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Control of Plant Diseases with Biological Components of Suppressive Soils

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ABSTRACT

Basic research has identified mechanisms associated with biocontrol by use of antagonists from suppressive soils. Mycoparasitism operates in suppression of Rhizoctonia solani induced by Trichoderma spp. and pathogenic Pythium spp. by the recently described P. nunn. These antagonists produced antibiotics. A "routing factor," which disorganized protoplasm in hyphal tips of Pythium spp., was the major factor inducing control of damping-off of seed treated with Trichoderma spp. When mechanisms involved in antagonism are known, it is possible to enhance activity of biocontrol agents more efficiently. Enhancement of agents was achieved by genetic improvement, providing appropriate substrates to improve saprophytic reproduction, management to increase competitive advantage, strategic placement of agents in appropriate infection courts, increasing rhizosphere competence, providing favorable environments for activity of agents and integration with other types of control. Some of the agents not only controlled plant diseases by enhanced growth. This has potential for not only paying for the cost of application but should lead to increased profits.

BODY OF REPORT

The only literature covered in this report will be that generated and supported wholly or in part by BARD Grant US-290-80. Literature supporting the basic assumptions and previous work done by our laboratories is also cited occasionally to provide rationale and background for logical presentation of results.

During the course of this research effort, reviews, invited symposium papers, theoretical articles, and chapters in books were written summarizing the ongoing research supported by BARD. They are not necessarily cited in this section of the report but are listed here as follows:

- Baker, R. 1983. State of the art: plant diseases. Pages 14-22 in: Proc. Natl. Interdisciplinary Biological Control Conf. S. L. Battenfield, ed. 15-17 Feb. 1983. Las Vegas, NV CSRS/USDA Washington, DC.
- Baker, R. 1984. Biological control of plant pathogens: definitions. Biocontrol workshop paper to be published by Academic Press.
- Baker, R. 1984. Survey of biological control strategies for plant diseases. Proceedings of the IV Congreso Asociacion Colombiana de Fitopatologia--ASCOLFI. (in press).
- Baker, R., and Chet, I. 1982. Induction of suppressiveness. Pages 35-50 in: R. W. Schneider, ed. Suppressive Soils and Plant Disease. The American Phytopathol. Soc., St. Paul, MN. 88 pp.
- Chet, I., and Elad, Y. 1982. Mechanism of mycoparasitism. La Selection des Plantes, Bordeaux (France), 21-26 mars 1982. Ed INRA Publ., 1982 (Les Colloques de l'INRA, n° 11.)
- Chet, I. and Y. Elad. 1983. Biological and Integrated Control of Soil-borne Plant Pathogens: Mechanism and Application. Proceedings of Gottingen Symposium.
- Elad, Y., and Chet, I. 1982. Prevention of plant infection by biological means. La Selection des Plantes, Bordeaux (France), 21-26 mars. 1982. Ed INRA Publ. 1982. 1982 (Les Colloques, de l'INRA, n° 11). Pp. 195-204.

Elad, Y., A. Sivan, O. Zeidan, O. Klaifeld and I. Chet. 1983. Biological and integrated control of soil pathogens as a means for reduction of fungicides. In: Shuval, H.I. (ed.) Developments in Ecology and Environmental Quality. Balaban Inter. Sci. Service Vol. 2:587-594.

Suppressiveness induced by *Trichoderma* spp. In previous studies, the mechanism associated with the induction of suppressiveness in soils to *Rhizoctonia solani* by *Trichoderma* spp. was associated with mycoparasitism. Suppressiveness to *Pythium* spp., however, was not observed even when these antagonists were mixed into soil at high population densities (Lifshitz, et al. 1984b). Seed treatment was effective (Table 1), however, and a series of experiments were initiated to determine the mechanism(s) associated with this phenomenon (Lifshitz, et al. 1985c).

TABLE 1. The effect of seed treatment with conidia of *Trichoderma* spp.^a and organic nutrients^b on the incidence^c of *Pythium* damping off

Seed treatment ^a	Incidence of damping-off (%) ^b			
	Inoculated ^c		Noninoculated	
	19 C	26 C	19 C	26 C
No nutrients				
T95 (<i>T. harzianum</i>)	97.2 ^v	50.0 ^{vw}	77.8 ^{vw}	37.8 ^{wxy}
T12 (<i>T. harzianum</i>)	75.0 ^v	41.4 ^w	58.3 ^w	33.3 ^{xyz}
T 8 (<i>T. koningii</i>)	47.2 ^w	33.3 ^w	41.7 ^w	27.8 ^{yz}
Pelgel-treated control	86.1 ^v	77.8 ^v	77.8 ^{vw}	58.3 ^{vw}
Nontreated control	88.9 ^v	88.9 ^v	75.0 ^{vw}	61.1 ^v
With nutrients ^d				
T95 (<i>T. harzianum</i>)	91.7 ^v	91.7 ^v	80.6 ^{vw}	75.0 ^{vw}
T 8 (<i>T. koningii</i>)	41.7 ^w	52.8 ^w	44.4 ^w	11.1 ^z
Control	97.2 ^v	30.6 ^w	94.4 ^v	22.2 ^{yz}

^a For each isolate 2.25 ml of conidial suspension in 10% Pelgel provided 1.4×10^7 conidia per g of seeds.

^b In each column, values followed by the same letter do not differ significantly ($P = 0.05$) according to FLSD.

^c Ten sporangia of *Pythium* sp. N1 were added to each g air dried soil.

^d Seeds were coated with 4.5 and 1.1 mg D-glucose and L-asparagin per g of seeds.

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 the agents. A fundamental observation leading to this conclusion is that seed treatment with isolates of *Trichoderma* spp.

was effective in control of both *Pythium* and *Rhizoctonia* damping-off; whereas, mixing these same biocontrol agents in soil induced suppressiveness to *R. solani* but not *Pythium* spp. How can this phenomenon be explained?

First, inoculum densities of the two pathogens found in nature are exceedingly different. *R. solani* is conventionally isolated from field soils below 1-10 propagules per g, whereas, inoculum densities of *Pythium* spp. are considerably higher. *R. solani* compensates for such low inoculum densities by having a relatively high linear growth rate. Evidence 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 touch the seed coat and, thus, avoid antagonism in the spermosphere. In contrast, propagules of *Pythium* spp. may have to touch the infection court to induce successful infection of the host; relatively high inoculum densities, in this case, insure that an adequate proportion of the total inoculum in the soil is adjacent to the seed. Therefore, biocontrol agents must be positioned to provide a barrier interposed between the propagules of the pathogen and the host, namely, a coating of the agent on the seed.

Trichoderma spp. parasitized hyphae of *Pythium* sp. isolate N1 in vitro; however, the time restraints are critical for mycoparasitism to occur. 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 (Table 2) confirm 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 period of time available for biocontrol agents to parasitize *Pythium* spp. also 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 (Table 3); but these isolates still protected seed from damping-off at this temperature (Table 1). Therefore, it is doubtful whether mycoparasitism provides a full explanation for the mechanism of antagonism involved in seed treatment with *Trichoderma* spp. for control of *Pythium* damping-off.

TABLE 2. The incidence of seed-coat and embryo colonization by *Pythium* sp. N1 during incubation in infested soil

Incubation (hr)	Incidence of isolation ^a			
	Seed Coat		Embryo	
	19 C	26 C	19 C	26 C
12	100	100	4.6 ^x	6.0 ^x
24	100	100	5.0 ^x	24.0 ^x
48	100	100	61.8 ^y	46.2 ^z

^a In each column, values followed by the same letter do not differ significantly ($P = 0.05$) according to FLSD.

TABLE 3. Germination of conidia of Trichoderma spp. during incubation on 2% water agar

Temperature or incubation (C)	Time (hr)	Germination (%)		
		<u>T. harzianum</u> T95	<u>T. harzianum</u> T12	<u>T. koningii</u> T8
26.	10	14.4	31.0	86.5
26	14	73.6	75.0	95.6
26	18	98.0	93.6	>95.0
19	10	0	0	0
19	14	0.3	0.9	14.5
19	18	8.7	13.4	78.0
19	22	14.8	26.5	91.4
19	26	26.5	54.2	91.2

^a Percentage of germinating conidia are means of 150-200 conidia observed on three different plates.

Competition for nutrients in seed exudates also is a candidate for the mechanism of biological control of Pythium damping-off by seed treatment. Since the correlation between the quantity of seed exudates and disease incidence is well established, it may be assumed that a partial removal of seed exudates by a biocontrol agent applied on seed could reduce the inoculum potential of Pythium spp. However, our study suggests that competition for seed exudates is not an important antagonistic mechanism between Trichoderma spp. and isolate N1 based on the following: addition of nutrients (glucose and asparagin) to the seeds did not affect successful disease control (Table 1), and the presence of Trichoderma spp. on seed did not reduce germination of sporangia of isolate N1 sporangia or chlamydospores of F. oxysporum f. sp. cucumerinum in the spermosphere (Table 4). 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.

TABLE 4. Germination of Fusarium oxysporum f. sp. cucumerinum chlamydospores^a and sporangia of Pythium sp. N1^b in soil under the influence of seeds treated with Trichoderma spp

Seed treatment	Germination (%) ^c	
	Chlamydospores	Sporangia
Nontreated	87.8 ^Y	94.0 ^Y
Pelgel-treated control	92.6 ^Y	90.1 ^Y
T95 (<u>T. harzianum</u>)	94.1 ^Y	92.4 ^Y
T12 (<u>T. harzianum</u>)	86.4 ^Y	89.6 ^Y
T 8 (<u>T. koningii</u>)	88.8 ^Y	96.4 ^Y
Raw soil only ^d	3.2 ^Z	6.0 ^Z

^a Chlamydospore density in raw soil was estimated at 2×10^5 cfu/g air dried soil, 2 g of soil per seed. Germination was assayed 18 hr after sowing.

^b Sporangium density in raw soil was ca 1.2×10^4 cfu/g air dried soil, 2 g of soil per seed. Germination was assayed 1.5 hr after sowing.

^c In each column, numbers followed by the same letter do not differ significantly ($P = 0.05$) according to FLSD.

^d Seed were not present in soil.

In early reports, antibiosis was considered to be a candidate mechanism for biological control induced by Trichoderma spp. Nevertheless antibiosis was not considered in later reports on biological control of Pythium damping-off by Trichoderma spp. In our study, toxic metabolites were produced in vitro by seven effective biocontrol isolates of Trichoderma (Table 5). In addition, observations revealed obvious stress signs in P. ultimum hyphae grown in dual culture 24 hr before mycoparasitism reaction was observed. This interaction, however, was only observed at a microscopic level and would not have been evident if macrobiotic observations, conventionally used in screening, were employed. Since the protoplasm in the hyphal tips of a pathogenic species of Pythium (isolate N1) responds to the Trichoderma-produced toxin by being "disorganized and thrown in confused flight" (Webster), we suggest that it be named "the routing factor."

TABLE 5. The effect of extracts of nutrient medium supporting growth of *Trichoderma* isolates on mycelial growth and sporangium germination of sporangia of *Pythium* sp. N1.

Trichoderma isolate	Fresh extract ^{ab}		Four-day-old extract ^{ac}	Autoclaved extract ^{ad}
	Linear growth rate ^e (mm/day)	Sporangium germination ^f (%)	Linear growth rate ^e (mm/day)	Linear growth rate ^e (mm/day)
T95	21 ^x	0 ^x	29 ^w	33 ^w
T 1	23 ^x	0 ^x	29 ^w	-
T1-R1	18 ^x	0 ^x	29 ^w	-
W 6	21 ^x	0 ^x	27 ^w	-
W6-15	17 ^x	0 ^x	28 ^w	-
T 8	13 ^y	0 ^x	27 ^w	32 ^w
T12	5 ^z	0 ^x	26 ^{wx}	30 ^w
Control ^g	30 ^w	96 ^w	23 ^x	32 ^w

^a In each column, values followed by the same letter do not differ significantly ($P = 0.05$) according to FLSD.

^b Fresh extract: *Trichoderma* spp. growth medium filtered through Nucleopore membrane (0.45 μ m pore size) and diluted unmediated 1:10 with 1% (w/v) water agar (49 C); for sporangia germination assay, 1 ml of medium filtrates were supplemented with 0.1 ml of sterile potato dextrose broth and with 0.1 ml of N1 sporangium suspension in sterile distilled water (10^4 sporangia per ml).

^c Four-day-old extract: *Trichoderma* spp. growth medium filtered as above but stored for 4 days at 4 C before being diluted in water agar (1:10).

^d Autoclaved extract: *Trichoderma* spp. growth medium autoclaved for 15 min at 43.9 g/cm² then diluted in water agar (1:10).

^e Linear growth rate based on two perpendicular diameters of colony of isolate after 24 hr growth at 26 C from 4 mm disk (cut from the margin of 2-day-old culture).

^f Sporangium germination based on three observations, each of at least 100 sporangia, incubated for 90 min at 26 C and 100% R.H.

^g Control: 1% (w/v) water agar only for linear growth; 1 ml sterile water with 0.1 ml potato dextrose broth and 0.1 ml sporangial suspension in sterile water for sporangium germination.

The lack of growth inhibition of *T. harzianum* T12 by metabolites derived from the same fungus suggests that the factors inhibitory to isolate N1 were not staling substances. Culture filtrates were inhibitory to radial growth of isolate N1 but not to *R. solani*, again suggesting that biological control mechanisms of *Trichoderma* spp. against the two pathogens are dissimilar.

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 has been attributed to its great sensitivity to toxic metabolites

produced by other microorganisms. Since the interaction between *Trichoderma* spp. and isolate N1 occurred on dead seed coats, and those isolates were capable of producing toxic metabolites to the pathogen, it is suggested that production of toxic metabolites by *Trichoderma* spp. on seed coats is the principle mechanism of biological control of *Pythium* damping-off.

There have been many reports that propose use of *Trichoderma* spp. for control of damping-off; however, extensive commercial application of the agents to control such diseases has not occurred and field results have not been spectacular (Ruppel et al. 1983). The predominant reason is that biocontrol agents have not obtained efficiencies matching those of currently available fungicides under all environmental conditions.

An integrated approach could provide solution to these problems. Pathogen-specific fungicides could be utilized to decrease detrimental effects on applied biocontrol agents and/or beneficial microorganisms already present in the soil. Further, should environmental conditions be temporarily unfavorable for activity of the biocontrol agent, an associated fungicide could provide an effective back-up system. Finally, there is opportunity for additive or synergistic control by a combination of chemical-biological control strategies.

A study was initiated to test possibilities for integrated chemical and biological control of *Rhizoctonia* preemergence damping-off (Fig. 1) based on the above postulates (Lifshitz et al. 1985b). *I. harzianum* was used for the biocontrol agent. Benadonil, a newly produced fungicide, was used by chemical control.

Fig. 1. Inoculum density-disease relationships for preemergence damping-off of radish induced by *Rhizoctonia solani*.

For integration of chemical and biological control of plant pathogens, the systems must be compatible. In this study, benadonil was not fungitoxic to *I. harzianum* (Fig. 2B) in vitro at rates far below those inhibitory to *R. solani* (Fig. 2A). The relatively low rate of benadonil inhibitory to radial growth of *R. solani* in vitro ($ED_{50} = 0.28$ g a.i./g of medium) was reflected in soil as measured by disease incidence, $ED_{50} = 1.3$ g a.i./g of soil (Fig. 3). Thus, the two systems are potentially compatible.

Fig. 2. Radial growth of *Rhizoctonia solani* (A) and *Trichoderma harzianum* (B) on potato dextrose agar containing various concentrations of benadonil.

Fig. 3. Dosage response relating the concentration of benadonil in soil to incidence of preemergence damping-off of radish induced by Rhizoctonia solani.

In past research, it was difficult to induce suppressiveness to R. solani in alkaline soils by amendments of conidia of T. harzianum. 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 as compared with inoculated controls (Table 6). The second strategy involved the implementation of monoculture in which repeated weekly plantings of radish induced suppressiveness in soil containing high population densities of T. harzianum (Fig. 4A, C). During these experiments, there was little change in the population density of Trichoderma spp. in the alkaline soil (Fig. 4B, D). Such phenomena suggest that propagules of R. solani were inactivated during monoculture.

TABLE 6. Mean percent emergence of radishes in soil containing 1 propagule/g soil of Rhizoctonia solani and treated with benodanil and/or Trichoderma harzianum^a mixed in the soil

Treatment ^b	Benodamil ($\mu\text{g a.i./g soil}$)				
	0	0.5	1.5	3.5	5
<u>Trichoderma harzianum</u>					
Mixed in soil ^c	20Y	52Y	92Z	98Z	100Z
On seed ^d	74Z	90Z	92Z	94Z	100Z
No treatment with antagonist	16Y	44Y	88Z	98Z	98Z

a Figures are based on emergence of 10 seeds in each of five replicates per treatment. Emergence in noninoculated the control was 90-94%.

b Numbers of each column followed by the same letter are not significantly different ($P = 0.05$) by use of FLSD analysis.

c *T. harzianum* applied at 2.5×10^6 conidia per g of soil.

d Seed were dipped in a conidium suspension with 3.4×10^8 conidia per ml of Peigel solution.

Fig. 4. Effect of *Trichoderma harzianum* and/or benadonil in soil infested with *Rhizoctonia solani* (1 propagule per gram soil) on emergence of radish (A, C) planted at weekly intervals and the population density of *Trichoderma* spp. (B, D) during this monoculture.

There was no evidence that benadonil enhanced the activity of *Trichoderma* spp. during monoculture (Fig. 4C, D); however, a possible interaction between seed treatment with *T. harzianum* and benadonil in soil was tested statistically by means of a factorial design (Table 7). The analysis of variance indicated a nonsignificant *T. harzianum* X benadonil interaction. This demonstrated that disease control by *T. harzianum* and benadonil 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.

TABLE 7. Mean percent emergence of radishes in soil infested with *Rhizoctonia solani* (1 propagule per g of soil) when benadonil was mixed into soil and *Trichoderma harzianum* seed treatment was used at various levels^a

Benadonil (mg a.i./g soil)	<i>Trichoderma harzianum</i> (conidia per ml of seed treatment suspension)			
	0	10 ⁷	10 ⁸	10 ⁹
	(%)	(%)	(%)	(%)
0	34	68	52	66
0.4	62	68	66	72
1.6	70	84	84	90
3.2	88	84	96	90

^a Figures are mean emergence of 10 seeds in each five replicates per treatment.

Suppressiveness induced by fluorescent pseudomonads. Fluorescent pseudomonads intimately associated with mycelial mats of pathogenic f. sp. of *Fusarium oxysporum* in suppressive soil induced disease suppressiveness when added to raw (previously) conducive soil (Scher & Baker 1980). Soil microorganisms isolated with the same techniques from conducive soil did not induce suppressiveness. Subsequent research suggested that competition for available iron (Fe) by fluorescent pseudomonads was responsible at least partially for suppressiveness (Scher & Baker 1982).

The Fe available in soil for plants and/or microorganisms is defined by the equilibrium reaction:

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 necessary for germ-tube elongation from spores of f. sp. of *Fusarium oxysporum* (Scher & Baker 1982). Indeed, when the suppressive Metz

sandy loam of the Salinas Valley in California (native pH ca 8.0) was reduced to 7.0, about half of the suppressiveness was lost. At a pH of 6.0 the soil was conducive (Scher & Baker 1980). A theoretical basis for induction of suppressiveness through manipulation of Fe availability by use of chelates and/or siderophores (Scher & Baker 1982) was established.

When *Pseudomonas putida*, ethylenediaminedi-O-hydroxyphenylacetic acid (EDDHA), or the ferrated form (FeEDDHA) was added to conducive soil, the soil became suppressive to *Fusarium* wilt of flax, cucumber, or radish. Suppressiveness was not induced by Fe-ethylenediaminetetracetic acid (FeEDTA), or Fe-diethylenediaminetetraacetic acid (FeDTPA). These results may be explained by the mechanism of competition for Fe illustrated in Fig. 5.

Fig. 5. Mechanisms and pathways involved in soil suppressive to *Fusarium* wilt pathogens through competition for iron (Fe).

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

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 the *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 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 the *Fusarium* wilt pathogens.

If soil suppressiveness is biologically induced, for example, during monoculture, current hypotheses suggest that one or more antagonistic microorganisms increase in population density over time (Baker & Chet 1982). These antagonists increase in some soils but not in others because the suppressive soils provide favorable ecological conditions for increase in their activity. The results reported by Scher et al. (1984) support this hypothesis.

With the interrelationships illustrated in Fig. 5 in mind, theory expansion suggested that a soil could be induced to become suppressive by managing the availability of Fe^{3+} so that the activity of antagonistic fluorescent pseudomonads with high Fe-binding properties was enhanced. If this were true, statistically significant interactive effects in the induction of suppressiveness between *P. putida* and FeEDDHA would be observed; however, additive, not synergistic, effects were noticed in short-term experiments (Scher and Baker 1982).

Long-term experiments, however, suggest that the manipulation of appropriate ecological factors in soil can induce suppressiveness of enhancement of the activities of antagonists (Scher et al. 1984).

If either *P. putida* or FeEDDHA induce suppressiveness because they produced fungitoxic substances, a reduction in the inoculum density of the pathogen is expected. This did not occur. Inoculum densities were the same regardless of treatment and were not correlated with suppressiveness (Table 8, Fig. 6). No changes in inoculum density are expected if the mechanism of competition (for Fe^{3+} in this case) is involved.

TABLE 8. Populations of *Fusarium oxysporum* f. sp. *conglutinans* in the rhizosphere of 30 day old radishes^a

TREATMENT	cfu/g soil
CONTROL	2.1×10^3
<i>Pseudomonas putida</i>	1.8×10^3
100 μg FeEDDHA/g soil	2.2×10^3
<i>P. putida</i> and 100 μg FeEDDHA/g soil	2.0×10^3

^a Figures are means from four pots, 10 plants each. Treatments are not significantly different from the control ($P = 0.05$).

Fig. 6. Population density of *Fusarium oxysporum* f. sp. conglutinans in the rhizospheres of radishes undergoing weekly replanting when A, *Pseudomonas putida* N1-R at 1×10^7 cfu/g soil or B, 100 or 1000 µg FeEDDHA/g soil were added to soil. Initial population densities of *F. oxysporum* f. sp. conglutinans were A, 700 or B, 200 propagules/g soil. Data points represent means from four pots, 10 plants each. Treatments were not significantly different from controls ($P = 0.05$) at any replanting times.

In contrast, population densities of *P. putida* were significantly affected by treatment with Fe chelators. Addition of FeEDDHA increased the density of *P. putida* in rhizosphere soil of cucumbers 30 days after application (Table 9) or when radishes were grown in monoculture (Fig. 7) while FeEDTA suppressed the development of *P. putida*.

TABLE 9. Populations of *Pseudomonas putida* in the rhizosphere of 30 day old cucumbers

Treatment	cfu/g soil
CONTROL	1.3×10^5
100 μ g FeEDDHA/g soil	3.7×10^5 *
1000 μ g FeEDDHA/g soil	4.0×10^6 *

^a Figures are means from four pots, five plants each.

* Significantly different than control ($p = 0.05$).

Fig. 7. Populations of *Pseudomonas putida* N1-R in the rhizosphere of radishes undergoing successive weekly replanting. *P. putida* (10^5 cfu/g soil) and FeEDDHA (100 or 1000 μ g/g soil) or FeEDTA (100 μ g/g soil) were initially added to soil. Data points represent means from four pots, 10 plants each. Populations in the FeEDTA and 100 μ g FeEDDHA/g soil treatments were significantly different than in the controls at all replanting times. Populations in the 100 μ g FeEDDHA/g soil treatment were significantly different from the control at replanting times 1 and 2.

Various hypotheses were explored to explain these phenomena. FeEDDHA could be utilized as a nutrient by *P. putida*; however, this was not demonstrated with in vitro experiments. It is also possible that microbes, other than *P. putida*, could break down FeEDDHA to a utilizable nutrient source for the antagonists. This could explain why the population density of *P. putida* increased by addition of FeEDDHA in fallow but not in autoclaved soil (Fig. 8).

Fig. 8. Effect of FeEDDHA (100 or 100 μ g/g soil) or FeEDTA (100 μ g/g soil) on the survival of *Pseudomonas putida* N1-R in fallow soil. A, raw soil, populations in the 1000 μ g FeEDDHA/g soil treatments were significantly different from the control at 3 and 21 days, whereas 100 μ g FeEDDHA/g soil had no significant effect. B, sterile soil, treatments were not significantly different from the control at any time ($P = 0.05$).

In our opinion, the more likely explanation involves the unique ability of *P. putida* to acquire Fe^{3+} to the detriment of the activity of other microorganisms. When EDDHA is added to soils, a high proportion of Fe^{3+} is bound at a stability constant of $\log_{10} = 33.9$. Only microorganisms, like *P. putida*, producing siderophores with a higher stability constant can utilize this trace element and increase in activity and population density. Thus, population densities of the antagonists increased over time in rhizospheres (Table 8, Fig. 7) or in raw fallow soil (Fig. 8A) when FeEDDHA was added; however, such increase was not observed when the soil was treated with FeEDTA which has a lower stability constant than the commonly produced hydroxamate siderophores. By this hypothesis, *P. putida* had no competitive advantage for acquisition of Fe^{3+} in autoclaved soil when FeEDDHA was added and, indeed, no effect due to treatment with chelates was observed (Fig. 3B).

These results suggest that management of the environment of conducive soils, in this case appropriate applications of an Fe chelate with a relatively high stability constant, can induce suppressiveness by selectively enhancing the activity of certain antagonistic components of soil in the rhizospheres of plants.

An attempt was made by Sneh et al. (1984) to identify other mechanisms than competition by Fe in the induction of suppressiveness in soil to *Fusarium* wilt. For example, lysis of germ tubes from chlamydospores could be involved. Thus, over 700 bacteria and actinomycetes were isolated from the *Fusarium*-suppressive Metz fine sandy loam collected from the Salinas Valley in California. Isolates were screened for their ability to produce the fungal cell-wall degrading enzyme chitinase, to lyse dead and living germ-tubes of chlamydospores and to produce fluorescent compounds (siderophores) in iron (Fe)-deficient nutrient medium. Representative isolates from groups with or without these attributes were added to conducive soil where they were evaluated for their influence on chlamydospore germination of *Fusarium oxysporum* f. sp. *cucumerinum* rhizosphere (Table 10) and nonrhizosphere soil (Table 11), and for their ability to induce suppressiveness to *Fusarium* wilt of cucumber (Table 12). Nonfluorescent bacteria generally were not inhibitory to chlamydospore germination except for *Enterobacter cloaca* and isolate 691 which induced lysis in vitro. Those isolates capable of inducing lyses in vitro did not induce more germ-tube lysis in rhizosphere (Table 13) or nonrhizosphere soil (Table 14) than in controls. There was a direct correlation ($r = 0.99$) between the amount of siderophores produced by various fluorescent pseudomonads and inhibition of chlamydospore germination by these isolates in soil (Fig. 9). Addition to soil of Fe^{2+} , Zn^{2+} , Co^{2+} , Mn and MoO_4 partially counteracted the inhibitory effect induced by fluorescent pseudomonads. The same concentration of micronutrients was not inhibitory to the pseudomonads in soil. A chelator (FeEDDHA), previously reported to induce *Fusarium* suppressiveness in soil, inhibited chlamydospore germination in the rhizosphere but not in nonrhizosphere soil. This confirmed the theory (Scher & Baker 1982) related to the role of Fe^{3+} in the mechanism of competition. Disease incidence suppression was stronger with the highly fluorescent siderophore-producing pseudomonads than in other isolates tested; however, some nonfluorescent bacteria also suppressed disease. Combinations of lytic and fluorescent bacteria did not increase suppressiveness (Table 12).

TABLE 10. Effect of bacterial isolates^a on germination of chlamydospores of *Fusarium oxysporum* f. sp. *cucumerinum* in the rhizosphere of cucumber seedlings^b

Isolate	Characteristic	Chlamydospore germination	
		%	% of control
Control		35	100
<i>Pseudomonas</i> sp.	61 Fluorescent	27	77
<i>Pseudomonas</i> sp.	346 Fluorescent	17	49
<i>Pseudomonas</i> sp.	712 Fluorescent	16	46
<i>Pseudomonas</i> sp.	N1R Fluorescent	23	65
<i>Serratia liquifasciens</i>	Chitinolytic	35	100
<i>Arthrobacter</i> sp.	P35 Germ-tube lytic	35	100
<i>Actinomyces</i>	227 Germ-tube lytic	35	100
not identified	691 Germ-tube lytic	25	71
not identified	710 Germ-tube lytic	27	77
not identified	711 Germ-tube lytic	27	77
<i>Enterobacter cloaca</i>	None ^c	17	49
<i>Bacillus</i> sp.	443 None	35	100
LSD ($P = 0.05$)		9	

^a Bacteria were added to soil at 1×10^8 cfu/g soil.

^b 0.5 mm layer of soil was collected around tap roots of 3 day old seedlings.

^c Characteristics in this column marked "None" designates isolates that were not fluorescent, chitinolytic or germ-tube lytic.

TABLE 11. Effect of bacterial isolates^a on germination of chlamydospores of *Fusarium oxysporum* f. sp. *cucumerinum* in soil amended with glucose and asparagine^b

Isolate	Characteristics	Chlamydospore germination	
		%	% of control
Control		82	100
<i>Pseudomonas</i> sp.	A12 Fluorescent	31	38
<i>Pseudomonas</i> sp.	B10 Fluorescent	43	52
<i>Pseudomonas</i> sp.	61 Fluorescent	64	78
<i>Pseudomonas</i> sp.	346 Fluorescent	13	16
<i>Pseudomonas</i> sp.	381 Fluorescent	14	17
<i>Pseudomonas</i> sp.	712 Fluorescent	13	16
<i>Pseudomonas</i> sp.	N1R Fluorescent	15	18
not identified	3c Chitinolytic	71	87
not identified	12c Chitinolytic	69	84
<i>Serratia liquifasciens</i>	Chitinolytic	74	90
<i>Arthrobacter</i> sp.	P35 Germ-tube lytic	72	88
<i>Arthrobacter</i> sp.	163 Germ-tube lytic	68	83
<i>Actinomycete</i>	227 Germ-tube lytic	82	100
not identified	496 Germ-tube lytic	68	83
not identified	691 Germ-tube lytic	59	72
not identified	710 Germ-tube lytic	62	76
not identified	711 Germ-tube lytic	70	86
<i>Enterobacter cloaca</i>	None ^c	16	20
<i>Erwinia herbicola</i>	None	61	75
<i>Bacillus</i> sp.	443 None	78	95
not identified	700 None	75	92
LSD ($P = 0.05$)		15	

^a Bacteria were added to soil at 1×10^8 cfu/g soil.

^b Glucose and asparagine were added to soil at 600 and 150 μ g/g soil, respectively.

^c Characteristics in this column marked "None" designate isolates which were not fluorescent, chitinolytic or germ-tube lytic.

TABLE 12. Effect of addition of bacterial isolates^a on disease induced by *Fusarium oxysporum* f. sp. *cucumerinum*

Isolate	Characteristic ^a	Disease incidence ^b (% of control) ^b
<i>Pseudomonas</i> sp.	B10	Fluorescent
<i>Pseudomonas</i> sp.	A12	Fluorescent
<i>Pseudomonas</i> sp.	N1R	Fluorescent
<i>Pseudomonas</i> sp.	8c	Fluorescent
<i>Pseudomonas</i> sp.	61	Fluorescent
<i>Pseudomonas</i> sp.	346	Fluorescent
<i>Pseudomonas</i> sp.	381	Fluorescent
<i>Pseudomonas</i> sp.	544	Fluorescent
<i>Pseudomonas</i> sp.	712	Fluorescent
<i>Pseudomonas</i> sp.	452	Fluorescent
<i>Pseudomonas</i> sp.	609	Fluorescent
Not identified	3c	Chitinolytic
Not identified	12a	Chitinolytic
Not identified	409	Chitinolytic
<i>Hafnia</i> sp.		Chitinolytic
<i>Serratia liquifasciens</i>		Chitinolytic
<i>Arthrobacter</i> sp.	P35	Germ-tube lytic
Not identified	163	Germ-tube lytic
Not identified	496	Germ-tube lytic
Not identified	710	Germ-tube lytic
<i>Actinomycete</i>	227	Germ-tube lytic
Not identified	406	Germ-tube lytic
Not identified	411	Germ-tube lytic
Not identified	632	Germ-tube lytic
Not identified	641	Germ-tube lytic
Not identified	682	Germ-tube lytic
Not identified	691	Germ-tube lytic
Not identified	711	Germ-tube lytic
<i>Bacillus</i> sp.	443	None
Not identified	509	None
Not identified	700	None
<i>Erwinia herbicola</i>		None
<i>Enterobacter cloacae</i>		None
<i>Pseudomonas</i> sp.	B10 and	Fluorescent
<i>Pseudomonas</i> sp.	346 and	Fluorescent
<i>Pseudomonas</i> sp.	712	Fluorescent
<i>Pseudomonas</i> sp.	61 and	Fluorescent
<i>Serratia liquifasciens</i>		Chitinolytic
<i>Pseudomonas</i> sp.	N1R and	Fluorescent
Not identified	163	Germ-tube lytic
<i>Pseudomonas</i> sp.	B10 and	Fluorescent
<i>Arthrobacter</i> sp.	P35	Germ-tube lytic
<i>Pseudomonas</i> sp.	712 and	Fluorescent
Not identified	691	Germ-tube lytic
<i>Pseudomonas</i> sp.	B10 and	Fluorescent
<i>Serratia liquifasciens</i>		Chitinolytic

<u>Pseudomonas sp.</u>	712 and	Fluorescent	60
<u>Serratia liquifasciens</u>		Chitinolytic	
<u>Pseudomonas sp.</u>	712 and	Fluorescent	60
<u>Not identified</u>	411	Germ-tube lytic	
<u>Pseudomonas sp.</u>	712 and	Fluorescent	60
<u>Not identified</u>	711	Germ-tube lytic	
<u>Pseudomonas sp.</u>	712 and	Fluorescent	60
<u>Enterobacter cloacae</u>		Germ-tube lytic	
<u>Pseudomonas sp.</u>	346 and	Fluorescent	49
<u>Serratia liquifasciens</u>		Chitinolytic	
<u>Pseudomonas sp.</u>	381 and	Fluorescent	70
<u>Not identified</u>	496	Germ-tube lytic	
<u>Pseudomonas sp.</u>	609 and	Fluorescent	55
<u>Not identified</u>	710	Germ-tube lytic	
<u>Pseudomonas sp.</u>	381 and	Fluorescent	37
<u>Not identified</u>	691	Germ-tube lytic	
<u>Pseudomonas sp.</u>	346 and	Fluorescent	43
<u>Not identified</u>	409	Chitinolytic	
LSD ($P=0.05$)			23

^a Characteristics in this column marked "None" designates isolates that were not fluorescent, chitinolytic or germ-tube lytic.

^b Disease incidence was calculated as percent incidence of nontreated control which had $\pm$ 13% incidence. Results are the average of three trials. Each pot contained five plants, treatments were randomized in three replications.

TABLE 13. Effect of bacterial isolates^a on germination and subsequent lysis of chlamydospores of Fusarium oxysporum f. sp. cucumerinum in the rhizosphere^b of cucumber seedlings^c

Isolate	Characteristic	Incubation period		
		2 days	8 days	
		Germi- nation (%)	Chlamydo- spores with germ- tubes (%)	Germ- tubes with lysis (%)
Control		43	23	12
<u>Pseudomonas</u> sp. N1R	Fluorescent	28	23	11
<u>Pseudomonas</u> sp. 61	Fluorescent	34	25	12
<u>Pseudomonas</u> sp. 346	Fluorescent	26	23	11
<u>Serratia liquifasciens</u>	Chitinolytic	47	24	14
not identified 691	Germ-tube lytic	28	19	8
not identified 710	Germ-tube lytic	40	23	12
not identified 711	Germ-tube lytic	38	20	10
<u>Enterobacter cloaca</u>	None ^d	19	16	7
<u>Pseudomonas</u> sp. 61 and	Fluorescent	29	25	12
<u>Serratia liquifasciens</u>	Chitinolytic			
<u>Pseudomonas</u> sp. N1R and	Fluorescent	33	26	18
not identified 691	Germ-tube lytic			
<u>Pseudomonas</u> sp. N1R and	Fluorescent	34	16	7
not identified 711	Germ-tube lytic			
LSD ($P = 0.05\%$)			11	7

^a Bacteria were added to soil at 1×10^8 cfu/g soil.

^b A 0.5 mm layer of soil was collected around tap roots of 3 days old cucumber seedlings.

^c Results are an average of three independent experiments each with three replications.

^d Characteristics in this column marked "None" designates isolates that were not fluorescent, chitinolytic or germ-tube lytic.

TABLE 14. Effect of bacterial isolates^a on lysis of pregerminated chlamydospores of Fusarium oxysporum f. sp. cucumerinum^b

Isolates		Characteristic	Incubation (8 days)	
			Chlamydo- spores with germ-tubes (%)	Germ tubes with lysis (%)
Control			24 ^b	10 ^b
<u>Pseudomonas</u> sp.	61	Fluorescent	22	11
<u>Pseudomonas</u> sp.	346	Fluorescent	22	11
not identified	691	Germ-tube lytic	21	13
not identified	710	Germ-tube lytic	23	13
not identified	711	Germ-tube lytic	24	14
<u>Enterobacter cloaca</u>		None ^d	21	10

^a Bacteria were added to soil at 1×10^8 cfu/g soil after 24 hr of incubation in soil amended with glucose (600 μ g/g soil) and asparagine (150 μ g/g soil). During the 24 hr incubation period chlamydospores were induced to germinate at a level of 78%.

^b Results of all treatments in each column are not significantly different ($P = 0.05$). The results are average of three independent experiments each with three replications.

^c After incubating for 8 days.

^d Characteristics in this column marked "None" designates isolates that were not fluorescent, chitinolytic or germ-tube lytic.

Fig. 9. The correlation between siderophore production by nine isolates of *Pseudomonas* sp. (as measured by optical density of a siderophore extract at 420 nm) and inhibition of chlamydospore germination of *Fusarium oxysporum* f. sp. *cucumerinum* in soil amended with glucose (600 $\mu\text{g/g}$) and asparagine (150 $\mu\text{g/g}$ soil).

If indeed, siderophores produced by the fluorescent pseudomonads compete for limiting Fe^{3+} , then the addition of available Fe to a (previously) suppressive system should result in a shift to conduciveness. This was the case (Sneh et al 1984), as reviewed above; however, addition of other cations also reduced suppressiveness to chlamydospore germination to a smaller extent. To investigate this, siderophores were partially purified by dialysis and freeze drying (Elad & Baker 1985a).

In vitro, the cations Mg^{2+} , Mn^{2+} , Ca^{2+} , Co^{2+} , Cu^{2+} , Zn^{2+} , Al^{3+} , K^{+} , Na^{+} , NH_4^{+} , or Ni^{+} competed with Fe^{3+} on binding sites of siderophores (Fig. 10, 11). Thus, an excess of these cations would alleviate competition for available Fe by fluorescent pseudomonads. These results also suggest, however, that the effect is temporary since the affinity of the siderophore to Fe^{3+} was higher than the other cations.

Fig. 10. Absorption curves at 410 nm (characteristic of Fe-siderophore absorption characteristics) for purified siderophore solution produced by *Pseudomonas putida* (isolates A12) where FeCl_3 was added at various concentrations after incubation for 1 hr of the solutions with the respective cations added at $0.33 \times 10^{-4} \text{ M/ml}$.

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TABLE 15. The influence of siderophore, EDDHA and iron on percentage of germination of chlamydospores of Fusarium oxysporum f. sp. cucumerinum, in vitro

Treatments	Germ tube length (μ m) 16 hr	Germination 16 hr	Germination 8 hr after addition of glucose and asparagine (g/a) -g/a ^b +g/a ^b
-			
-			
-Fe ³⁺	>100	54.0 c ^f	56.8 b
+Fe ³⁺	>100	61.6 c	81.7 b
Siderophore ^c	10-18	30.5 b	35.5 a
Siderophore	10-95	30.7 b	54.3 a
EDDHA ^d	5-15	25.4 a	57.3 a
EDDHA	5-15	27.5 a	54.3 a
			53.4 a

^a 5 x 10³ chlamydospores per ml were stimulated to germinate by 250:62 μ g/ml glucose and asparagine, respectively.

^b All chlamydospore suspensions were centrifuged and washed in distilled water after 16 hr incubation. Glucose and asparagine were added at 250:62 μ g/ml.

^c Lyophilized and freeze dried siderophores of Pseudomonas sp. 346 was mixed in final dose of 128 μ g protein/ml.

^d 5 x 10⁻⁴ M/ml.

^e 1 x 10⁻⁴ M/ml.

^f Number of each column followed by the same letter are not significantly different ($P = 0.05$).

Two hypotheses have been advanced to explain the mechanism(s) associated with suppressiveness. The first hypothesis (elaborated at some length, above) suggests that the activity of fluorescent pseudomonads in soils (of high pH) produce siderophores that compete for available Fe required by the pathogen for germination and penetration. The other hypothesis is based on observations that more nutrients were required to induce chlamydospore germination in suppressive than in conducive soils. This, and other observations lead French workers to hypothesize that the total biomass in suppressive soils reduced nutrient concentrations to the extent that the ability of chlamydospores to germinate and maintain subsequent activity during the infection process was reduced. Evidence related to the validity of these two hypotheses and the relative roles of Fe and carbon competition in suppressive soils induced by fluorescent pseudomonads was accumulated by Elad & Baker (1985b).

Growth rates of pseudomonads in soil were not significantly different (Fig. 12). Thus, the biomass of each was the same. Yet their ability to inhibit chlamydospore germination in soil, in the rhizosphere (Table 17) and/or to decrease disease (Fig. 13) varied. Such responses were directly correlated with their ability to produce siderophores. Thus, no evidence for substantial induction of pathogen or disease suppressiveness by biomass alone was obtained.

Fig. 12. Growth curves, in chlamydospore-enriched soil, of *Pseudomonas* spp. isolates 346 (▲), 2ZA (○), A1/UV/AB-6 (▼), *P. putida* A12 (●) and *Enterobacter cloacae* (□).

Table 17. The effect of *Pseudomonas* spp.^a with (isolate 346) and without (isolate A1/UV/AB-6) the ability to produce siderophores and Fe^b on germination of chlamydospores of *Fusarium* spp. in rhizosphere soil^c

Species of <i>Fusarium</i>	Plant rhizo-sphere ^d	Chlamydospore germination (%)					
		Control		A1/UV/AB-6		346	
		No Fe ³⁺ added	Fe ³⁺ added	No Fe ³⁺ added	Fe ³⁺ added	No Fe ³⁺ added	Fe ³⁺ added
<i>F. oxysporum</i>							
<i>lini</i>	flax	34.3	36.1	29.1	31.0	12.9*	28.4*
<i>conglutans</i>	radish	41.2	47.3	41.2	43.7	20.5*	37.5*
<i>cucumerinum</i>	cucumber	46.3	43.4	31.4*	31.7*	23.9*	31.7*
<i>pisi</i>	pea	42.1	51.3	37.3	41.2	25.3*	39.1*
<i>F. solani</i> f. sp.							
<i>pisi</i>	pea	50.0	48.1	38.4*	40.0*	35.1*	34.4*
<i>cucurbitae</i>	cucumber	38.1	39.4	32.3*	30.1*	34.0	30.4*
<i>phaseoli</i>	bean	27.1	26.4	28.3	26.9	22.3*	23.6*
<i>F. roseum</i> ^e							
'Graminearium' I	wheat	27.2	33.4	34.1	32.2	25.0	26.4
'Graminearium' II	corn	36.7	35.7	32.0	33.3	29.6	31.0

^a Population density of both *Pseudomonas* spp. isolates was adjusted to 9×10^6 cfu/g soil initially.

^b Fe³⁺ concentration was 2×10^{-4} M/g soil.

^c Values marked * are significantly different from the control ($P = 0.05$).

^d Roots of pregerminated plants were placed on chlamydospore-enriched soil between two glass slides. Germination of chlamydospores in the rhizosphere of each plant was observed after a 36-hr incubation period at 28 C.

^e Culture I of *F. roseum* was isolated from wheat. Culture II was isolated from carnation.

Fig. 13. Correlations between siderophore production by various isolates of *Pseudomonas* spp. and suppression of *Fusarium* wilts induced by: A. *F. oxysporum* f. sp. *cucumerinum* in cucumber ($r = 0.747$ -) or f. sp. *conglutinans* in radish ($r = 0.825$ - 0) and B. *F. oxysporum* f. sp. *pisii* in peas ($r = 0.872$).

Inhibition by any of the isolates used in these investigations due to antibiosis in vitro was not observed. However, competition for nutrient(s), other than Fe, may be involved in inhibiting chlamydospore germination. Evidence for this was obtained by use of the mutant (AI/UV/AB-6) which did not produce siderophores. When this isolate was added to soil, chlamydospore germination of *F. solani* f. sp. *pisii* and *cucurbitae*, and *F. oxysporum* f. sp. *cucumerinum* in rhizospheres of host plants was inhibited in comparison to nontreated controls (Table 17). However, in all these cases, available iron added to systems did not influence inhibition by isolate AI/UV/AB-6. In contrast, where Fe was added to soil containing the siderophore-producing isolate (346), inhibition of chlamydospore germination of f. sp. of *F. oxysporum* was partially nullified (Tables 17, 18). In this case iron was limiting--the element involved in competition. Clearly, in systems involving inhibition induced by the mutant (not producing siderophores), the limiting factor was not Fe. Rather some other nutrient essential for complete germination of chlamydospores was not available. Such observations may provide information on the impact of biomass on suppressiveness but it is obvious from a study of the data in Table 17 that such effects are quantitatively lower than the more specific mechanism of Fe- competition.

Table 18. The influence of *Pseudomonas* sp. (346) on germination of chlamydospores of *Fusarium oxysporum* f. sp. *cucumerinum* in soil when glucose and asparagine or Fe were applied in equal (total) amounts at the beginning of the experiments or in pulses^a

Applications of Fe	Application of glucose and asparagine	Germination of chlamydospores (%) ^b	
		Control	<i>Pseudomonas</i> sp. (346) ^c
None	One	49.3 w ^d	17.5 z
None	Six pulses	35.3 x	26.0 z
One	One	52.0 w	41.2 wx
Six pulses	Six pulses	23.8 z	22.2 z

^a Glucose and asparagine or Fe were applied once or applications were divided equally in six applications at intervals of every 2 hr in pulses. Total application of glucose:asparagine was 125:41 µg/g of soil. Total application of Fe³⁺ was 1×10^{-4} M/g soil.

^b Germination of chlamydospores was observed after a 24 hr incubation period at 27 C.

^c Population density dynamics of isolate 346 during the course of the experiment are presented in Fig. 4.

^d Numbers followed by the same letter are not significantly different ($P = 0.05$). There were four replications and 200 chlamydospores counted in each replication.

What nutrient is likely to be limiting if not Fe? Other factors essential for germination of propagules of *Fusarium* spp. in soil are carbon and nitrogen. Since nitrogen was in excess in the soil and systems involved in this research, the most likely limiting factor is carbon.

In the past, experimenters have applied one application of various organic nitrogen and carbon sources to induce chlamydospore germination of *Fusarium* spp. in soil. Obviously, this abstraction does not reflect the concrete situation found in soils where root exudates triggering germination are continuously being 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 between this treatment and a one-time application of nutrients at the beginning of the experiment in the presence of fluorescent pseudomonads. Except at either 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 (Fig. 14). 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 tests in which soils were supplied with one application of nutrients.

Fig. 14. Influence of different concentrations of glucose and asparagine (1/4 of each glucose concentration) on percentage of reduction in germination of chlamydospores of *Fusarium oxysporum* f. sp. *cucumerinum*, by *Pseudomonas* sp. (346). Glucose and asparagine were applied one time at the beginning of the 24 hr incubation (0) or in six pulses (o), every 2 hr in the same total concentration as in one application.

The explanation for these results may be complex but certain hypotheses may be advanced. First, it is apparent that nutrient concentration has a profound effect on the production of siderophores; the less nutrient the lower the proportion of siderophores produced per unit of multiplication of a pseudomonad (Fig. 15). This is not surprising since siderophores are secondary metabolites and require adequate carbon levels to sustain production. It follows that small pulses of nutrients applied experimentally to soil or in the rhizosphere would supply less carbon to support activity of pseudomonads during the first critical period of chlamydospore germination induction than a one-time application. This is reflected by results presented in Fig. 16; one application of glucose-asparagine resulted in a higher growth rate (especially 2-6 hr after application) than when applied in pulses. In this same experiment (Table 18), inhibition of chlamydospore germination by isolate 346 in a one-time application of nutrients was 65%; when pulsed it was 26%. This difference in inhibition was complexed and based on two variables: first, chlamydospore germination level in soil not supplied with isolate 346 and amended once with glucose-asparagine was higher than when these nutrients

were applied in pulses, and, second, the reverse was true for soil infested with this siderophore-producing isolate. This apparent correlation was also noticed in experiments involving many isolates of *Fusarium* spp. germinating in rhizospheres in soil infested or not infested with the siderophore-producing isolate 346 (Table 19). There was a significant direct correlation between the germination of chlamydospores in soil to which isolate 346 had not been added and the inhibition induced when the isolate was applied. This suggests that the more nutrients in host exudates (as reflected by higher levels of chlamydospore germination) the more inhibition induced by the pseudomonad. Therefore, increased inhibition results in soil when more nutrients are available for the production of siderophores (Fig. 15).

Fig. 15. Siderophore concentration (----) and growth (—) during 16 hr incubation of *Pseudomonas* sp. 346 (○) and *P. putida* A12 (Δ).

Fig. 16. Growth curves of *Pseudomonas* sp. (346) in chlamydospore-enriched soil. Glucose:asparagine (125:41 μ g/g soil) were applied one time (O, Δ) whereas Fe^{3+} (10^{-4} M/g soil) was applied one time (O), pulsed with the glucose and asparagine (P) or was not applied (O, Δ).

TABLE 19. Germination of chlamydospores of various *Fusarium* spp. in the rhizospheres^a of plant-roots in soil with or without the addition of *Pseudomonas* sp. (isolate 346)^b

Species of <i>Fusarium</i>	Average inhibition (%) ^b	Correlation coefficient between germination and inhibition ^c
<i>F. oxysporum</i> f. sp.		
<u>vasinfectum</u>	59.2	0.80
<u>dianthi</u>	63.2	0.69
<u>conglutinans</u>	58.2	0.79
<u>melonis</u>	67.1	0.92
<u>lini</u>	61.9	0.81
<u>cucumerinum</u>	59.9	0.76
<u>pisi</u>	59.0	0.78
<i>F. solani</i> f. sp.		
<u>pisi</u>	32.9	0.65
<u>cucurbitae</u>	29.5	0.49
<u>phaseoli</u>	37.1	0.70
<i>F. roseum</i> ^d		
'Graminearum' I	49.1	0.92
'Graminearum' II	41.9	0.85

a Roots of pregerminated plants were placed on chlamydospore-enriched soil between two glass slides. Germination of chlamydospores in the rhizosphere of each plant was observed after a 36-hr incubation period at 28 C.

b Average inhibition in rhizospheres of cucumber, corn, tomato, radish, flax, sugarbeet, bean, wheat and pea. Germination among nontreated plants rhizospheres varied from 12.2-64.3%. Figures are averages over all observations for a single Fusarium spp. isolate.

c Numbers represent correlation coefficients between chlamydospore germination in nontreated rhizosphere soil and the inhibition induced by application of isolate 346. All r values are significant ($P = 0.05$) except for F. solani f. sp. cucurbitae.

d Culture I of F. roseum was isolated from wheat. Culture II was isolated from carnations.

A further test of these conclusions may be performed by varying the Fe-availability in soil. Fluorescent pseudomonads produce siderophores only in low Fe environments. Fe supplied either in pulses or at one time, nullified inhibition of chlamydospore germination induced by isolate 346 (Table 18). Thus, even though chlamydospore germination was increased by one application of glucose-asparagine plus Fe over that in the pulsed treatment, one application of Fe depressed activity of isolate 346 more than any other treatment (Fig. 20). Depression of pseudomonads by Fe was also noticed in rhizospheres or radishes in monoculture when a chelated Fe compound with a low stability constant was added to the soil (Scher et al. 1984).

Suppressiveness to the f. sp. of F. oxysporum has been noticed in the field but not to other species of Fusarium. Low levels of inhibition of chlamydospore germination were noticed in this study for some isolates of F. solani attributable to competition for carbon but not Fe. Again, addition of the ligand EDDHA (that binds Fe in soil so that it is not available to the hydroxamate siderophores of Fusarium spp. [Scher & Baker 1982] suppressed germination of isolates of F. oxysporum but not solani or roseum (Table 20).

Table 20. The effect of EDDHA on germination of chlamydospores^a of Fusarium spp. in soil

Species of <u>Fusarium</u>	Germination (%) ^b	
	EDDHA not applied	EDDHA applied
<u>F. oxysporum</u> f. sp.		
<u>vasinfectum</u>	32.3	15.7*
<u>conglutinans</u>	48.2	24.3*
<u>melonis</u>	45.4	25.4*
<u>lini</u>	41.3	12.4*
<u>cucumerinum</u>	31.4	20.2*
<u>pisi</u>	47.3	27.2*
<u>F. solani</u>		
<u>pisi</u>	38.3	31.7
<u>cucurbitae</u>	26.3	28.9
<u>phaseoli</u>	31.8	30.8
<u>F. roseum</u> ^d		
'Graminearum' I	26.7	28.3
'Graminearum' II	44.1	38.3

^a Chlamydospores were induced to germinate by adding 125:41 μ g glucose: asparagine to 1 g of soil. Measurements of germination were obtained 20 hr after initiation of the experiment. Incubated at 28 C.

^b Numbers designated * were significantly ($P = 0.05$) different from controls where EDDHA was not supplied. There were four replications and 200 chlamydospores were counted in each replication.

^c EDDHA was applied at 3×10^{-4} M/g soil.

^d Culture I of F. roseum was isolated from wheat, Culture II was isolated from carnation.

Why did low availability of Fe affect germination of f. sp. of F. oxysporum but not F. solani and roseum? In general, chlamydospores of F. oxysporum are smaller than in the other species. Indeed, when chlamydospores of F. solani f. sp. phaseoli and F. oxysporum f. sp. cucumerinum were compared, the ratios of volume were 4.99:1, weight 4.6:1, and Fe 3:1. This suggests that the larger chlamydospores of F. solani contained more Fe per propagule than F. oxysporum and, thus, did not require an exogenous supply of this element.

What information is currently available leading to the identification of mechanisms associated with the induction of suppressiveness in soil to the Fusarium wilt pathogens? Fluorescent pseudomonads intimately associated with mycelial mats of pathogenic f. sp. of F. oxysporum in suppressive soil induced disease suppressiveness when added to raw (previously) conducive soil (Scher & Baker 1980). Soil microorganisms isolated with the same techniques from conducive soil did not induce suppressiveness. Available Fe was necessary for complete germination of chlamydospores of F. oxysporum and evidence accumulated by Scher and Baker (1982) suggested that siderophores produced by fluorescent pseudomonads competed for Fe on the rhizoplane of the host root. Low Fe availability was necessary for siderophore production and, therefore, biological control. Low Fe

status was found in the *Fusarium* suppressive alkaline soil in the Salinas Valley of California. Soil pH was 7.3 or above and Fe was available at 3.2 g/g soil. When this soil was acidified, it became conducive (Scher & Baker 1980). Suppressiveness (induced by activity of fluorescent pseudomonads) was induced to become conducive when Fe available to the pathogen was applied (Scher & Baker 1982, Sneh et al. 1984). A chelator (FeEDDHA) with higher stability (Fe-binding) constants than the hydroxamate siderophores of the f. sp. of *F. oxysporum* induced suppressiveness (Scher & Baker 1982). Indeed, the rhizosphere competence of *P. putida* was increased significantly upon the application of FeEDDHA presumably because the siderophores produced by pseudomonads were able to obtain Fe from the chelate, whereas, other groups of potentially competing rhizosphere colonists were not (Scher et al. 1984). In every case Fe-competition by pseudomonads was responsible for inhibition of chlamydospore germination in soil or in the rhizosphere (Elad & Baker 1985b). Finally, there was correlation between chlamydospore germination in soil (Sneh et al. 1984) or disease suppressiveness and production of siderophores (Elad & Baker 1985b).

Mechanisms, other than Fe-competition, were explored that could implicate additional factors potentially capable of inducing suppressiveness. The possibility of cross protection induced by nonpathogenic microorganisms in the suppressive soil was tested with negative results (unpublished data). As mentioned above, there is no evidence of antibiosis among our antagonists; indeed, there was no correlation between in vitro antibiosis and suppression by isolates from our laboratory of take-all diseases of wheat and *Ophiobolus* patch in turfgrass (Wong & Baker 1984). While numerous bacteria capable of fungal-cell wall degradation and/or lysis were isolated from the *Fusarium*-suppressive soil, none of these added to conducive soil hastened or increased lysis in the rhizospheres of host plants (Sneh et al. 1984). Finally, carbon competition induced by increases in soil biomass may occur in small magnitude but competition for this element was not correlated with suppressiveness (Elad & Baker 1985b).

The only mechanism so far clearly identified for the induction of *Fusarium*-suppressiveness in soil is competition for Fe.

Effective induction and maintenance of disease suppression by an introduced microbial antagonist, like *P. putida*, may be largely dependent on the survival of these agents. Thus, establishment and domination of these microorganisms in the infection court could be a prerequisite to biological control. Thus, the survival of *P. putida* in raw soils was followed (Dupler & Baker 1984).

In this study, survival of an antagonistic *P. putida*, capable of significantly reducing *Fusarium* wilt incidence (Fig. 17), was examined as affected by various factors in nonsterile soil. Addition of *P. putida* (isolate N-1R) to soil at favorable matric potentials did not result in rapid death as often occurs when bacteria are added to soil.

In contrast, after 5 wk, numbers were often higher than the initial population densities (Figs. 18, 19).

Fig. 17. Effect of addition of *Pseudomonas putida* (isolate N-1R) at 10^7 cfu/g of soil on flax wilt incidence. At the end of the experiment wilt incidence differed significantly ($P = 0.05$) from the control.

Fig. 18. A, Survival curves of *Pseudomonas putida* (isolate N-1R) added to fallow, nonsterile soil (10^7 cfu/g dry soil). B, Moisture levels during the experiment. Initial levels corresponded to -0.3, -2, and -15 bars matrix potential.

Fig. 19. Survival curves of *Pseudomonas putida* (isolate N-1R) added at either 10^5 or 10^7 cfu/g dry soil at moisture levels initially at -0.3, -2, and -15 bars matric potential.

Pseudomonas spp. are considered to be drought susceptible with rapid loss of viability when exposed to desiccated soils. However, when *P. putida* was added to soil, which was allowed to dry slowly over a 5-wk period to -100 bars, over 5×10^5 cfu/g soil could be recovered (Fig. 18).

The ability of *P. putida* to survive low soil matric potentials might have been due to an increase in internal osmotic tension of the cells. It has been suggested that there is a relationship between the ability to persist throughout prolonged drought and high internal osmotic tension. When the internal osmotic potential of bacterial cells of drought-susceptible species was raised by growth on media with low water activity, the isolates were more tolerant to dry soil conditions. Perhaps, *Pseudomonas* spp., existing in soils undergoing

slow drying, may be able to increase cell osmotic tension, and thus, become more resistant to desiccation. In addition, it is possible that clay colloids could form favorable microenvironments protecting bacteria in dry soil.

When different initial population densities of *P. putida* were added to nonsterile soil, subsequent increase of the bacteria was greater when soil received the lower inoculum level. This effect was evident when the bacteria multiplied in fallow soil (Fig. 19), or colonized the rhizosphere of radish (Fig. 20). It is possible that multiplication was restricted when *P. putida* was added initially at 10^7 cfu/g dry soil by a soil-imposed limit. It has been postulated that in sterile soil the maximum colonization of *Pseudomonas* was probably due to nutrient availability, space availability, cell migration to new colonizing sites, or a combination of those factors. However, when soil received an initial population density of 10^5 cfu/g soil, maximum numbers reached were always well below those attained in soil that had received the higher inoculum level (Fig. 19). This implies that, in nonsterile soil, there is a microbially imposed limit as well.

Fig. 20. Influence of biologically active soils on colonization of *Pseudomonas putida* (isolate N-1R) in the radish rhizosphere. Soil was incubated 1 wk at near -0.3 bars to stimulate biological activity. This treatment, before the introduction of N-1R (10^5 cfu/g soil), decreased subsequent rhizosphere colonization compared to soil which was air-dry at the beginning of the experiment. A similar reduction in colonization occurred when bacteria were added at 10^7 cfu/g soil.

Population density of *P. putida* was significantly lower when air-dry soil was moistened 1 wk before planting than when moistened at the time of planting (Fig. 20). This reduction in rhizosphere colonization might reflect differences in the nutritional status of the soil, as well as the presence of competitors, predators, and other organisms detrimental to *Pseudomonas*.

P. putida, which existed in soil under ambient environmental conditions in Colorado, were subject to a wide variety of interacting factors that affected their survival (Fig. 21). Season of year had a pronounced effect on population densities over the course of the experiment. As was considered typical of bacteria in soils in temperate regions, numbers diminished in winter. Bacteria surviving in winter soils were thought to be in a state of biochemical inactivity, ready for reactivation when conditions became more favorable. Cell numbers were greatest in the spring, perhaps due to increased soil temperature and availability of organic materials.

Soil type affected numbers of *P. putida* recovered, with counts of the bacteria in the NSL (Fig. 21) often 10 X higher than found in the FCCL (Fig. 21). However, this might reflect a difference in the ability to recover the bacterium rather than survival. Bacteria which existed in the clay loam, may have been more tightly bound by soil colloids, thus lowering plate counts.

Fig. 21. Survival of *Pseudomonas putida* (isolate N-1R) under ambient environmental conditions in fallow A, Fort Collins clay loam or B, Nunn sandy loam, at three depths.

Survival in the Nunn sandy loam was greater at a 25-cm depth than at 5- or 10-cm (Fig. 21). This possibility reflected a more favorable matric potential at the lower depth. However, survival of the bacterium in the Fort Collins clay loam could not be correlated similarly (Fig. 21).

In the colonization of cucumber (Fig. 22) and radish (Fig. 20) plant roots, *P. putida* persisted throughout the field trials.

The ability of certain *Pseudomonas* spp. to induce suppressiveness to Fusarium wilts may be largely dependent on their root-colonizing ability. Therefore, an understanding of how alterations in the soil environment affect the survival and activity of *Pseudomonas* spp. could lead to more successful applications of the biological control agent to soil.

Fig. 22. Number of cfu of *Pseudomonas putida* (N-1R) in the rhizosphere of cucumber plants grown under field conditions in a 9-wk trial.

A new antagonist -- *Pythium nunn*. Lifshitz et al. (1984a) tested the ability of *Trichoderma* spp. to induce suppressiveness to *Pythium* spp. in soil. Weekly application of bean meal to soil is known to increase activity and inoculum density of this pathogen and this treatment was combined with introduction of high population densities of antagonistic *Trichoderma* spp. No effect of the addition of these antagonists was observed and inoculum densities of a pathogenic *Pythium* sp. (designated N1 and tentatively identified as *P. ultimum*) was increased in all treatments and/or controls except soil incubated at 26°C at pH 7.3 where suppression of N1 occurred (Fig. 23). This suppression was associated with the increase in population density of a *Pythium* sp., isolates designated N2 and N3 (Fig. 24).

Fig. 23. Effect of incubation temperature and soil pH on propagule density of a pathogenic species of *Pythium* (isolate N1) during six weekly incorporations of dried bean leaf meal (0.3% w/w). Bars indicate *P. ultimum* propagule density range (not significantly different) of isolate N1.

Fig. 24. Effect of incubation temperature and soil pH on *Pythium* spp. propagule densities, as determined by soil dilutions on a selective medium after six weekly repeated incorporations of dried bean leaf meal to the soil. Colonies were counted on the plates after 4 days. Isolate N1 formed colonies of >50 mm diameter whereas *Pythium* sp. isolates N2 and N3 formed colonies <30 mm diameter.

When isolates N2 and N3 were added to soil conducive to activity of N1, the competitive saprophytic ability of the pathogen (N1) (Table 21) and also its capacity to induce preemergence damping-off of cucumber (Table 22) was decreased. Therefore N2 and/or N3 induced both pathogen and disease suppressiveness.

TABLE 21. Population densities of a pathogenic species of Pythium (isolate N1) and N2 or N3 in soil incubated with two applications of dried bean leaf meal at 0 and 7 days over a 2-wk period

Soil batch ^a	Temperature	Pythium sp. isolate added ^b	Incubation time				
			1 wk		2 wk		
			Small colonies ^c	Large colonies ^d	Small colonies ^c	Large colonies ^b	
(C)			(cfu/g x 100)	(cfu/g x 100)	(cfu/g x 100)	(cfu/g x 100)	(cfu/g x 100)
A	19	None	NDe z	52 w	4 y	46 w	
		N2	100 v	12 x	380 st	ND z	
		N3	160 u	10 x	360 st	ND z	
A	26	None	0.4 z	5 y	43 w	9 xy	
		N2	160 u	0.2 y	460 s	ND z	
		N3	190 u	1 z	430 s	0.1 z	
B	19	None	ND z	160 u	ND z	150 u	
		N2	110 v	4 y	510 s	ND z	
		N3	110 v	8 xy	450 s	2.4 yz	
B	26	None	ND z	50 w	ND z	60 w	
		N2	130 v	4 y	430 s	0.7 z	
		N3	110 v	2 yz	310 t	ND z	

^a A-Nunn sandy loam collected April 1983; B-Nunn soil collected in July 1982.

^b Pythium isolates N2 and N3 were added to soil so that the population densities were ca 100 cfu/g soil.

^c Small colonies <30 mm diameter after 4 days on the Pythium selective medium--characteristic of the colony morphology of N2 and N3.

^d Large colonies >50 mm diameter after 4 days on the Pythium selective medium--characteristic of isolate N1.

^e ND-not detectable; numbers followed by the same letters are not significantly different ($P = 0.05$) use of FLSD.

TABLE 22. Preemergence damping-off of cucumbers induced by *Pythium* sp. (isolate N1) in soil to which isolates N2 and N3 had or had not been added

Soil ^a batch	Raw soil ^b in mixture	Temperature of incubation	Healthy plants		
			Control	N2	N3
A	(%)	(C)	(%)	(%)	(%)
	90	19	46.7 y	86.7 v	90.0 v
	99	19	70.0 wx	83.3 v	90.0 v
	90	26	60.0 x	90.0 v	86.7 v
B	99	26	83.3 v	83.3 v	90.0 v
	90	19	30.0 z	90.0 v	70.0 wx
	99	19	66.7 x	90.0 v	63.3 x
	90	26	36.7 z	76.7 v	76.7 w
A (nontreated)	99	26	63.3 x	90.0 v	83.3 v
			90.0 v		

^a A-Nunn soil collected April 1983; B-nunn soil collected in July 1982.

^b Isolates N2 and N3 were added to raw soils A and B and leaf meal was added at two weekly intervals during the 2 wk of incubation. In controls, N2 and N3 were not added. These soils were added to raw soil (A) at concentrations of either 1 or 10% by weight.

^c Numbers followed by the same letters are not significantly different ($P = 0.05$) by use of FLSD.

Isolates N2 and N3 had morphological features which were not characteristic of any previously described species of *Pythium*. Therefore, the isolates were described as a new species (Lifshitz et al. 1984b). Since they were isolated from soil in the vicinity of Nunn, Colorado the species was named *P. nunn* Lifshitz, Stanghellini & Baker. Morphological characteristics are illustrated in Fig. 25.

Fig. 25. *Pythium nunn*. Camera lucida drawings.
a-p) Oogonia, antheridia, and oospores.
q-t) Sporangia.

The mechanisms by which *P. nunn* induced suppressiveness were explored. The decrease in inoculum density of pathogenic *Pythium* spp. by activity of the antagonist, suggested mycoparasitic activity. Therefore, the modes of hyphal interaction between the mycoparasite, *Pythium nunn*, and several soil fungi were studied by both phase contrast and scanning electron microscopy (Lifshitz et al. 1985a). In the zone of interaction, *P. nunn* massively coiled around and subsequently lysed hyphae of *P. ultimum* and *P. vexans*. In contrast, *P. nunn* penetrated and eventually parasitized hyphae of *Rhizoctonia solani*, *P. aphanidermatum*, *Phytophthora parasitica*, and *P. cinnamomi* by forming appressorium-like structures. However, *P. nunn* was not mycoparasitic against *F. oxysporum* f. sp. *cucumerinum* or *Trichoderma*

koningnii, and was destroyed by T. harzianum and T. viride. These observations demonstrated that P. nunn was a necrotrophic mycoparasite, with a limited host range and differential modes of action among susceptibles.

These fine structure observations of hyphal interactions between P. nunn and plant pathogenic species suggested that enzymatic dissolution of cell walls of host fungi was involved. This was studied by Elad et al. (1985c). The fluorescence, localized about cell wall lysis at points of interaction when stained with Calcofluor White M2R New (Scher & Baker 1984) suggested enzymatic activity since this stain binds to oligomers in regions of incomplete cell wall polymers. Again, mycoparasitic activity of P. nunn released $^{14}\text{CO}_2$ from labeled cell walls of a plant pathogenic species of Pythium and Rhizoctonia solani. P. nunn had high cellulase and -1,3-glucanase activity when grown on cell walls of Pythium spp. (Tables 24, 25). Chitinase and -1,3-glucanase were produced by P. nunn in cultures containing cell walls of R. solani and Sclerotium rolfsii (Table 25) but low activity was associated with cell walls of Fusarium oxysporum f. sp. cucumerinum (Table 26). These hydrolytic enzymes were produced in dual cultures of P. nunn with several Pythium spp., Phytophthora spp., Mucor sp., Rhizopus sp., R. solani and S. rolfsii but was not detected with ten nonhost fungi in the Deuteromyces. This may be due to the outer cell wall layer of mucilaginous material associated with these nonhost fungi since trypsin- or KOH-treated hyphae of F. oxysporum f. sp. cucumerinum released N-acetyl-D-glucosamine in the presence of crude enzymatic preparations of P. nunn. This suggests that the potential host range of P. nunn is limited by components on the outer layer of fungal cell walls.

TABLE 24. β -1,3-Glucanase (GU) production by Oomycetes fungia

	Substrate - 2.0 mg ml			liquid medium - as sole carbon source	
	Glucose	Laminarin	Pythium sp. (N1) cell walls	R. solani cell walls	
<u>Pythium nunn</u> (N2)	0	1.49 ab	1.35 a	1.54 a	
<u>P. aphanidermatum</u>	0.28	0.41 b	0.58 b	0.21 b	
<u>P. vexans</u>	0	0.54 b	0.31 b	0.23 b	
<u>P. paroecandrum</u>	0	0.53 b	0.06 b	0.16 b	
<u>P. oligandrum</u>	0	0.16 b	0.20 b	0.39 b	
<u>P. ultimum</u> (67.1)	0	0.19 b	0.22 b	0.11 b	
<u>Pythium</u> sp. (N1)	0	0.31 b	0.42 b	0.27 b	
<u>Phytophthora parasitica</u>	0	0.04 c	0 c	0 c	
<u>P. cinnamomi</u>	0	0 c	0 c	0 c	

^a Activity (GU) was tested after incubation of fungi with the above mentioned inducers in liquid SM for 58 hr at 27° C.

^b Numbers in each column followed by the same letter are not significantly different at $P = 0.05$.

TABLE 25. Enzymatic activity of *Pythium nunn* (N2) excreted into liquid dual culture in the presence of different host fungi^a

Host fungus	β -1,3-Glucanase		Cellulase		Chitinase	
	HF	HF+N2	HF	HF+N2	HF	HF+N2
<i>Pythium aphanidermatum</i>	0	0.11 b	0	0.05 a	0	0
<i>P. vexans</i>	0	0.75 b	0.21 a	0.71 b		
<i>P. paroecandrum</i>	0	2.80 b	0.04 a	0.34 b		
<i>P. oligandrum</i>	1.02 a	1.81 b	0.34 a	0.96 b		
<i>P. ultimum</i> (67.1)	0.75 a	3.64 b	0.44 a	1.13 b		
<i>Pythium</i> sp. (N1)	1.17 a	2.70 b	0.21 a	0.67 b		
<i>Phytophthora parasitica</i>	0	1.40 b	0.11 a	0.49 b		
<i>P. cinnamomi</i>	0	0.22 b	0	0.31 b		
<i>Rhizoctonia solani</i>	0	2.51 b	0	0.99 b		
<i>Sclerotium rolfsii</i>	0.94 a	6.07 b	0.01 a	0.02 a	0.13 a	1.47 b
<i>Mucor</i> sp.	0.51 a	1.08 b	0.04 a	0.01 a	0.17 a	0.61 b
<i>Rhizopus</i> sp.	0.09 a	0.21 a	0	0.03 a	0.11 a	0.85 b
<i>Fusarium oxysporum</i>	0.05 a	0.24 b	0	0.04 a	0.09 a	0.57 b
<i>f. sp. cucumerinum</i>	0.49 a	0.57 a	0.08 a	0.08 a	0.33 a	0.35 a

^a *Pythium nunn* (N2) or host fungi (HF) were grown in SM for 24 hr, washed in sterile distilled water and inoculated simultaneously or separately into a liquid SM without any carbon source. Specific enzymatic activity was tested 48 hr later.

^b Numbers of each pair in a row followed by the same letter are not significantly different at $P = 0.05$.

TABLE 26. Release of cell wall monomers ($\mu\text{g ml}^{-1}$) by a crude enzyme solution of *Pythium nunn*^a from hyphae of *Fusarium oxysporum* f. sp. *cucumerinum*^b

Origin of Enzymes	Treatment of <i>Fusarium</i> hyphae					
	None		KOH		Trypsin	
	Glucose	N-acetyl-D-glucosamine	Glucose	N-acetyl-D-glucosamine	Glucose	N-acetyl-D-glucosamine
<i>P. nunn</i> (N2)	0	37 a ^c	0 a	37 a	0	36 a
<i>P. nunn</i> (N3)	0	37 a	45 c	47 b	0	37 a
	0	40 a	27 b	120 d	0	36 a

^a *Pythium nunn* (N2 or N3) were grown for 58 hr on 2 mg ml⁻¹ mixture of *R. solani* cw, laminarin, and chitin (1:1:1-w/w/w), centrifuged and dialyzed for 24 hr to eliminate glucose and N-acetyl-D-glucosamine. β -1,3-Glucanase and chitinase activities in the crude enzyme solution of *P. nunn* (N2) were 0.91 and 0.90 respectively, and 1.64 and 4.30 in *P. nunn* (N3) solutions.

^b *F. oxysporum* f. sp. *cucumerinum* was grown in liquid synthetic medium for 48 hr, harvested and washed in autoclaved distilled water. Hyphae were either nontreated, or given one of the following treatments: KOH for 2 hr, Trypsin (1%) for 20 min in 37° C, or boiling for 20 min. Hyphae were washed three more times and exposed to crude enzyme solution.

^c Numbers of each monomer followed by the same letter are not significantly different at $p = 0.05$.

P. nunn also produced factors inhibiting growth and/or propagule germination of a plant pathogenic Pythium sp. (N1) in vitro (Table 27). Also, lysis of sporangial germ tubes of Pythium sp. (N1) occurred earlier when sporangia on membranes were placed on a repeatedly-amended soil than on raw soil (Lifshitz et al. 1984b and Table 28). Similar lysis occurred in autoclaved soil infested with Pythium types N2 and N3, but N3 was less effective. Sporangia on membranes placed on soil previously amended with bean leaf meal formed less new typical sporangia than on raw soil. Moreover more aborted sporangia were observed on the former treatment and on sterile soil infested with isolate N2 and N3. Sporangia of isolate N1 added to soil did not affect new sporangium formation on membranes indicating that simple competition for nutrients was not involved. However, the effect of N2 and N3 was apparent through the membranes suggesting that a diffusible substance (enzymatic or toxic) is also involved in the destruction of the mycelium of isolate N1 after germination.

TABLE 27. Mycelial growth of *Pythium* sp. (N1) and *Rhizoctonia solani* in liquid medium previously supporting growth of *P. nunn* (N2), *R. solani* and *Pythium* sp. (N1)^a

Fungus grown 1st cycle	Portions of medium in second cycle growth medium mixture (%)			Mycelium dry wt. (mg plate ⁻¹)-second cycle	
	1st cycle medium	PDB	H ₂ O	<i>Pythium</i> sp. (N1)	<i>R. solani</i>
---	0	100	0	87.50 ab	47.30 a
---		0	25	75	35.15 b
<i>P. nunn</i>		75	25	0	11.50 d
"		75	0	25	11.33 d
"		100	0	0	1.20 e
Heat treated <i>P. nunn</i> medium		75	25	0	34.50 b
<i>Pythium</i> sp. (N1)		75	25	0	22.50 c
"		75	0	25	24.20 c
<i>R. solani</i>		100	0	0	26.37 c
"		75	25	0	37.41 b
"		75	0	25	20.40 c
Heat treated <i>R. solani</i> medium		100	0	0	31.75 bc
		75	25	0	34.52 b

^a Fungi were grown in PDB (half concn) for 3d during the first cycle. After aseptic removal of mycelium, the tested fungi were introduced separately into the medium previously supporting growth of various fungi and grown for 3 days in the second cycle. The resulting thalli were collected, oven dried, and weighed.

^b Numbers in each column followed by the same letter are not significantly different at $p = 0.05$.

TABLE 28. Effect of six weekly incorporations of bean leaves (0.3% w/w) to soil on germination and new sporangia formation by sporangia of *Pythium* sp. (isolate N1) placed on membranes on soil

Soil treatment ^a	Germination (%)	Number sporangia/membrane ^b				
		Typical		Ratio		
		24 hr	Empty	New 20-24 μ m	New 10-15 μ m	New:Empty
Nonamended	98		675 z ^d	1564 z	245 x	0.4:1 x
Amended with bean leaves only	98		603 z	230 y	842 y	1.4:1 y
<i>Pythium</i> sp. N1 ^c	98		775 z	1742 z	302 x	0.4:1 x
<i>Pythium</i> sp. N3	98		627 z	132 x	1110 z	1.8:1 z
<i>Pythium</i> sp. N2	98		639 z	49 w	837 y	1.3:1 y

^a Bean leaves (0.3%) were added to all treatments before placing the Nucleopore membranes on soil (batch A).

^b All sporangia were counted on each membrane after 48 hr incubation. Empty sporangia were the germinated ones and the new were the newly formed ones. Two membranes were counted for each treatment. The experiment was repeated three times.

^c Sporangia of isolate N1 added to raw soil (10⁴ spoorangia/g soil).

^d Numbers in each column followed by the same letters are not significantly different ($\bar{p} = 0.05$) by use of FLSD.

Pathogenicity tests of *Pythium* isolates N1, N2 and N3 indicated that while N1 was pathogenic to the plants tested except wheat and barley, the other two isolates were not. These findings suggest the potential use of *P. nunn* isolates N2 and N3 as biocontrol agents against pathogenic *Pythium* sp.

Successful control of *Pythium*-induced damping-off has been achieved by seed coating with a variety of antagonistic microorganisms. However, seed treatment generally provides a temporary protection for only a short period of time and requires repeated applications for every crop. Conversely, control of *Pythium* spp., by direct application of antagonists to raw soil requires relatively large amounts of a biocontrol agent. The present study suggests a procedure to induce suppressiveness by an initial application of a relatively small density of biocontrol agent followed by organic amendment to provide a propagule build up of the biocontrol agent in soil. Such a procedure also has potential for a long-term effect.

The increased growth response. While increased growth responses induced by fluorescent pseudomonads have been observed, the first report of induction of enhanced growth by *Trichoderma* spp. was published jointly by the elements of our BARD cooperating groups (Baker et al. 1984). Increases in dry weight of radishes approaching 300% were observed by mixing a peat-bran culture or conidia of *T. harzianum* in raw soil (Fig. 26).

Fig. 26. The effects of *Trichoderma harzianum* previously grown in peat-bran medium applied at various dosages and various manipulations of this treatment on oven dry weight of leaves of radish after 6 wk of incubation. Each treatment at each dosage contained five replications and there were 10 plants per pot. A. Dosages were plotted according to weight of peat-bran or as relative amounts of conidia added on the abscissa as appropriate. B. The same data plotted by matching cfu dosages on the abscissa. By regression analysis, there were no significant differences ($P = 0.5$) in slope values among the various manipulations. When means of each manipulation were compared, only *T. harzianum* grown in peat-bran was significantly different ($P = 0.05$, FLSD) from the control (horizontal line at 100%) at each level of application.

Extensive experimentation with numerous plant species suggests that Trichoderma spp. can enhance plant growth. Details (supported by data analysis) will not be reviewed here since manuscripts are just now being prepared (e.g., Windham et al. 1985). A factor involved in increased growth response is the production of a secondary metabolite that induces responses in germ-free plants. This metabolite has not been detected in the fluorescent pseudomonads which may explain why responses are greater when Trichoderma spp. are used. The possibility of patenting this phenomenon is presently under study.

Parasitism of sclerotia of Sclerotium rolfsii by Trichoderma harzianum. The possible connection between the enzymatic activity of Trichoderma and its ability to parasitize S. rolfsii sclerotia was examined. Among three T. harzianum isolates, only one (No. 203) was capable of attacking S. rolfsii. All three were inoculated into autoclaved soil containing sclerotia. Enzymatic activity was tested in extracts of both the attacked sclerotia and the soil. T. harzianum isolate 203, capable of attacking S. rolfsii, excreted more β -(1,3)-glucanase and chitinase than isolates (No. 250 and 110) incapable of attacking this pathogen (Table 29).

With the SEM it was seen that S. rolfsii sclerotia parasitized by T. harzianum had lost their regular shape and that holes had appeared in them (Figs. 27 and 28). The Trichoderma mycelium grew over the sclerotia and appeared to penetrate inbetween rind cells (Fig. 29). Over the surface of the sclerotium it is possible to observe aggregates of conidia of the mycoparasite (Fig. 29). Parasitized sclerotia were cut and observed under SEM, and hyphae of Trichoderma were detected inside the cut sclerotium (Fig. 30) while many conidia of T. harzianum were also found on the cut surface of a sclerotium (Fig. 31).

The mode of parasitism by T. harzianum was further investigated by examining parasitized sclerotia with TEM. T. harzianum hyphae penetrated medullar sclerotial cells (Fig. 32) and this mycelial invasion is apparently associated with hydrolysis of the host cell walls. A progressive invasion resulted in loss of cytoplasm in host cells. In general, the cells of the sclerotial host which are relatively large, contained or were surrounded by a few cells of the mycoparasites. It should be noted that osmiophilic inclusions were observed in the Trichoderma mycelium which interacted with the host cells.

The enzymes β -(1,3)-glucanase and chitinase were shown to be excreted by T. harzianum when grown on cell walls of the soilborne plant pathogens S. rolfsii and R. solani (Elad et al., 1982). Hyphal cell wall degradation was previously observed by SEM and TEM (Elad et al., 1983). However, S. rolfsii sclerotia have melanin in their rind, which apparently increases resistance of the rind to biodegradation. The data presented here (Table 29) show that T. harzianum No. 203 decomposed not only hyphal walls but also contents of sclerotia of S. rolfsii. These data show that significantly greater activity of hydrolytic enzymes was detected in the surrounding soil, and in the parasitized S. rolfsii sclerotia in the presence of the pathogenic Trichoderma isolate No. 203, compared with the non-pathogenic isolates (Table 29). This phenomenon may explain the variability in the antagonistic activity of T. harzianum isolates.

TABLE 29. Enzymatic activity of I. harzianum isolates in the soil and in S. rolfsii sclerotia^a

Enzymatic activity	<u>I. harzianum</u> isolate		
	-	203 ^b	250 ^c 110 ^c
β -(1,3)-glucanase activity in soil (mg glucose ml ⁻¹ x h ⁻¹) (GA)	0.05 ^{cd}	4.36a	2.96b 3.56ab
Chitinase activity in soil (mg GlcNAc ml ⁻¹ x h ⁻¹) (CA)	0.08c	5.88a	2.12b 1.53c
GA in sclerotial extract	1.28c	6.00a	4.80b 4.40b
CA in sclerotial extract	1.34c	6.80a	4.20b 4.40b

^a Extracts were made from soil and sclerotia. Enzymatic activity of β -(1,3)-glucanase and chitinase was tested by following the release of glucose and N-acetylglucosamine from laminarin and chitin, respectively.

^b I. harzianum 203 is capable of parasitizing S. rolfsii and R. solani.

^c I. harzianum 250 and 110 parasitize only R. solani.

^d Numbers of each line followed by the same letters are not significantly different ($P = 0.05$).

Fig. 27. Scanning electron micrograph (SEM) of non-parasitized *S. rolfsii* sclerotium. (Bar marker = 100 μ m).

Fig. 28. SEM micrograph of a sclerotium attacked by *I. harzianum* (Bar marker = 100 μ m).

Fig. 29. SEM micrograph of *T. harzianum* hyphae growing over a *S. rolfsii* sclerotium and penetrating in between its cells. (Bar marker = 10 μ m). Note the *T. harzianum* conidia over the sclerotium surface.

Fig. 30. SEM micrograph of a broken sclerotium showing many mycoparasitic hyphae (arrows) inside the sclerotium. (Bar marker = 10 μ m).

Fig. 31. SEM micrograph of *T. harzianum* conidia growing inside a broken sclerotium. (Bar marker = 1 μ m).

Fig. 32. TEM micrographs of a thin section of an attacked sclerotium showing extracellular and intercellular mycoparasitic hyphae of *T. harzianum* (TH). (Bar marker = 0.2 μ m).

The antagonistic *T. harzianum* has indeed already proved its potential as a biological control agent (Elad et al., 1982). This fact is explained here by the high lytic activity of *T. harzianum* associated with its ability to invade sclerotia. Sclerotia invasion and cell wall biodegradation was observed by both SEM and TEM (Figs. 27-32). Sclerotial cell wall degradation indicates the possible activity of extracellular lytic enzymes, which also cause the loss of cytoplasmic content in the attacked cells. Since *T. harzianum* excretes proteases and lipases (Elad et al., 1982) it may be assumed to utilize the host cell contents, which probably enables it to grow and sporulate intensively (Figs. 29 and 31). However, the emptiness of host cells could be resulted from disintegration of cell walls and leakage of the cytoplasm. Enzymatic decomposition of host cell walls has already been demonstrated with other mycoparasites such as *Gliocladium virens*, which attacks the mycelium and sclerotia of *Sclerotinia sclerotiorum* and *T. harzianum* attacking hyphae of *R. solani* and *S. rolfsii* (Elad et al., 1983). SEM and TEM techniques enabled us to demonstrate how the mycoparasite *T. harzianum* attacks not only mycelium but also resistant resting structures of *S. rolfsii*.

Integrated control of *Rhizoctonia solani* Kuhn by methyl bromide and *Trichoderma harzianum* rifai aggr. Methyl bromide (MB) is one of the conventional and effective means of controlling soil-borne plant pathogenic fungi. It is toxic to a large range of microorganisms in soil and has some beneficial side effects, such as an increase in growth and yield of crops planted in treated soil.

Former findings could support the idea of integrating soil fumigation and biological control for controlling *R. solani* and *P. aphanidermatum*. This integration would enable the farmer to reduce the amount of MB; to decrease the damage it may cause and to prolong its efficacy in soil.

T. harzianum and *R. solani* were grown on SM plates for 24 hr. Colonies were then exposed to MB vapor for 24 hr at rates of 0-30,000 ppm (v/v). Neither *Rhizoctonia* nor *Trichoderma* grew during exposure of 9,000 - 30,000 ppm MB. When colonies were aerated, *T. harzianum* taken from 9,000 - 20,000 ppm MB dessicators continued to grow, whereas *R. solani* did not grow even after exposure to 9,000 ppm (Fig. 33). Mycelial samples taken from colonies which did not continue growing after exposure to MB were inoculated on fresh SM plates and were incubated in 30 C for 7 days in order to test its viability. It was concluded that *R. solani* was killed at a rate of 9,000 ppm MB, whereas *T. harzianum* was found dead only after exposure to 30,000 ppm MB. Susceptibility of *Pythium aphanidermatum* to MB was found to be similar to that of *R. solani*.

Dual cultures of *T. harzianum* and *R. solani* were exposed to MB at a rate of 6,000 ppm for 24 hr after colonies meeting on SM plates. In non-exposed cultures, *T. harzianum* grew into *R. solani* and colonized all its mycelium within 27 hr. When dual culture was exposed to MB, *T. harzianum* did not colonize *R. solani* colony but after aeration of plates it grew over the pathogen's mycelium at a rate similar to that of non-exposed culture.

General fungal population in a wet sandy soil was 4.10^3 CFU/g soil, whereas *Trichoderma* spp. population in it was $2.4.10^2$ CFU/g soil. The soil was fumigated with 500 kg/ha MB and general fungal population in it decreased to $2.1.10^2$ CFU/g soil. However, *Trichoderma* was not affected by fumigation and its relative amount in the general population in soil increased from 6% before fumigation up to 86% 16 days post fumigation (Fig. 34). Wheat-bran/peat preparation of *T. harzianum* was mixed with soil (5

g/kg) two weeks before fumigation with 500 kg/MB/ha. *Trichoderma harzianum* population in non-fumigated soil was $5.2 \cdot 10^6$ CFU/g and $4.6 \cdot 10^6$ CFU/g soil after fumigation.

Fig. 33. Growth of *Trichoderma harzianum* (□) and *Rhizoctonia solani* (○) in petri dishes. Period of exposure to methyl bromide at the mentioned concentration for 24 h is indicated by the diagonal lines. Cultures were then aerated and the cultures which did not continue to grow were transferred into fresh medium and were not alive (*).

Fig. 34. The influence of methyl bromide on the general fungal and *Trichoderma* spp. populations in soil. General fungal population in natural ($\square$) and fumigated (III) soil and *Trichoderma* spp. population in the same soils (/// , XX). Numbers adjacent to each column indicate the percentage of *Trichoderma* spp. propagules in the soil compared with the general fungal population.

Soils which were fumigated with methyl bromide (at rates which correspond to 0, 100 and 500 kg/ha) were treated with *Trichoderma* preparation before and after MB fumigation. Table 1 shows that 500 kg MB/ha was more effective in reducing *R. solani* disease index than fumigation with the reduced rate. *T. harzianum* reduced disease incidence in fumigated soil when it was applied either before or after soil fumigation (Table 30).

TABLE 30. The influence of methyl-bromide and *T. harzianum* (5 g/kg soil) on *R. solani* disease index in beans under greenhouse conditions

Methyl bromide dose (Kg/ha)	Trichoderma application time		
	No.	2 weeks before fumigation	1 week after fumigation
0	1.5 A ^a	0.3 A	0.7 A
100	0.7 B	0 A	0.4 A
500	0 C	0 A	0 A

^a Numbers in each column followed by a common letter are not significantly different ($P = 0.05$).

TABLE 31. The influence of soil fumigation and *Trichoderma* application (1500 kg/ha) on the commercial yield of carrots (kg/m²)

Methyl bromide dose (kg/ha)	Trichoderma application	
	-	+
0	3.5 A ^a	3.9 A
200	4.3 B	4.6 B
500	4.5 B	4.5 B

^a Numbers in each column followed by a common letter are not significantly different ($P = 0.05$).

Sandy soil which showed *R. solani* index of 1.8 in beans, was fumigated with 500 kg MB/ha and divided into two parts. *Trichoderma* preparation was mixed with half of the soil (5 g/kg soil) and soils were planted with beans. No disease could be identified in either *Trichoderma* applied or non-applied soils.

Soil naturally infested with *R. solani* was mixed with both soils (1:20, v/v) and was replanted twice with beans. The results (Fig. 35) show that *Trichoderma* preparation reduced disease index in the fumigated soils by 80-83% in both planting cycles.

Fig. 35. Prevention of reinfestation by *T. harzianum* of fumigated soil with *R. solani*. *T. harzianum* preparation was either mixed (▨) or not mixed (□) in fumigated soil. Three growth cycles of beans were carried out in the soil and *R. solani* disease index was assessed after each three weeks growth in the beans hypocotyl. Columns of each pair followed by the same letter are not statistically different ($P = 0.05$).

Naturally *R. solani* infested soil was fumigated with 0, 200 or 500 kg MB/ha and *T. harzianum* preparation (1500 kg/ha) was applied in it a week later. Another week later, carrot seeds were planted in the field plots and during the first month of growth pre- and post-emergence disease incidence was recorded (Fig. 36). *T. harzianum* reduced preemergence disease incidence by 47% whereas fumigation with 200 and 500 kg MB/ha reduced preemergence disease incidence by 69% and 84%, respectively. Combination of *T. harzianum* with both rates of MB reduced preemergence by 97% and 100%, respectively (Fig. 36). *R. solani* postemergence damping-off was reduced by 500 kg MB/ha by 78%. However, postemergence disease reduction was 74% and 97% when *Trichoderma* was applied after soil fumigation at a rate of 200 and 500 kg MB/ha, respectively.

We have found that MB in soil fumigation caused mortality to the general fungal populations whereas it did not reduce *Trichoderma* populations and the fungus even multiplied after fumigation up to 86% of the soil fungal population.

Fig. 36. Integrated control of *R. solani* in carrot seedlings. Preemergence (□, |||) and postemergence (□, ×××) damping-off percentages were calculated in fumigated field plots which were treated with *T. harzianum* (×××) or untreated (□). Factorial statistical analysis was made ($P = 0.05$). The influence of *Trichoderma* treatments is indicated by capital letters whereas the influence of methyl bromide treatments is indicated by lower case.

Moreover, mixing preparation of Trichoderma in soil before fumigation did not result in fungal decline after treatment. The antagonist colonized fumigated soil even better than natural one, within the two weeks of testing. These may be due to "microbial vacuum" which exists after application of the eradicant or is due.

It was concluded that fumigation with the sub-lethal dose of the fumigant is sufficient for disease control when it is followed by application of Trichoderma. Although soil disinfestation with MB is an effective way to control severe fungal diseases including R. solani, nevertheless, reinfestation of the fumigated soil often occurs. The degree of reinfestation is proportional to the reduction in antagonistic microorganisms and the alteration of the original ecosystem which follows disinfestation. Eradicants such as MB cause 'microbial vacuum' which may soon be followed by a build-up in the population of pathogens. This effect was also noted in our work with R. solani in fumigated soil. T. harzianum prevented disease build-up in fumigated soil which was reinfested and replanted with beans.

DESCRIPTION OF COOPERATION

For three years cooperative research has been actively carried out between the group at Colorado State University in Fort Collins and the group at The Hebrew University of Jerusalem in Rehovot. This cooperation began in 1979-1980 while Prof. Chet visited at CSU and studied mechanisms involved in induction of soil suppressiveness, and that was the basis for the BARD project. During the collaborative project excellent working relationship have been established. Prof. Baker visited in Israel; he gave a lecture in a symposium organized by Prof. Chet dedicated to biological control and its abstracts appeared in the international journal "Phytoparasitica." During the visits of both Baker in Rehovot and Chet in Fort Collins they met with the students, gave seminars and taught new techniques.

Good isolates of the biocontrol agent Trichoderma were transferred between the two labs and were tested in both countries.

During 1983/84 Dr. Y. Elad from Chet's group did his post doctoral work Prof. Baker and had the opportunity to study and develop new biocontrol systems.

This collaboration was a stimulating opportunity to study and to exchange ideas. This resulted in many publications which were all published in international journals some of which were jointly authored. The research led to the development of preparations which are tested now for application for farmers.

BENEFIT TO AGRICULTURE

While numerous biocontrol systems have been discovered and attempts have been made to exploit and develop these in agricultural applications, success has rarely been achieved. Cook and Baker, in a recently published treatment of the subject, list no less than 40 biocontrol agents described in the literature. Thus, there are many natural enemies. The challenge is to increase their efficiency so that they can be applied in agricultural situations.

In our opinion research strategy should focus on enhancing the activity of biocontrol agents to the detriment of pathogens. In the research under BARD for the past 3 yr, this has been the basic approach. The strategies are briefly presented below:

- i. **Genetic improvement.** There is always the possibility that another biocontrol agent may be discovered with improved attributes. Such an agent could be P. nunn. The agent is capable of reducing the inoculum density of pathogenic species of Pythium by appropriate manipulations of the environment. Mutation of proven agents may also be exploited to provide tolerance to chemicals routinely used in agriculture and/or to improve efficiency of biocontrol. This has been accomplished with T. harzianum resulting in a benomyl-tolerant isolate identified as T-95.
- ii. **Providing appropriate substrate.** Substrates may be added to soil that favor saprophytic activity of biocontrol agents. This was realized for P. nunn by addition of bean meal. Cellulose substrate enhanced competitive saprophytic ability of T. harzianum. Formulation of the agents on peat-bran medium affords better biocontrol and/or increased growth response over individual propagules (conidia).
- iii. **Management to increase competitive advantage of biocontrol agents.** As reviewed above, by proper management of available Fe, it is possible to enhance the rhizosphere competence of fluorescent pseudomonads. An experiment involving Fusarium wilt of carnations was designed to exploit this phenomenon under simulated commercial conditions (Fig. 27). Enhanced control by use of P. putida and FeEDDHA was not relayed; however, this may have been due to FeEDDHA being leached from the raised beds. With FeEDDHA alone, control was achieved early but subsequently disease incidence was not significantly different from the control.
- iv. **Placement--protection of infection court.** If pathogens (like Pythium spp.) are in high enough inoculum density so that a considerable portion of the inoculum touches the infection court, biocontrol agents must be present in this area to be effective. Seed treatment with agents meets this requirement for pathogens inducing preemergence damping-off.
- v. **Increasing rhizosphere competence.** Trichoderma spp. have not proved rhizosphere competent according to reports from two other laboratories. T. harzianum (T95) proved rhizosphere competent in our tests and we confirmed that isolates from the other two laboratories were not. T-95 is tolerant to benomyl. If benomyl was mixed into the soil, the population density of T95 was substantially increased in the rhizospheres of corn, radish and cucumber plants in comparison with nontreated controls.

- vi. **Providing favorable environments for activity.** As reviewed above, soil treatments, soil pH adjustments and organic amendments have been used to increase efficiency of biocontrol agents. Temperature relationships are exceedingly critical and these can be optimized by use of biotic agents in covered environmentally-controlled structures found in the ornamental industry. Yet to be explored are adjustments in dates of planting.
- vii. **Integration with other controls.** Control of *Rhizoctonia* preemergence damping-off was additive by use of benadonil and seed treatment with *T. harzianum* (reviewed above).
- viii. **Increasing cost effectiveness--the increased growth response.** Through the use of biocontrol agents, diseases are suppressed; however, associated growth enhancement not only pays for the cost of application but increases profits.

Throughout these investigations the emphasis has been placed on identification of mechanisms involved in biological control. Only through such basic research can strategies for enhancement and more intelligent management of biocontrol strategies be achieved.

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