-28% of plant samples taken from the rest of the treatments yielded the pathogen. Melon fruits were collected twice a week between 70-91 days after planting. Whereas the four Trichoderma treatments increased total yield by 5.8-8.9%, no increase in yield was observed in the PCNB-treated plots. Moreover, in the first three harvests, yields of the Trichoderma-treated melon seeds increased by 61, 41 and 28% over the untreated control.

The second field experiment, carried out in a commercial corn field, naturally infested with M. phaseolina, consisted of untreated control, seed coating with T. harzianum (203 or 134) and broadcast application with peat:bran preparation of T-203 or T-134. The first symptoms of "charcoal root rot" were observed 131 days after planting

(20 days after irrigation was stopped). On harvest day (150 days after planting) disease incidence, which reached 45% in the untreated plots, was significantly reduced by all Trichoderma treatments (Fig. 28). The untreated control yielded 76250 corn ears/ha, weighing 8435 kg/ha, and the weight of kernels was 6741 kg/ha. Trichoderma treatments increased both number and weight of ears and the weight of kernels by up to 6.9, 6.2 and 6.7%, respectively.

Trichoderma harzianum is an effective biocontrol agent of Sclerotium rolfsii, Rhizoctonia solani and Pythium aphanidermatum. This is the first report of its effectiveness against M. phaseolina.

Antagonism of T. viride against M. phaseolina in culture was shown. The increase in T. viride population was associated with disease control in cotton. However, so far, potential biocontrol agents were not applied in infested fields. We have isolated an effective, antagonistic T. harzianum, isolate which inhibited microsclerotia production and growth of M. phaseolina. Moreover, Trichoderma proliferated in the presence of the host fungus, when grown together in culture, utilizing cell wall products of M. phaseolina, as previously shown with the pathogenic fungi S. rolfsii and R. solani. Applying antagonistic Trichoderma isolates to soil infested with the plant pathogen, significantly reduced disease incidence in beans and melons, under greenhouse conditions. Control effectiveness varied with the isolate but not with the method of application.

T. harzianum was less susceptible to PCNB and Dazomet in agar media. However, when the fungus was combined with one of these fungistates in soil, no additive effect was observed.

Diseased plants were not observed during the melon growth season. This was achieved by trickle irrigation which did, indeed, prevent plant stress. However, M. phaseolina invaded roots and was isolated from stems of melon plants sampled in all field plots. Trichoderma decreased the percentage of plants from which the pathogen was isolated. This was also reflected in the earlier yield collected from coated-seed plants. All Trichoderma treatments significantly decreased (by 16.5-28.6%) "charcoal root rot" incidence in the corn field. This reduction was also reflected in a moderate yield increase.

"Charcoal root rot" may be a difficult disease to control because of the nature of the causal pathogen. M. phaseolina invades the roots of the host at an early stage, 1-2 weeks after planting, but the symptoms appear only in mature plants. Moreover, once the pathogen is inside the roots, the biocontrol agent cannot reach or affect it thus giving the pathogen a free-hand without any interaction with the antagonist during most of the growth season.

Trichoderma may have an advantage in controlling "charcoal root rot" in combination with cultural practices, especially management of water availability in the field. The biocontrol agent reduces root invasion by the pathogen, early in the growth season, while later, plants will be kept without water stress.

Fig. 26: Population dynamics in mixed submerged culture of *M. phaseolina* and *T. harzianum* (134).
 A. *T. harzianum* conidia were added to a viable culture of *M. phaseolina* (Δ), heat killed *M. phaseolina* (▲) or control basal medium (□).
 B. Counts of *M. phaseolina* in the control medium (○) or when grown with *T. harzianum* (●).

Fig. 27: The effect of *T. harzianum* (243) seed coating on disease incidence induced by *M. phaseolina* in melons under greenhouse conditions. Untreated control (○) and *T. harzianum* application (●).

Fig. 28: Incidence of "charcoal rot" of corn during growth in a naturally infested field. Plots were subjected to the following *Trichoderma* treatments: untreated control (O), seed coating with either T-203 (▲) or T-134 (■) or application to soil of peat:bran cultures of T-203 (△) or T-134 (□).

B. Trichoderma harzianum: an effective biocontrol agent of Fusarium spp.

An isolate of Trichoderma harzianum was found to be highly effective in controlling three formae speciales of Fusarium oxysporum in cotton, muskmelon and tomato and F. culmorum in wheat by up to 83% disease reduction. When tested against F. oxysporum f. sp. vasinfectum the antagonist persisted well in soil and reduced the disease incidence in cotton throughout three consecutive plantings. Under field conditions T. harzianum effectively controlled F. oxysporum f. sp. melonis and Fusarium oxysporum f. sp. radicis lycopersici by 53 and 37% increasing the yield of melons and tomatoes by 34 and 19%, respectively.

a. Application techniques of Trichoderma harzianum

T. harzianum was applied as a seed coating or as a wheat bran/peat preparation when tested against several Fusaria on four crops (Table 3).

Table 3: Biological control of Fusarium spp. under greenhouse conditions

Pathogen	Crop	(1) Disease reduction (%)
<u>F. oxysporum</u> f. sp. <u>vasinfectum</u>	cotton	80
<u>F. oxysporum</u> f. sp. <u>melonis</u>	melon	60
<u>F. oxysporum</u> f. sp. <u>radicis lycopersici</u>	tomato	80
<u>F. culmorum</u>	wheat	83

(1) Percentages of disease reduction (DR) were calculated according to the following formula:

$$DR = \left[1 - \frac{DT}{DC} \right] \times 100$$

where, DC and DT are percentages of disease control and treatment, respectively.

I. Seed coating

Seeds were soaked in a concentrated conidial suspension of Trichoderma (5×10^9 conidia/ml) amended with an adhesive. After 30 sec, the seeds were dried by warm ventilation.

II. Bran/Peat preparation

Under greenhouse conditions, wheat bran/peat (1:1, v/v) preparation of T. harzianum was mixed with soil at a rate of 5 gr/kg soil. However, when tested in the field, this preparation was applied to the rooting mixture of "speedlings" in the nursery (10% by volume). At the end of the rooting period (30 days), the Trichoderma loaded "speedlings" were transplanted in the field.

b. Greenhouse experiments

Under greenhouse conditions, sandy loam soil was artificially infested with microconidia of F. oxysporum f. sp. vasinfectum, F. oxysporum f. sp. melonis or F. culmorum. Another biological control test of F. oxysporum f. sp. radicis-lycopersici was carried out in a naturally infested soil. Table 3 shows the efficiency of T. harzianum in controlling several Fusaria.

Fig. 29: Biological control of Fusarium crown rot in tomatoes.
A - Infested and untreated control
B - Seed treatment with T. harzianum

c. Field experiments

All experiments were carried out in naturally infested fields. The biological control effects on Fusarium wilt of melon were tested by two experiments conducted in two parts of the same field infested with F. oxysporum f. sp. melonis in central Israel. The disease control is shown in Fig. 30

Fig. 30: Biological control of *F. oxysporum* f. sp. *melonis* by *T. harzianum* under field conditions

In the first experiment (A) *Trichoderma* was applied as a seed coating ○-----○, while in the second (B) it was mixed with the rooting mixture of melon seedlings, as a wheat bran/peat preparation ▲-----▲. *Trichoderma* treatments were compared with naturally infested and untreated control ○---○ and ▲---▲, in both experiments A and B, respectively.

Both application techniques of *T. harzianum* delayed the disease first appearance by 4 weeks and significantly ($P = 0.05$) reduced the total incidence of disease by 53 and 46% in experiment A and B, respectively as can be observed in Table 4.

Table 4: Biological control of Fusarium wilt in melons under field conditions

Experiment	Treatment	<u>Trichoderma</u> application	Disease incidence (%)	Yield (Ton/ha)	Average fruit weight (kg)
A	Control	None	30.6 a	18.88 b	1.387 b
	<u>T. harzianum</u>	Seed coating	14.3 b	23.11 a	1.531 a
B	Control	None	24.2 a	15.57 b	1.245 a
	<u>T. harzianum</u>	Bran/peat preparation	13.0 b	20.94 a	1.36 a

Numbers in columns followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

T. harzianum applied to seeds or to the rhizosphere of melons as a wheat bran/peat preparation significantly increased the total yields by 22 and 34% in both experiments, respectively. Moreover, Trichoderma treatments increased the average fruit weight of melons. We have a negative correlation ($r = 0.93$) between the incidence of wilt and the fruit weight.

Biological control experiment of F. oxysporum f. sp. radicis lycopersici was carried out in southern Israel. The results showed that Trichoderma preparation applied to tomato seedlings reduced the incidence of disease by 37% and increased the total tomato yield by 19%.

T. harzianum may be applied as a bran/peat preparation mixed with the rooting medium of the plant or as a seed coating where minimal amounts of antagonist are needed. Nevertheless, this study shows that seed treatment can be effective for controlling wilts on mature plants.

After testing the antagonist under greenhouse conditions we conducted several field experiments in which T. harzianum decreased the disease incidence in melons by up to 53% and simultaneously increased the total yield by 34%. Since wilt symptoms induced by Fusarium on mature plants led to smaller fruits, the beneficial effect of Trichoderma on the average fruit weight indicates its influence on both disease incidence and yield as well.

The usefulness of Trichoderma versus Fusarium can be viewed from another perspective: The chemical control of Fusarium wilt diseases must take place before planting by means of soil disinfection, mostly methyl bromide which induces many ecological problems. Soil disinfection promotes, an ecological vacuum which enables the pathogen to recolonize the plant habitat which might cause a higher disease incidence.

Trichoderma applied to seeds or soil together or not with soil disinfestation may protect the plant through a long period.

It can be concluded, therefore, that T. harzianum is a promising biocontrol agent of Fusarium spp. However, the mechanisms which are involved in the antagonistic activity still must be studied.

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The cooperating principal investigators interacted personally (by visits to Israel or the U.S.), by frequent letter communication and occasionally by phone. This resulted in no less than three joint publications (Baker et al. 1984, Chang et al. 1986, and Elad et al. 1986). The contents of these articles reflect the in-depth cooperative approach to research development, strategies and problem solution.

Three Israeli research scientists also spent a year or more (each) in the U.S. laboratory (Y. Elad, R. Lifshitz, B. Sneh). The extremely prolific results of their research activity is reflected in at least 13 publications generated during their visits.

g. Evaluation of research achievements

Objective i. Four biocontrol systems have been developed during the term of the project. These are: 1) fluorescent pseudomonads especially as they influence *Fusarium* wilt diseases; 2) *Trichoderma* spp. effective against *Rhizoctonia solani*, *Pythium* spp. and *Fusarium* wilt disease pathogens; 3) a new antagonist (*Pythium nunn*) effective at low densities in control of *Pythium* spp. and, potentially, *Phytophthora* spp.; and 4) development of suppressiveness to *Fusarium* wilt diseases in raw soil by use of nonpathogenic isolates of *Fusarium oxysporum*. Identification of mechanisms in all these systems, except the nonpathogenic fusaria, has been the subject of extensive research publications.

Preliminary research suggests that the nonpathogenic fusaria operate by induction of resistance reactions in the host. When applied to seed, they colonize interior root tissue, not the rhizosphere. But the root eventually is not colonized to lower depths due to the slower growth rates of the biocontrol agents. By mutation, slow-growing spp. of *Trichoderma* were induced to grow at higher growth rates and were rhizosphere-competent and this could be accomplished with the *Fusarium* agents. Unpublished results with these fusaria suggests that spectacular control, especially in conjunction with other biocontrol systems, is possible.

Rhizosphere-competence has been induced in four species of *Trichoderma*. The profound increases in efficiency of rhizosphere-competent mutants over the incompetent wild-types is documented above. The exploitation and use of the mutants was explored in commercial, environmentally controlled structures (see above) and in the field (see below) with spectacular results. The applications to agriculture have already become apparent and a number of companies have expressed interest and are drawing up contracts for exploitation of the mutants. Only a fraction of the potential for application to agriculture has been explored, however.

Objective ii. Strategies for enhancement were reviewed (Baker and Scher 1987). These include: 1) management of potentially limiting factors; 2) alteration of genetic characteristics so that agents can operate in wider ranges of temperature and soil reaction; 3) identification of agricultural systems that have environments (e.g., greenhouse industry), climatic conditions, or planting dates favorable for activity of biocontrol agents; 4) alteration of quantity or quality of nutrients (e.g., by formulation, addition of crop residues, chelating agents) that selectively increase activity of the agents; 5) manipulation of the biocontrol agent, for example, by mutation to rhizosphere-competence; 6) increasing mycoparasitic, antibiotic or competitive activity by biotechnology; 7) integration with chemical control (agents have been mutated for resistance to fungicides), other conventional strategies in plant disease management, or by combination with

other biocontrol agents; and 8) making biocontrol by use of Trichoderma spp. more attractive by exploiting its ability to increase growth and yield of treated crops.

The aggressive exploitation of substrates by P. nunn is a particularly interesting strategy for enhancing biological control. As reviewed above, P. nunn displaced P. ultimum from crop debris and has the potential for eradication of such pathogens through appropriate manipulation of crop residues.

The review section of this proposal indicates considerable progress on the eight approaches to enhancement listed above; however, the potentials for use in agriculture have not been fully explored.

Objective iii. The mechanisms associated with increased growth responses induced by Trichoderma spp. were identified (Baker 1988). The quantitative aspects associated with control of minor pathogens vs stimulation by the hormone-like substance produced by the agents are currently being investigated as far as possible with the substantially reduced funding support. The growth-promoting substance has been extracted and bioassays developed. The biological and chemical properties of the substance do not match those of any known plant hormone (i.e., indoles, abscisic acid, ethylene, cytokinins or gibberellins, and it may be a novel compound. Our research support did not allow further exploration of the chemical nature of the compound and we have negotiated a contract with Food-Machinery Corp. for further research on this aspect. Should the substance turn out to be novel, the application to agriculture and the patenting potential are obvious.

The latest (unpublished) report indicated an increase in potato yield by seedpiece treatment with one of our mutants of 41 sacks/acre over controls of treatment with a wild-type (J. Davis, personal communication). This approaches an additional income approaching \$200 per acre.

Objective iv. Again, the research reviewed above provides exciting insights into the potential of combining different biological agents as well as the combination of biological and chemical control. Even if the agents are sensitive to certain fungicides, they can be mutated so that they are resistant. The strategy for combination of the biocontrol agents has been to use those that have different mechanisms and/or occupy different ecological niches. For example, research (in press) suggests that combination of a fluorescent pseudomonad that protects the rhizoplane with non-pathogenic fusaria that occupy interior tissues and (presumably) induce host resistance give additive control of Fusarium wilt diseases. Again, rhizosphere-competent Trichoderma spp. could protect the root against attack by Pythium spp., whereas P. nunn could eradicate these pathogens in regions distant from the root (unpublished results). These examples suggest that the often-advanced idea that combinations of biocontrol agents may be used to advantage has validity.

Note: It is obvious from the summary and review of research supported by this grant that substantial progress was made toward the attainment of the objectives. In this evaluation, the potentially exciting extension and application of this research are stated. Yet, our proposal to develop these potentials was turned down by BARD because of the "the low prospects of success in developing the use of a useful soil system." Further, the reviewers were critical of "the use of mutagens rather than natural selection to develop new strains" when it was demonstrated that wild-types do not possess the attributes necessary for enhancement. This report clearly establishes that the reviewers were wrong on all counts!

h. List of publications

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