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Factors Affecting Biological Control of Pythium Ultimum on Alfalfa using Seed Treatment with Gliocladium Virens, Trichoderma Harzianum and T. Hamatum

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FACTORS AFFECTING BIOLOGICAL CONTROL OF PYTHIUM ULTIMUM ON ALFALFA USING SEED TREATMENT WITH GLIOCLADIUM VIRENS, TRICHODERMA HARZIANUM AND T. HAMATUM

by

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PYTHIUM ULTIMUM ON ALFALFA USING

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TRICHODERMA HARZIANUM AND T. HAMATUM

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Doctor of Philosophy, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ABSTRACT

Gliocladium virens Millar et al. applied as a seed treatment controlled alfalfa (*Medicago sativa* L. 'Travois') damping-off in natural soils artificially infested with high levels of *Pythium ultimum* Trow, but *Trichoderma hamatum* (Bon.) Bain and *Trichoderma harzianum* Rifai were not effective at relatively high soil pH levels. Biocontrol with *G. virens* was nearly as effective as metalaxyl seed treatment, and better than Captan in most environments. Biocontrol was optimum at soil temperatures between 15 and 30 C, soil moistures of 15% to 30%, soil pH of 6 to 8 and when seeds had been treated with conidial concentrations of 10^9 spores/ml or greater. In a parallel study in steamed soils infested with *P. ultimum*, biocontrol was most effective between 25 and 35 C. at relatively low soil moistures, and at pH levels of 6 to 8. Amendment of the biocontrol spore suspension with bleached chitin did not affect the ability of the antagonists to protect alfalfa against *P. ultimum*, while unbleached chitin consistently destroyed effective biocontrol. *G. virens* seed treatment also effectively controlled damping-off in field tests. In vitro, 30 C and lower pH levels were optimum conditions for growth and sporulation of all three biocontrols.

INTRODUCTION

This study deals with biological control of alfalfa (Medicago sativa L. Travois) seedling blight or damping-off caused by Pythium ultimum Trow. The disease is distributed worldwide and causes losses in stand which range from zero to 100%. In South Dakota, one third of soil samples collected from 149 fields developed between 50% and 91% preemergence damping-off when planted to alfalfa in the greenhouse (66). Stand failures are not unusual in South Dakota and the heavy planting rates needed to give the desired stand have resulted in increased production costs.

Biological control of plant diseases can be achieved by using other microorganisms that either are antagonistic to the pathogen or parasitize the pathogen. Antagonism can be effected by competition for nutrients, or by the production of toxic metabolites by the antagonist which inhibit growth or may even kill the target organism. Man's increasing disenchantment with pesticides due to their inherent environmental hazard, coupled with the potential of nonchemical or biological control to provide relatively inexpensive, safe, energy efficient and long lasting protection have led to increased interest in this field.

Recently several examples of successful biological control of soil borne diseases have been reported. These have given impetus to renewed research efforts in an area which has previously been one of considerable academic interest but of little practical significance. The credit for these successful biocontrol systems can be attributed,

at least in part, to the discovery of better biocontrol agents, to genetic improvement in their activity and to development of more effective systems of delivering biocontrol agents into the appropriate infection courts.

The research reported herein evaluated the effects of certain environmental factors, notably temperature, moisture and pH on biocontrol of damping-off of alfalfa by three antagonistic or mycoparasitic fungi, Gliocladium virens, Millar et al. Trichoderma hamatum (Bon.) Bain and Trichoderma harzianum Rifai. Experiments designed to field test the effectiveness of such control also are presented.

LITERATURE REVIEW

Pythium spp. are ecologically versatile and physiologically unique. They are ubiquitous in soils and aquatic environment, world-wide in distribution and have an extremely broad and diverse host range. They are among the most important and destructive plant pathogens inflecting serious economic losses on a wide variety of crops by destroying seeds, storage organs, root tissues and other plant parts (119).

The *Pythium spp.* associated with legumes occur throughout the world, especially in the finer textured acid soils (16, 29). Seed rotting, and pre- and postemergence blighting (damping-off) reduce stands in the small seeded legumes, especially alfalfa and sweet clover. The disease is primarily associated with seedlings, although rootlet rot and stem rot occur in established plants, especially in wet locations. Soft rot of the tissues and presence of sporangia and oospores in freshly rotted tissues are the most certain symptoms and signs. Several species of *Pythium* are associated with the disease on small seeded legumes. These species also occur on a wide range of cultivated and wild plants. Middleton (84) described this group of morphologically similar species with somewhat spherical nonproliferous sporangia. They differ primarily in the number and origin of antheridia attached to the oogonia. In *Pythium ultimum* Trow, terminal sporangia and oogonia are common, and usually one monoclinous antheridium is attached to each oogonium. The sporangia germinate by germ tubes attached to each oogonium. No zoospores are produced. The etiology of

diseases caused by various members this group is similar. Seed and seedling attack from soil-borne mycelium is associated with the maturity and general condition of the seed and with the soil environment in which the seeds germinate. Fully matured seed, a well prepared seed bed, balanced fertility, including liming of acid soils, and seed treatment under some conditions are the best control measures. (3)

Alfalfa seedling rot in South Dakota, as in other parts of the United States and the world, is a destructive alfalfa disease. In many fields its abundance prevents the establishment of adequate alfalfa stands, requiring the use of excessive amounts of seeds or chemical seed treatments to establish such stands. (66) The absence or near absence of seedling rot in other fields sown with the normal amount of seeds often results in stands that are too dense for best growth of plants under semi-arid conditions. Soil inhabiting fungi of the genus Pythium generally have been responsible for this disease; among these, P. debaryanum, P. irregulare, P. splendens and P. ultimum are most destructive. To account for the difference in incidence of seedling rot, several possible explanations may apply, namely: (a) the soil and weather factors were sufficiently different in the various soils to affect disease development; (b) that antagonistic microorganisms were more suppressive in one soil than another; (c) that the quantity of Pythium inoculum was more abundant in one soil than another.

Platings on agar media of diseased seedlings from a number of different soils in South Dakota yielded P. ultimum as the predominant

pythiacious species inducing the disease. (66)

Biological control of plant pathogens accomplished through host plant resistance and cultural practices has been working for decades and continues to be a predominant disease control strategy. In contrast, biological control accomplished through introduction or encouragement of microorganisms antagonistic to plant pathogens has been slow to develop (10).

Extensive efforts were made in the 1920s and 1930s to introduce alien, antibiotic-producing microorganisms into soil for root disease control. This was first attempted by Hartley in 1921 against damping-off of coniferous seedlings, Millard and Tayler in 1927 against common scab of potato, and Sanford and Broadfoot in 1931 against take-all of wheat (12). These unsuccessful attempts led to the realization that little was known about the ecology of soil microorganisms and that such knowledge was fundamental to the manipulation of populations of soil organisms. Unfortunately, the failures in these earlier years also fostered negative attitudes among plant pathologists toward biological control.

A landmark for research on biological control of plant pathogens occurred in 1963 when, at Berkeley, California, an international symposium was held on "Ecology of soil borne plant pathogens -- prelude to biological control" (12). At least 12 international symposia have been held on this topic since 1963, and the first book devoted wholly to the subject of biological control was published in 1974 (10). The first Standing Committee on Biological Control was established within the

American Phytopathological Society in 1976.

Numerous reviews have covered the status of biological control (25, 26, 34, 35, 36, 37, 76, 77, 94, 96, 113, 114, 120, 129) and its principles and mechanisms (11, 88, 128). Many ecological aspects of propagule survival in soil also have been reviewed (15, 24, 82, 92). It is now widely recognized that biological control of plant pathogens is a distinct possibility for the future (10, 25, 26, 34, 35, 36, 95, 96) and can be successfully exploited in modern agriculture especially within the framework of integrated pest management systems (80, 96, 97).

Some successful examples of biological control via hyperparasitism or antagonistic action between microorganisms are briefly presented in the following paragraphs.

Sporidesmium sclerotivorum is an unusual, dematiaceous hyphomcete first found on sclerotia of Sclerotinia minor in soil at Beltsville, MD (123). S. minor causes a disease on lettuce called 'Sclerotinia lettuce drop'. The mycoparasite, S. sclerotivorum was shown to parasitize sclerotia of S. minor in natural soil as well as in vitro (8). In moist sand, steamed soil, and in natural soil, the mycoparasite infected and destroyed more than 95% of Sclerotia of S. minor within 10 weeks or less. Sclerotia of Sclerotium cepivorum, the cause of white rot of onion, and Sclerotinia sclerotiorum also were parasitized by S. sclerotivorum (7, 8). This fungus has been patented and is in the early stages of commercial development.

The only example of a commercial application of an organism to

control a pathogen in a freshly wounded plant surface is the use of Peniophora gigantea on pine stumps to control root rot caused by Fomes annosus [=Heterobasidion annosum (Fr.) Bref.] (104). Both the pathogen and the antagonist colonize live stumps of felled trees early (83), probably because of their broad distribution in nature. Rishbeth (103) found that massive doses (10,000 spores) of P. gigantea per 16-cm diameter stump surface prevented infection from airborne spores and checked growth of F. annosus in host tissue at or after the time of felling. This method is effective mainly in intensively managed pine plantations, where thinning and clear cutting are practiced and little if any root infection is present. In heavily infected stands, P. gigantea is better than fungicides because it hastens stump decay. In 1962, the Forestry Commission introduced P. gigantea as a stump inoculant in Britain, and by 1973 the antagonist had replaced chemicals in the thinning and clear cutting operations in about 30 forests (62,000 ha) (126). In 1966, Ecological Laboratories Ltd., in Dover, Kent, began the manufacture of the inoculant, which is currently used only in Britain. The antagonist, manufactured commercially, is packed in sachets of oidia suspended in a sucrose solution. The contents of the sachet are added to water, and this suspension is brushed onto stump surfaces of freshly felled pines. Artman (5) showed that oidia can be mixed with chain-saw oil, and stumps can be inoculated during cutting. In Britain, Greig (39) reported that this technique gave results comparable to those from conventional methods. New strains of P. gigantea are added from time to time to ensure high antagonistic

ability, consistent production of oidia, and activity in wood decay. Sequential sampling systems have been developed to maintain quality control (99).

The first commercial use of an organism to control a plant disease in soil is Agrobacterium radiobacter to control crown gall in rosaceous hosts. The initial discovery was made by Kerr (61), who isolated strain 84 that was antagonistic to A. tumefaciens (Smith & Town.) Con, when applied to peach seeds. Kerr (61) and New and Kerr (91) inoculated peach seeds with the nonpathogenic strain 84 and planted them in soil infested with A. tumefaciens, the strain that causes crown gall. After three months, 31% of plants from seeds inoculated with strain 84 had galls, compared to 97% in plants from nontreated seeds. Strain 84 produces an antibiotic called "agrocin 84," which is one of a group of highly specific antibiotics known as nucleotide bacteriocins. Only nonpathogenic agrobacteria that produce agrocin 84 control crown gall; nonproducers are ineffective (63). Since 1973, the Waite Agricultural Research Institute has supplied commercial growers with cultures of strain 84, but now the organism is marketed commercially in New South Wales, New Zealand, California, and Oregon. Seeds, cuttings, or roots of young plants are dipped into a bacterial suspension; in order for the nonpathogenic strain to be effective, the ratio of pathogen to nonpathogen at the root surface cannot exceed one. The treatment of seeds or cuttings with strain 84 offers continuing protection for up to two years, which is the period of greatest need for protection of seedlings. Seed treatment with

strain 84 is inexpensive, simple, and effective. It is harmless to humans and other mammals; inoculum can be stored in peat and retain a half-life of six months at room temperature, and it costs less than a cent per treatment to treat barerooted Prunus (63). This strain has been used in many countries of the world on thousands of plants of Prunus, Rubus, Malus, Salix, Vitis, Libocedrus, Chrysanthemum, Crataegus, Carya, Rosa, Pyrus, and Humulus (61). Although strain 84 controls crown gall in several countries (63), in Greece it was ineffective against three strains of crown gall isolated from peach (62). This has not been a problem in Australia, where there is only a single strain of the crown gall pathogen.

The biological control agents used in the present study also have been successfully used in other studies. Wells, Bell, and Jaworski in 1972 (127) obtained impressive control of Sclerotium rolfsii Sacc. diseases of lupines, tomatoes and peanuts by the use of Trichoderma harzianum Rifai in greenhouse tests and on tomatoes in the field. Although they showed that T. harzianum parasitized S. rolfsii in vitro, they did not investigate the mechanism of plant disease control in soil.

Backman and Rodriguez-Kabana in 1975 (9) developed a system of culture and delivery of T. harzianum for biological control, utilizing diatomaceous earth granules impregnated with molasses as a growth medium for the fungus. This inoculum applied to peanut fields 70 to 100 days after planting reduced southern blight (S. rolfsii) of peanuts 42% and increased crop yields. Wheat bran preparations of T. harzianum

applied in the field gave 38% reduction of peanut disease caused by S. rolfsii (42).

Trichoderma harzianum reduced seedling diseases caused by Rhizoctonia solani Kuhn (44), cucumber fruit rot caused by R. solani (72), and damping-off of tobacco caused by Pythium aphanidermatum (Edson) Fitzp. (32).

Mechanisms of biological control by T. harzianum were studied in soils suppressive to R. solani in 1980 (51) and T. harzianum was isolated from mycelial mats of R. solani incubated in suppressive soils in which radish and cucumber were grown.

An antagonistic strain of T. harzianum capable of controlling both S. rolfsii and R. solani was isolated from pathogen infested soil 1980 (30) and its behavior was studied under laboratory, greenhouse and field conditions.

In 1979 (110), effect of T. harzianum on wood invading fungi was studied and in 1981 (111) it was postulated that a mechanism of biological control of decay in red maple trees by T. harzianum was the replacement of pioneer colonizing fungi by T. harzianum.

Wheat bran cultures of T. harzianum were tested for control of R. solani in carnation fields treated with methyl bromide in 1981 (31) and a linear relation was obtained between the amount of T. harzianum preparation applied to soil and the degree of disease control.

Efficacy of T. harzianum seed treatment against damping-off of snap beans was studied in 1982 by Marshall (81) in two acidic soils and the results suggested that T. harzianum seed treatment of snap bean reduced incidence of R. solani damping-off in acidic soils.

Recently Hadar et al. in 1984 (45) isolated, characterized and tested T. harzianum against Pythium seed rot of peas. The isolate used was found to be effective.

On a different aspect of biocontrol, Papavizas et al. in 1982 (98) produced several mutant biotypes of T. harzianum that tolerated high concentrations (100-500 ug/ml) of the fungicide benomyl. The biotypes, induced by ultraviolet light, also differed considerably from the wild strain in appearance, growth habit, survival ability in the soil, fungitoxic metabolite production and ability to suppress damping-off of peas induced by Pythium ultimum, and damping-off of cotton and radish induced by Rhizoctonia solani.

Unlike T. harzianum, which was studied extensively in the period 1972-1983, the characterization of T. hamatum as a biocontrol agent began in 1980. The effectiveness of T. hamatum when applied as a seed treatment against Pythium spp. or Rhizoctonia solani which induced damping-off of peas and radishes, respectively, was studied by Harman et al. (48). Their results demonstrated that seed treatments with T. hamatum were nearly as effective as chemical seed treatment. Moreover this agent became established in soil and protected subsequent generations of seedlings from attack. In another study, Harman et al. (49) studied the effects of temperature, conidial concentration and amendments on the efficacy of seed treatment with T. hamatum against R. solani on radish and Pythium spp. on pea. Chet and Baker in 1981 (21) studied a Colombian soil that appeared to be suppressive to R. solani, and were able to demonstrate that T.

hamatum was the microorganism responsible for this phenomenon. Recently it was also demonstrated that seed colonizing pseudomonads were largely responsible for the failure of T. hamatum as a seed protectant in soils in New York (56, 86).

Aluko in 1968 (4) showed that application of the antagonist G. virens to seed potatoes in storage offered bright prospects for biological control of Corticium (Rhizoctonia) solani. In 1970, he showed that direct parasitism was unimportant compared with antibiosis in the antagonistic activities of G. virens against the pathogen on potato tubers. Culture filtrates of the antagonist contained both gliotoxin and viridin and were active against hyphae of the pathogen. The filtrates killed sclerotia even at low concentrations (4).

In vitro, J.C. Tu (121) demonstrated that G. virens was a destructive mycoparasite of sclerotia of Sclerotinia sclerotiorum, and in 1981 (122), he reported the hyperparasitism of G. virens on Rhizoctonia solani.

An isolate of G. virens found parasitizing a cotton strain of R. solani was used to protect cotton seedlings from damping-off incited by R. solani Kuhn and Pythium ultimum Trow. by Howell in 1982 (53). He demonstrated that when the antagonist was placed in the furrow with cottonseed, G. virens more than doubled the number of surviving cotton seedlings grown in soil infested with either pathogen. In vitro, he showed that G. virens parasitized R. solani by coiling around and penetrating the hyphae. P. ultimum was not parasitized by G. virens but was strongly inhibited by antibiosis.

Howell et al. in 1983 (55) isolated a new antibiotic, gliovirin, from G. virens and studied its role in biological control of P. ultimum. He concluded that G. virens did not parasitize P. ultimum, but appeared to kill it through the production of an antibiotic. The effects of gliovirin on P. ultimum and a number of different fungi and bacteria indicated that its activity might be limited to the members of the fungus class Oomycetes. He did not find evidence that G. virens produces gliovirin in the soil. The antibiotic was never isolated from the soil where the fungus was present. However two arguments indicated that the antibiotic was important to the antagonist-pathogen interaction in the soil. First, mutations which resulted in loss of gliovirin production also resulted in loss of its effectiveness as an antagonist. Second, mutations which enhanced gliovirin production allowed the mutant to maintain its antagonistic activity in spite of reductions in growth and parasitism.

In addition to the previous work, other biocontrol agents have been used to control Pythium spp. These are briefly listed in Table 1.

Table 1. Additional organisms used to control *P. ultimum*.

Antagonist	Pathogen	Crop	Location	Citation
		Fungi		
Chaetomium globosum	*Pythium spp.*	corn	field	Chang and Kommedahl 1968 (19)
	P. aphanidermatum *P. ultimum*	squash	laboratory	Harman Eckenrode & Webb, 1978 (47)
Penicillium frequentans	*P. ultimum*	table beet	greenhouse	Liu and Vaughan, 1965 (74)
Trichoderma viride	*P. ultimum*	table beet	greenhouse	Liu and Vaughan, 1965 (74)
	Pythium spp.	white mustard	greenhouse	Wright, 1956 (130)
Corticium (sensu lato)	*P. ultimum*	table beet	greenhouse	Hoch and Abawi, 1979 (52)
		Bacteria		
Arthrobacter spp.	*P. debaryanum*	tomato	greenhouse	Mitchell and Hurwitz, 1965 (90)
Pseudomonas fluorescens Mig. strain Pf-5	*P. ultimum*	cotton seedlings	greenhouse	Howell and Stipanovic, 1980 (54)
Pythium oligandrum	*P. ultimum*	cress seed	greenhouse	AL-Hamdani, A.M., R. S. Lutchmeah and R. C. Cooke, 1983 (2)

MATERIALS AND METHODS

MICROORGANISMS

Isolate 67-1 of Pythium ultimum isolated and identified by J. G. Hancock, University of California, was the plant pathogen used in this study. Gliocladium virens, strain G.V-P was provided by C. R. Howell, College Station TX. Trichoderma hamatum was provided by R. Baker, Colorado State University. Trichoderma harzianum was supplied by Carole E. Windels, University of Minnesota.

Isolates of all microorganisms were maintained on slants of potato dextrose agar (PDA). The PDA contained the following ingredients: infusion from 200 g sliced potatoes, 20 g dextrose, 12 g agar and water to make 1 liter. Periodic transfer was done monthly and stock cultures were kept under constant refrigeration at 4 C.

All biocontrols grew and sporulated readily on PDA when incubated at 25-30 C for 1 week. To obtain spore suspensions, the spores were gently rubbed from the flooded surface of a culture using a sterilized rubber 'policeman' and were transferred to a small sterile beaker. Then they would be shaken with a little sterile distilled water and strained through a new clean double cheesecloth into another sterilized beaker. The latter was then covered with parafilm and refrigerated if not used immediately.

When alfalfa seeds were to be treated, 0.5 ml or 1 ml of a spore suspension of each biocontrol agent was diluted with distilled water and counted under the microscope using a haemacytometer. Accordingly, the original spore suspensions were diluted to the required

concentration using sterile distilled water. Extremely high concentration levels were obtained by centrifugation for 30 minutes at 13,000 rpm, after which the pellet was resuspended in a smaller amount of sterile distilled water. A final spore count was made on the final dilution prior to application to the seed.

Two or three week old inoculum of P. ultimum was used in experiments that required infested soil. This was prepared by moistening 500 cc vermiculite with 250 ml nutrient solution (potato dextrose broth, PDB) in one liter Roux bottles. After autoclaving, a small block of agar containing the pathogen, P. ultimum, was introduced into the medium; bottles then were laid flat to allow good aeration. P. ultimum propagule counts were made with left-over inoculum. The actual number of propagules was determined by the quantitative method for isolation of P. ultimum from the soil by Stangellini and Hancock (117). Briefly, this method consisted of preparing an appropriate suspension of the soil sample in 0.2% water agar. Then 40 drops of the suspension were spotted in petri plates containing solidified 2% water agar, and the number of drops supporting growth of P. ultimum was determined microscopically after 24 hours at 24 C.

SOIL

The soil used routinely was a Vienna Loam obtained near Brookings, SD, mixed 3:1 with screened sand (v/v). When steamed soil was required, soil was placed in canvas bags and treated with free flowing steam for 4 hours. When cool, soil was moistened with a spray of soil extract solution, which was prepared by steeping 15 cc soil in

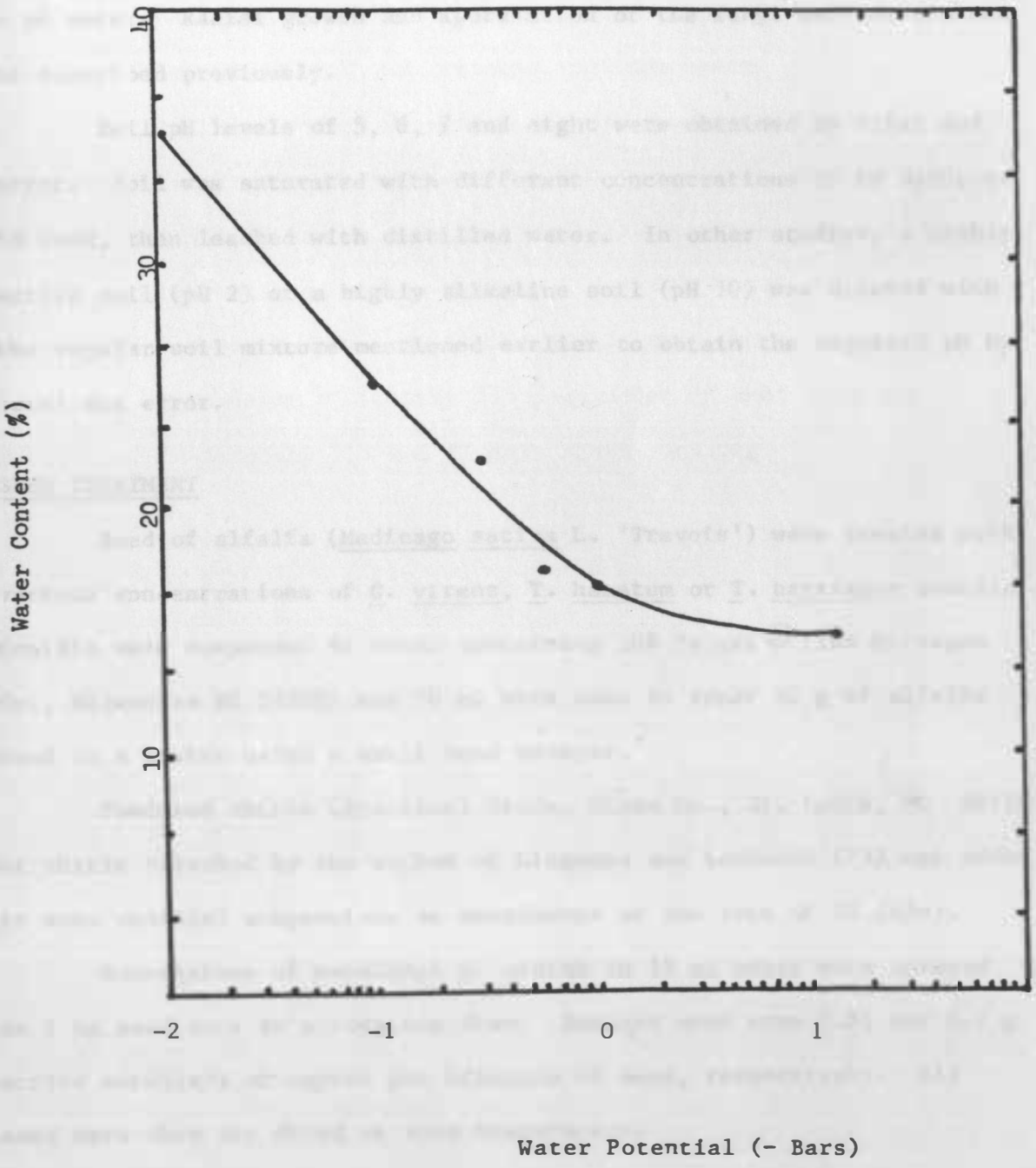
1 liter H_2O for 3 days. The liquid was decanted and filtered through filter paper, and the filtrate was added to steamed soil to reestablish a competitive microflora. A soil drying curve was determined for this soil mixture using the pressure plate method (Fig. 1). All pot experiments were conducted using this soil. Its characteristics as determined by the SDSU Soil Testing Laboratory were: pH 7.2, conductivity (soluble salts), 2.5mmho/cm; 213 ppm nitrate N; 5.8 ppm P; 29 ppm K; 273 ppm Ca; 70 ppm Mg. Steamed soil had the following characteristics: pH 7.5; conductivity (soluble salts) 2.7 mmho/cm; 208 ppm nitrate N; 7.8 ppm P; 34 ppm K; 351 pm Ca; 76 ppm Mg.

ENVIRONMENT

Effect of temperature on radial growth and sporulation of the biocontrols and pathogen was determined in vitro by incubating G. virens, T. hamatum, T. harzianum and P. ultimum isolates on PDA in 9 cm diameter petri dishes, at temperatures of 15, 20, 25, 30 and 35 C in the dark using 4 replicates. Colony diameter was measured daily for 6 days. After six days of growth, sporulation also was determined using a haemocytometer. This was done by flooding the plates with sterile water and as many spores as possible were removed with a single flooding, using a rubber policeman. No spore counts were made for P. ultimum, but presence or absence of sporulation noted. In pot experiments, the effect of temperature on seedling blight and its control were studied in similar incubators. Groups of pots were sealed in polyethylene bags until an experiment was terminated.

In vitro, pH of the PDA medium was varied by adding 0.1 N HCl or

Fig. 1. Water characteristic curve for Vienna Loam Soil.


40
30
20
10
Water Content (%)
-2
-1
0
1
Water Potential (- Bars)

0.1 N NaoH. After the medium was autoclaved, final pH was verified on a pH meter. Radial growth and sporulation of the fungi were determined as described previously.

Soil pH levels of 5, 6, 7 and eight were obtained by trial and error. Soil was saturated with different concentrations of 1N H_2SO_4 or 1N NaOH, then leached with distilled water. In other studies, a highly acidic soil (pH 2) or a highly allkaline soil (pH 10) was diluted with the regular soil mixture mentioned earlier to obtain the required pH by trial and error.

SEED TREATMENT

Seed of alfalfa (Medicago sativa L. 'Travois') were treated with various concentrations of G. virens, T. hamatum or T. harzianum conidia. Conidia were suspended in water containing 10% Pelgel ® (The Nitragen Co., Milwaukee WI 53209) and 10 ml were used to treat 60 g of alfalfa seed in a beaker using a small hand sprayer.

Powdered chitin (Practical Grade, Sigma Co., St. Louis, MO 68178) or chitin bleached by the method of Lingappa and Lockwood (73) was added to some conidial suspensions as amendments at the rate of 3% (W/v).

Suspensions of metalaxyl or captan in 15 ml water were sprayed on 1 kg seed lots in a rotating drum. Dosages used were 0.31 and 0.7 g active metalaxyl or captan per kilogram of seed, respectively. All seed were then air dried at room temperature.

POT CULTURE

An experimental unit consisted of a 200 ml styrofoam cup con-

taining 50 seeds of alfalfa planted at depth of 1 cm. Factorial experiments of four replications were arranged in randomized complete block designs. Each experiment was repeated at least twice.

For all experiments plastic bags were used to maintain constant moisture levels. Temperature levels of 30 C, moisture content of 20% and regular soil pH of 7.4 were used for all experiments except when studying different levels of one factor (variable). In such cases, the factor under investigation was varied, while the others remained constant. The number of healthy and the number of dead or dying plants were determined 3-5 and 12 days after planting.

RESULTS

TEMPERATURE

Growth and Sporulation of the Fungi

The maximum growth of *P. ultimum* on Potato Dextrose Agar (PDA) occurred at 25 to 30 C (Table 2, Fig. 2). Growth rate of *P. ultimum* increased more rapidly between 15 and 25 C than did the biocontrols. At all temperatures, *P. ultimum* grew most rapidly, followed by the *Trichoderma* spp., and finally *Gliocladium virens*. Growth and sporulation of the biocontrols peaked at 30 C, and *G. virens* generally produced fewer spores and had a lower growth rate than either of the *Trichoderma* spp. (Table 3, Fig. 3).

Effect of temperature on seedling blight and its control

At temperatures between 15 and 25 C, untreated alfalfa failed to emerge in steamed soil artificially infested with *P. ultimum*, but emerged well at 35 C (Table 4, Fig. 4-A). Seed treatment with either species of *Trichoderma* did not improve emergence. Between 15 and 25 C, Captan and *Gliocladium virens* resulted in equal but moderately effective control; but above 25 C, *G. virens* performed better than Captan and equal to metalaxyl. The latter provided excellent emergence throughout the temperature range tested. No post-emergence damping-off was observed on plants grown from metalaxyl treated seed at any temperature tested. Little post emergence damping-off was recorded for *G. virens* and Captan treatments, and was negligible at higher temperature of 30 C or above. No plants emerged from seed treated with *Trichoderma*

Table 2. Effect of temperature on rate of growth of fungi on PDA at pH 6.1.

Fungus	Temperature °C					
	15	20	25	30	35	Mean
	growth - mm/24 hours[a]					
1. *Pythium ultimum*	15.1	30.5	38.5	38.5	7.3	27.1
2. *G. virens*	4.5	8.1	11.3	16.1	5.3	9.0
3. *T. hamatum*	6.6	12.5	16.5	22.5	9.1	13.4
4. *T. harzianum*	6.9	12.7	16.3	22.3	8.9	13.4
Mean	8.3	15.9	21.4	25.6	7.6	

[a]$FLSD_{.05}$: Treatment = .08, temperature = .09, interaction = .18.

Figure 2. Effect of temperature on rate of growth of fungi on PDA.

★——— *P. ultimum*, ■——— *G. virens*, ▲——— *T. hamatum*,

□——— *T. harzianum*.

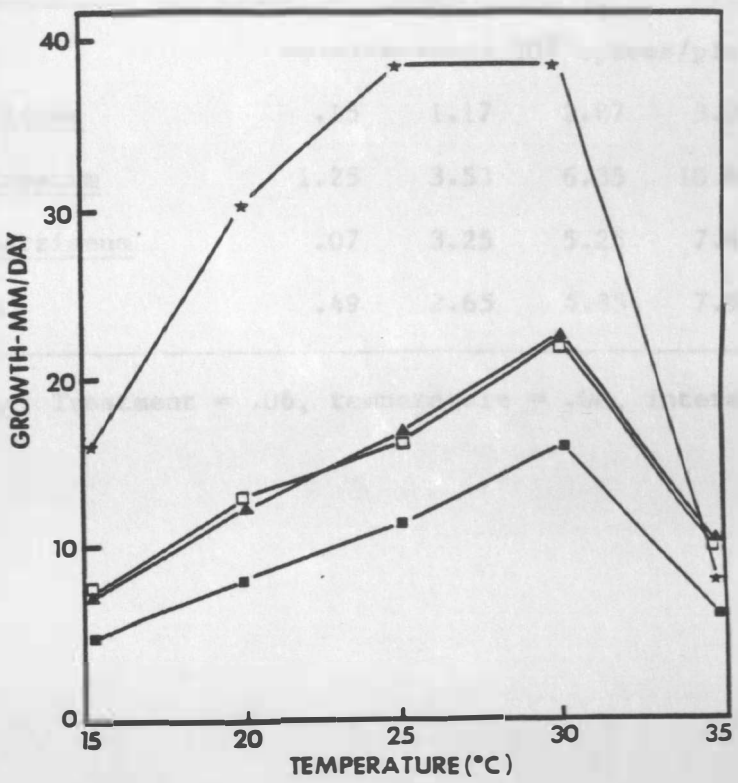

40
30
20
10
0
GROWTH-MM/DAY
15
20
25
30
35
TEMPERATURE (°C)

Table 3. Effect of temperature on sporulation of fungi on PDA at pH 6.1.

Fungus	Temperature °C					
	15	20	25	30	35	Mean
	10^9 spores/plate[a]					
1. G. virens	.15	1.17	2.87	3.06	1.51	1.75
2. T. hamatum	1.25	3.53	6.35	10.44	5.25	5.36
3. T. harzianum	.07	3.25	5.25	7.41	4.24	4.05
Mean	.49	2.65	4.83	7.97	3.67	

[a]$FLSD_{.05}$: Treatment = .06, temperature = .08, interaction = .13.

Figure 3. Effect of temperature on sporulation of fungi on PDA.

■——— G. virens, ▲——— T. hamatum,

□——— T. harzianum.

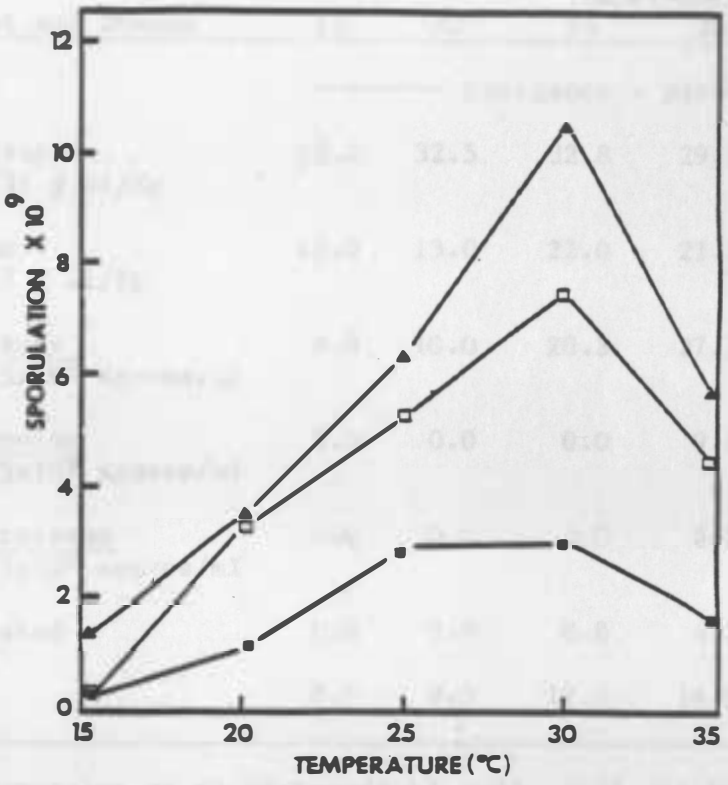

SPORULATION X 10⁹
0
2
4
6
8
10
12
15
20
25
30
35
TEMPERATURE (°C)

Table 4. Effect of temperature on emergence of Travois alfalfa 3-5 days after planting in steamed soil artificially infested with P. ultimum.[a]

Treatment and Dosage	Temperature °C 15	20	25	30	35	Mean
	-------- Emergence - plants/pot[bc] ----------					
1. Metalaxyl 0.31 g ai/Kg	30.0	32.5	32.8	29.3	26.3	30.2
2. Captan 0.7 g ai/Kg	12.3	13.0	22.0	21.3	18.0	16.9
3. G. virens $1.5x10^9$ spores/ml	8.8	10.0	20.3	27.3	30.5	19.4
4. T. hamatum $1.5x10^9$ spores/ml	0.0	0.0	0.0	9.8	25.8	7.1
5. T. harzianum $1.5x10^9$ spores/ml	0.0	0.0	0.0	6.3	23.8	6.0
6. Untreated	0.0	0.0	0.0	4.5	18.8	4.9
Mean	8.5	9.3	12.5	16.0	24.0	

[a]5000 propagules of P. ultimum/g of soil, soil moisture 20%, pH 7.4.

[b]$FLSD_{.05}$: Treatment = 2.22, temperature = 2.03, interaction = 5.00.

[c]Perfect stand = 33 plants/pot.

Table 5. Effect of temperature on stand establishment of Travois alfalfa 12 days after planting in steamed soil artificially infested with *P. ultimum*.[a]

Treatment and Dosage	Temperature °C 15	20	25	30	35	Mean
	Stand - plants/pot[bc]					
1. Metalaxyl 0.31 g ai/Kg	30.0	32.5	32.8	29.3	26.3	30.2
2. Captan 0.7 g ai/Kg	10.3	10.5	20.8	18.8	17.3	15.5
3. *G. virens* 1.5×10^9 spores/ml	7.3	8.3	19.8	26.3	30.0	18.4
4. *T. hamatum* 1.5×10^9 spores/ml	0.0	0.0	0.0	7.8	24.8	6.5
5. *T. harzianum* 1.5×10^9 spores/ml	0.0	0.0	0.0	5.0	22.8	5.6
6. Untreated	0.0	0.0	0.0	1.8	17.3	4.0
Mean	7.9	8.5	12.2	14.8	23.2	

[a]5000 propagules of *P. ultimum*/g of soil, soil moisture 20%, pH 7.4.

[b]$FLSD_{.05}$: Treatment = 2.04, temperature = 1.87, interaction = 4.57.

[c]Perfect stand = 33 plants/pot.

spp. or from untreated seed planted in soil at 25 C or below, yet surviving plants at 30 C and 35 C suffered little post-emergence damping-off (Table 5).

A similar experiment was conducted in natural (unsteamed) soil that had been artificially infested with Pythium ultimum. Throughout the range of temperature tested, emergence from untreated alfalfa seed was very poor, and seed treatment with Trichoderma spp. did not consistently improve it (Table 6, Fig. 4-B). However T. hamatum treatment slightly improved emergence at 25 C. At these temperatures metalaxyl treatment resulted in excellent emergence, and Gliocladium virens was only slightly less effective. The performance of G. virens and Captan were about equal up to 25 C, but Captan was inferior at 30 C. All three controls became less effective at 35 C. Throughout the temperature range tested, no postemergence damping-off was observed on plants treated with metalaxyl, and was neglibible for G. virens and Captan treatments. The few plants that emerged from Trichoderma treated or from untreated seed suffered heavy postemergence losses (Table 7).

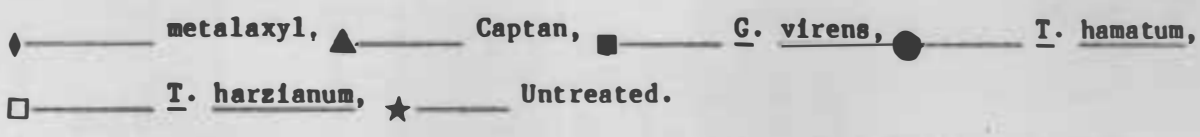
PH

Growth and Sporulation

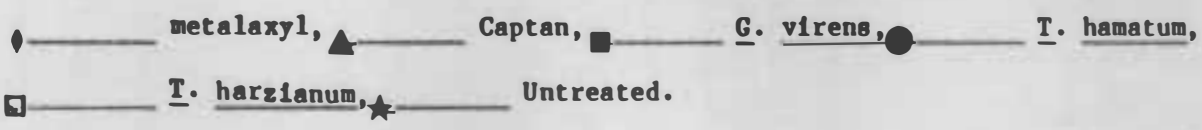
P. ultimum grew well on PDA over a range of pH levels from 4-9 with optimum pH levels of 5 to 7. Growth rate increased rapidly from pH 4 to pH 5 and reached a maximum at pH 6. Trichoderma hamatum, Trichoderma harzianum and Gliocladium virens grew most rapidly at pH levels between 4 and 5. Above pH 5 growth rate decreased as the pH was

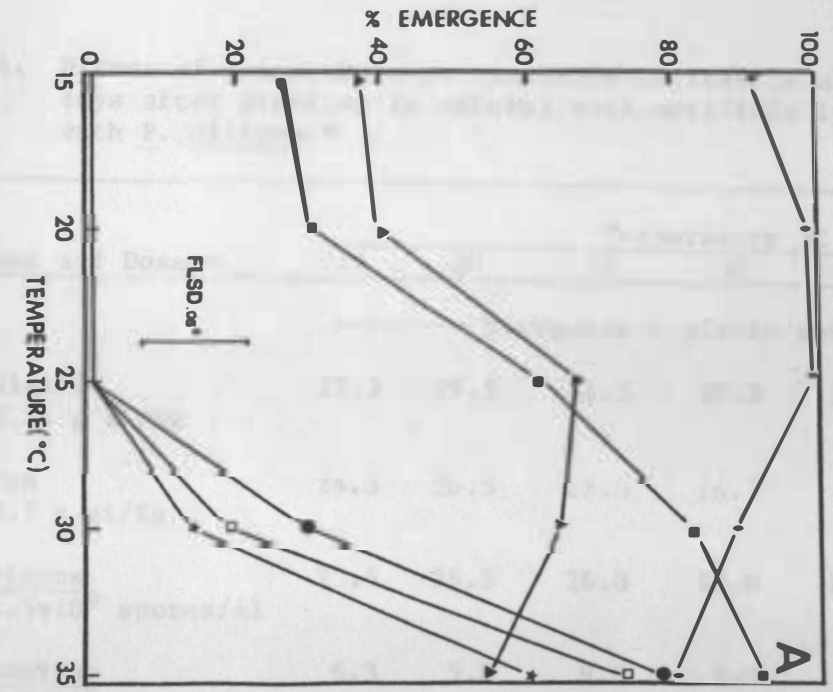
Figure 4. Effect of soil temperature on emergence of Travois alfalfa 3-5 days after planting.

(A) In steamed soil artificially infested with P. ultimum. Seed treated with:



(B) In natural soil artificially infested with P. ultimum. Seed treated with:




% EMERGENCE
0
20
40
60
80
100
15
20
25
30
35
TEMPERATURE(°C)
FLSD .05
A

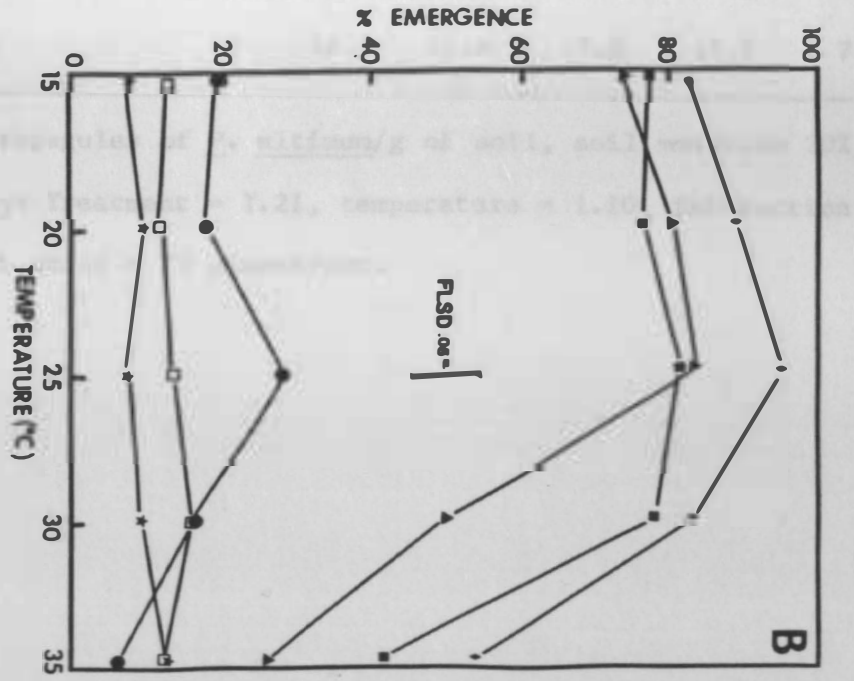

% EMERGENCE
0
20
40
60
80
100
15
20
25
30
35
TEMPERATURE (°C)
FLSD .05
B

Table 6. Effect of temperature on emergence of Travois alfalfa 3-5 days after planting in natural soil artificially infested with *P. ultimum*.[a]

Treatment and Dosage	Temperature °C 15	20	25	30	35	Mean
	-------- Emergence - plants/pot[bc] ----------					
1. Metalaxyl 0.31 g ai/Kg	27.3	29.5	31.5	27.3	17.0	26.5
2. Captan 0.7 g ai/Kg	24.3	26.5	27.5	16.3	7.8	20.5
3. *G. virens* 1.5×10^9 spores/ml	25.5	25.3	26.8	25.0	12.5	23.0
4. *T. hamatum* 1.5×10^9 spores/ml	6.3	5.8	9.3	5.0	1.5	5.6
5. *T. harzianum* 1.5×10^9 spores/ml	4.0	3.8	4.5	2.8	2.3	3.5
6. Untreated	2.3	3.0	2.3	2.5	2.8	2.6
Mean	14.9	15.6	17.0	13.1	7.3	

[a]5000 propagules of *P. ultimum*/g of soil, soil moisture 20%, pH 7.4.

[b]$FLSD_{.05}$: Treatment = 1.21, temperature = 1.10, interaction = 2.70.

[c]Perfect stand = 33 plants/pot.

Table 7. Effect of temperature on stand establishment of Travois alfalfa 12 days after planting in natural soil artificially infested with P. ultimum.[a]

Treatment and Dosage	Temperature °C 15	20	25	30	35	Mean
	Stand - plants/pot[bc]					
1. Metalaxyl 0.31 g ai/Kg	27.3	29.5	31.5	27.3	16.3	16.4
2. Captan 0.7 g ai/Kg	23.0	24.5	25.8	15.3	7.5	19.3
3. G. virens $1.5x10^9$ spores/ml	25.0	25.0	25.8	24.3	12.5	22.5
4. T. hamatum $1.5x10^9$ spores/ml	2.8	2.0	3.8	2.0	0.3	2.2
5. T. harzianum $1.5x10^9$ spores/ml	1.8	1.3	1.5	1.3	0.8	1.3
6. Untreated	0.3	0.3	0.5	0.3	0.5	0.4
Mean	13.3	13.8	14.8	11.8	6.3	

[a]5000 propagules of P. ultimum/g of soil, soil moisture 20%, pH 7.4.

[b]$FLSD_{.05}$: Treatment = 1.24, temperature = 1.13, interaction = 2.77.

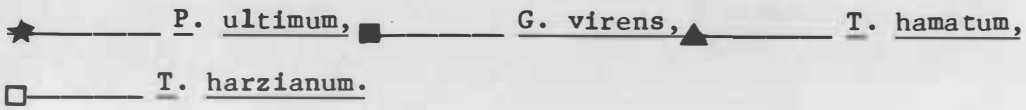
[c]Perfect stand = 33 plants/pot.

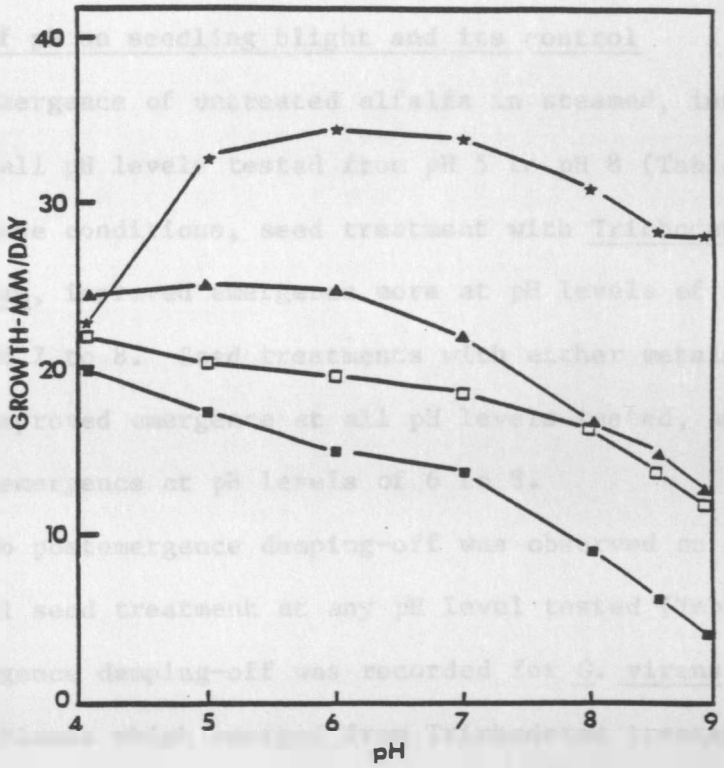
Table 8. Effect of pH on rate of growth of fungi on PDA at pH 6.1.

Fungus	pH 4	5	6	7	8	8.5	9	Mean
	growth - mm/24 hours[a]							
1. G. virens	19.9	17.5	15.3	13.6	9.0	6.0	3.0	12.1
2. T. hamatum	24.4	25.3	24.9	21.9	16.8	15.1	11.6	20.0
3. T. harzianum	21.9	20.3	19.5	18.5	16.3	13.8	11.3	17.3
4. P. ultimum	22.5	33.0	34.5	33.9	30.9	28.3	28.1	30.2
Mean	22.2	24.0	23.5	22.0	18.2	13.6	15.8	

[a]$FLSD_{.05}$: Treatment = .35, pH = .46, interaction = .9.

Figure 5. Effect of pH of rate of growth of fungi on PDA.




GROWTH-MM/DAY
40
30
20
10
0
4
5
6
7
8
9
pH

increased to pH 9 (Table 8, Fig. 5).

Sporulation of the biocontrols peaked at pH 5 and then decreased as the pH was increased up to pH 8.5 (Table 9, Fig. 6).

Effect of pH on seedling blight and its control

Emergence of untreated alfalfa in steamed, infested soil was poor at all pH levels tested from pH 5 to pH 8 (Table 10, Fig. 7-A). Under these conditions, seed treatment with Trichoderma spp., especially T. hamatum, improved emergence more at pH levels of 5 and 6 than at pH levels of 7 to 8. Seed treatments with either metalaxyl or Gliocladium virens improved emergence at all pH levels tested, and resulted in maximum emergence at pH levels of 6 to 8.

No postemergence damping-off was observed on plants grown from metalaxyl seed treatment at any pH level tested (Table 11). Little postemergence damping-off was recorded for G. virens and captan treatments. Plants which emerged from Trichoderma treated seeds had less postemergence damping-off at low pH levels of 5 to 6 than at pH levels of 7 to 8. Untreated alfalfa produced few plants, most of which damped off.

In natural soil artificially infested with P. ultimum, metalaxyl and Gliocladium virens seed treatments provided excellent emergence from pH 6 to pH 8, whereas captan seed treatment provided only moderate disease control (Table 12, Fig. 7-B). Effectiveness of metalaxyl, G. virens and captan treatments was very much reduced at pH 5. Trichoderma treatments provided slight protection at pH 5. Untreated alfalfa emerged poorly at all pH levels tested.

Table 9. Effect of pH on sporulation of fungi on PDA at 30 C.

Fungus	pH 5	6	7	8	8.5	Mean
	10^9 spores/plate[a]					
1. *G. virens*	5.04	3.52	3.23	3.24	1.04	3.22
2. *T. hamatum*	7.53	5.42	5.33	5.02	3.53	5.37
3. *T. harzianum*	6.22	5.03	4.13	3.78	2.85	4.39
Mean	6.27	4.66	4.23	4.00	2.47	

[a]$FLSD_{.05}$: Treatment = .0082; pH = .0111; Interaction = .0183.

Figure 4. Effect of pH of sporulation of fungi on PDA.

■——— G. virens, ▲——— T. hamatum,

□——— T. harzianum.

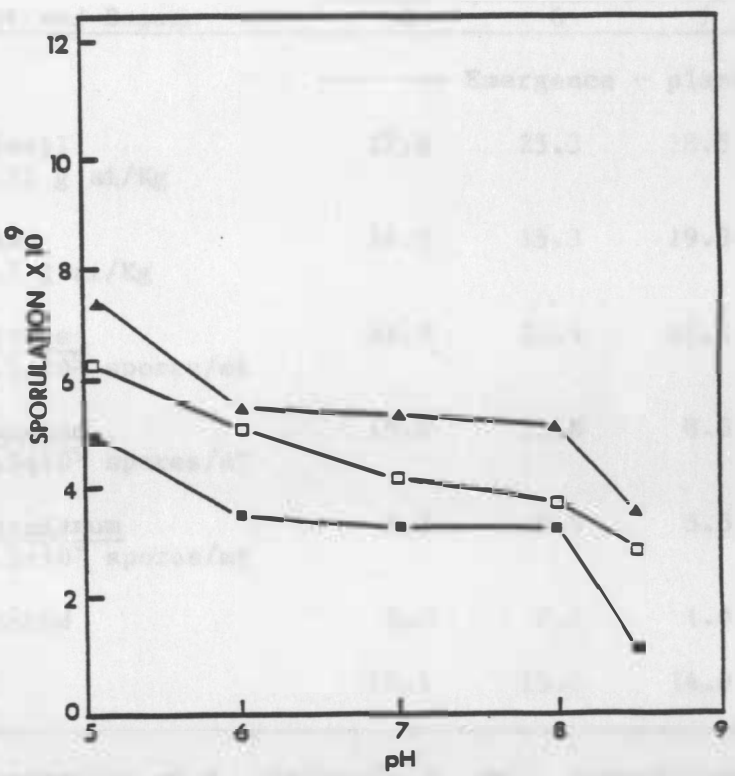

SPORULATION X 10^9
12
10
8
6
4
2
0
5
6
7
8
9
pH

Table 10. Effect of pH on emergence of Travois alfalfa 3-5 days after planting in steamed soil artificially infested with *P. ultimum*.[a]

Treatment and Dosage	soil pH 5	6	7	8	Mean
	-------- Emergence - plants/pot[bc] ----------				
1. Metalaxyl 0.31 g ai/Kg	17.8	25.3	28.5	26.8	24.6
2. Captan 0.7 g ai/Kg	14.3	15.3	19.3	17.8	16.6
3. *G. virens* 1.5×10^9 spores/ml	21.5	23.5	21.5	22.3	22.2
4. *T. hamatum* 1.5×10^9 spores/ml	15.0	13.8	8.5	4.8	10.5
5. *T. harzianum* 1.5×10^9 spores/ml	8.3	9.5	5.5	3.8	6.8
6. Untreated	2.0	2.5	1.0	1.0	1.7
Mean	13.1	15.0	14.0	13.2	

[a]5000 propagules of *P. ultimum*/g of soil, temperature 30 C, moisture 20%.

[b]$FLSD_{.05}$: Treatment = 3.43, pH = 2.8, interaction = 6.87.

[c]Perfect stand = 33 plants/pot.

Table 11. Effect of pH on stand establishment of Travois alfalfa 12 days after planting in steamed soil artificially infested with *P. ultimum*.[a]

Treatment and Dosage	Soil pH 5	6	7	8	Mean
	Stand - plants/pot[bc]				
1. Metalaxyl 0.31 g ai/Kg	17.8	25.3	31.0	26.3	25.1
2. Captan 0.7 g ai/Kg	13.3	14.3	18.3	17.0	15.7
3. *G. virens* 1.5×10^9 spores/ml	21.3	22.5	21.3	21.0	21.5
4. *T. hamatum* 1.5×10^9 spores/ml	11.8	10.8	4.8	2.3	7.4
5. *T. harzianum* 1.5×10^9 spores/ml	5.8	7.0	3.5	1.3	4.4
6. Untreated	1.0	0.0	0.3	0.0	0.5
Mean	11.8	13.4	13.2	11.8	

[a]5000 propagules of *P. ultimum*/g of soil, temperature 30 C, moisture 20%.

[b]$FLSD_{.05}$: Treatment = 2.98, pH = 2.43, interaction = 5.96.

[c]Perfect stand = 33 plants/pot.

Table 12. Effect of pH on emergence of Travois alfalfa 3-5 days after planting in natural soil artificially infested with *P. ultimum*.[a]

Treatment and Dosage	Soil pH 5	6	7	8	Mean
	-------- Emergence - plants/pot[bc] ----------				
1. Metalaxyl 0.31 g ai/Kg	13.3	28.0	29.0	28.5	24.7
2. Captan 0.7 g ai/Kg	13.0	20.0	17.3	19.3	17.4
3. *G. virens* 1.5×10^9 spores/ml	14.0	26.0	25.5	26.3	22.9
4. *T. hamatum* 1.5×10^9 spores/ml	13.3	10.3	9.5	8.8	10.4
5. *T. harzianum* 1.5×10^9 spores/ml	15.0	7.5	6.5	5.5	8.6
6. Untreated	2.0	1.8	2.0	1.8	2.0
Mean	11.8	15.6	15.0	15.0	

[a]5000 propagules of *P. ultimum*/g of soil, temperature 30 C, moisture 20%.

[b]$FLSD_{.05}$: Treatment = 2.43, pH = 1.99, interaction = 4.87.

[c]Perfect stand = 33 plants/pot.

Figure 7. Effect of soil pH on emergence of Travois alfalfa 3-5 days after planting.

(A) In steamed soil artificially infested with *P. ultimum*. Seed treated with:

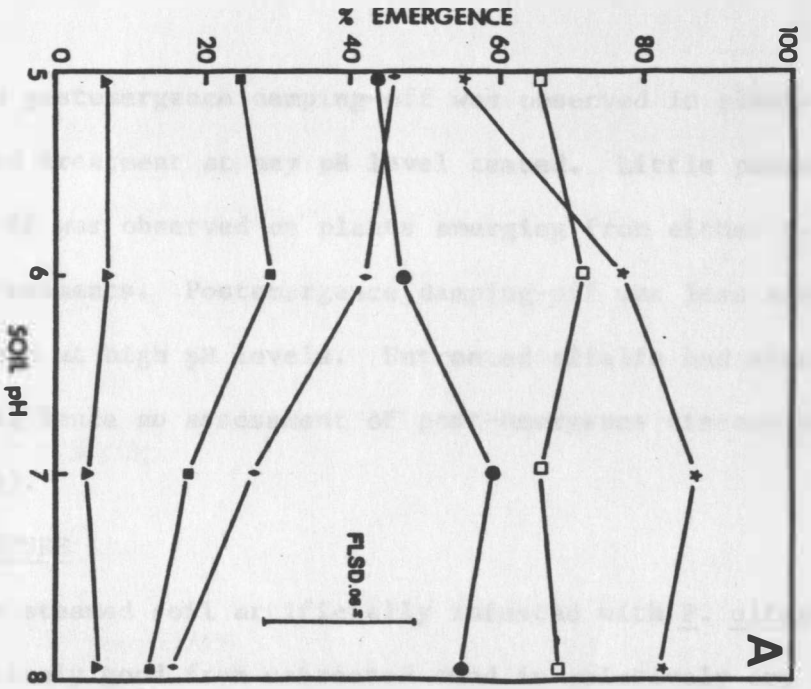
★——— metalaxyl, ●——— Captan, □——— *G. virens*, ◆——— *T. hamatum*,

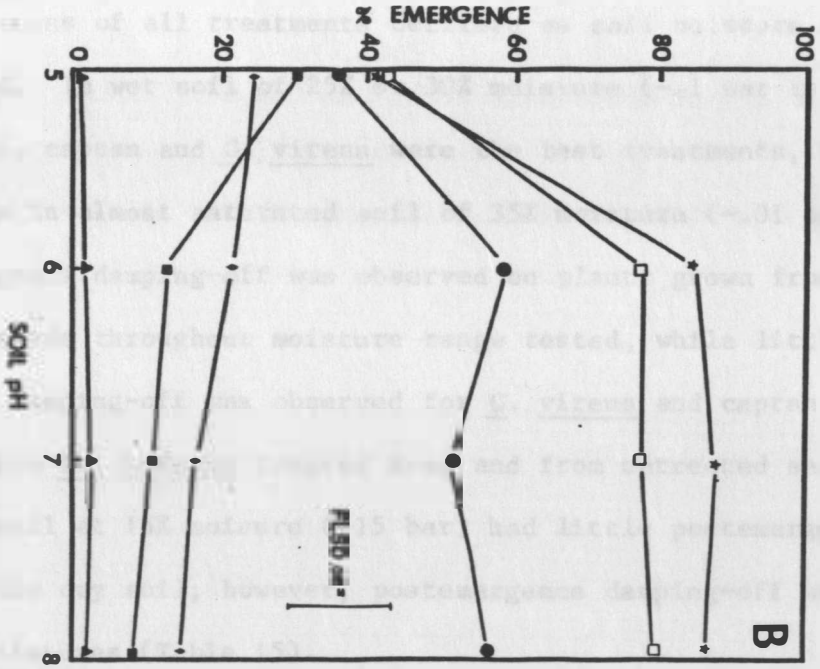
■——— *T. harzianum*, ▲——— Untreated.

(B) In natural soil artificially infested with *P. ultimum*. Seed treated with:

★——— metalaxyl, ●——— Captan, ⊟——— *G. virens*, ◆——— *T. hamatum*,

■——— *T. harzianum*, ▲——— Untreated.


% EMERGENCE
0
20
40
60
80
100
5
6
7
8
SOIL pH
FLSD.05 =
A


% EMERGENCE
0
20
40
60
80
100
5
6
7
8
SOIL pH
FLSD.05 =
B

No postemergence damping-off was observed in plants from metalaxyl seed treatment at any pH level tested. Little postemergence damping-off was observed on plants emerging from either G. virens or captan treatments. Postemergence damping-off was less severe at low pH levels than at high pH levels. Untreated alfalfa had virtually failed to emerge, hence no assessment of post-emergence disease was possible (Table 13).

SOIL MOISTURE

In steamed soil artificially infested with P. ultimum, emergence was relatively good from untreated seed in relatively dry soil of 15% moisture, but dropped sharply as soil moisture was increased (Table 14, Fig. 8-A). At 20% moisture (-.35 bar) only Trichoderma harzianum failed to improve emergence significantly over that of untreated seed. The effectiveness of all treatments declined as soil moisture was further increased. In wet soil of 25% or 30% moisture (-.1 bar to -.03 bar), metalaxyl, captan and G. virens were the best treatments, but none were effective in almost saturated soil of 35% moisture (-.01 bar). No postemergence damping-off was observed on plants grown from metalaxyl treated seeds throughout moisture range tested, while little postemergence damping-off was observed for G. virens and captan treatments. Plants from Trichoderma treated seed and from untreated seed which emerged well at 15% moisure (-15 bar) had little postemergence damping-off in this dry soil; however, postemergence damping-off was severe at higher moistures (Table 15).

When untreated and Trichoderma treated seeds were planted in

Table 13. Effect of pH on stand establishment of Travois alfalfa 12 days after planting in natural soil artificially infested with *P. ultimum*.[a]

Treatment and Dosage	Soil pH 5	6	7	8	Mean
	Stand - plants/pot[bc]				
1. Metalaxyl 0.31 g ai/Kg	13.3	28.0	28.8	28.3	24.6
2. Captan 0.7 g ai/Kg	11.8	19.3	16.8	18.5	16.6
3. *G. virens* 1.5×10^9 spores/ml	13.8	25.5	25.5	26.0	22.7
4. *T. hamatum* 1.5×10^9 spores/ml	8.0	7.0	5.3	4.5	6.2
5. *T. harzianum* 1.5×10^9 spores/ml	10.0	4.0	3.3	2.3	4.9
6. Untreated	0.5	0.3	0.5	0.3	0.4
Mean	9.5	14.0	13.3	13.3	

[a]5000 propagules of *P. ultimum*/g of soil, temperature 30 C, moisture 20%.

[b]$FLSD_{.05}$: Treatment = 2.23, pH = 1.82, interaction = 4.46.

[c]Perfect stand = 33 plants/pot.

Table 14. Effect of moisture on emergence of Travois alfalfa 3-5 days after planting in steamed soil artificially infested with *P. ultimum*.[a]

Treatment and Dosage	Soil Moisture % 15	20	25	30	35	Mean
	Emergence - plants/pot[bc]					
1. Metalaxyl 0.31 g ai/Kg	26.8	29.0	21.5	14.8	1.5	17.4
2. Captan 0.7 g ai/Kg	26.0	23.0	18.5	13.5	1.3	16.4
3. *G. virens* 1.5×10^9 spores/ml	25.5	28.3	20.0	10.5	1.3	17.1
4. *T. hamatum* 1.5×10^9 spores/ml	21.8	14.5	11.8	4.3	1.3	10.7
5. *T. harzianum* 1.5×10^9 spores/ml	17.3	9.8	9.3	4.3	1.0	8.3
6. Untreated	19.3	5.8	3.8	3.8	.8	6.7
Mean	22.7	18.4	13.0	8.5	1.2	

[a]5000 propagules of *P. ultimum*/g of soil, temperature 30 C, pH 7.4.

[b]$FLSD_{.05}$: Treatment = 4.21, moisture = 2.7, interaction = 6.65.

[c]Perfect stand = 33 plants/pot.

Table 15. Effect of moisture on stand establishment of Travois alfalfa 12 days after planting in steamed soil artificially infested with P. ultimum.[a]

Treatment and Dosage	Soil Moisture % 15	20	25	30	35	Mean
	Stand - plants/pot[bc]					
1. Metalaxyl 0.31 g ai/Kg	26.8	29.0	21.5	14.8	1.5	17.4
2. Captan 0.7 g ai/Kg	24.8	22.5	17.3	11.5	0.0	15.2
3. G. virens $1.5x10^9$ spores/ml	25.5	27.8	19.3	10.0	0.0	16.5
4. T. hamatum $1.5x10^9$ spores/ml	18.3	6.0	9.8	3.0	0.0	7.4
5. T. harzianum $1.5x10^9$ spores/ml	13.3	4.3	6.3	2.0	0.0	5.2
6. Untreated	14.0	1.8	1.0	0.8	0.0	3.5
Mean	20.4	15.2	11.4	7.0	.3	

[a]5000 propagules of P. ultimum/g of soil, temperature 30 C, pH 7.4.

[b]$FLSD_{.05}$: Treatment = 2.55, moisture = 2.32, interaction = 5.7.

[c]Perfect stand = 33 plants/pot.

Figure 8. Effect of soil moisture on emergence of Travois alfalfa 3-5 days after planting.

(A) In steamed soil artificially infested with *P. ultimum*. Seed treated with:

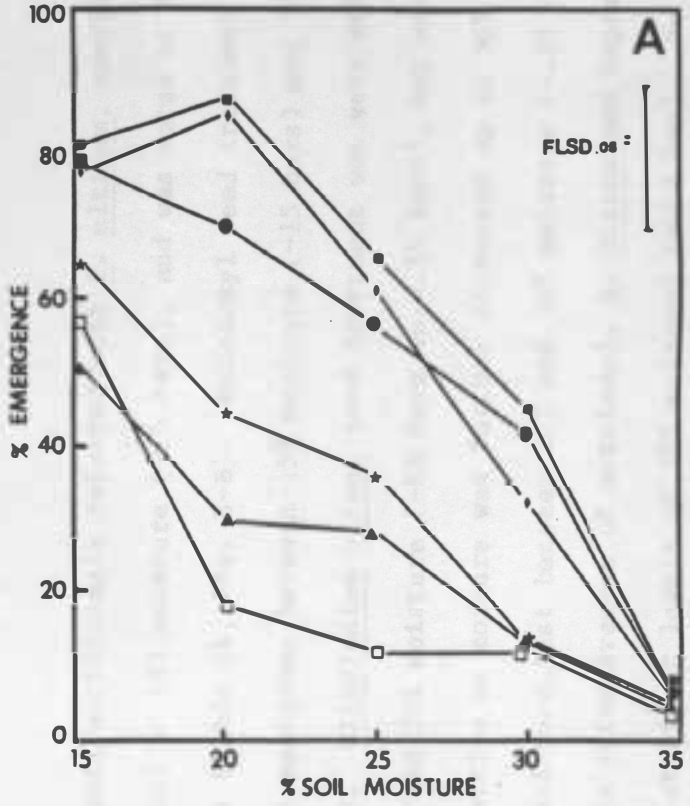
■——— metalaxyl, ●——— Captan, ◆——— *G. virens*, ★——— *T. hamatum*,

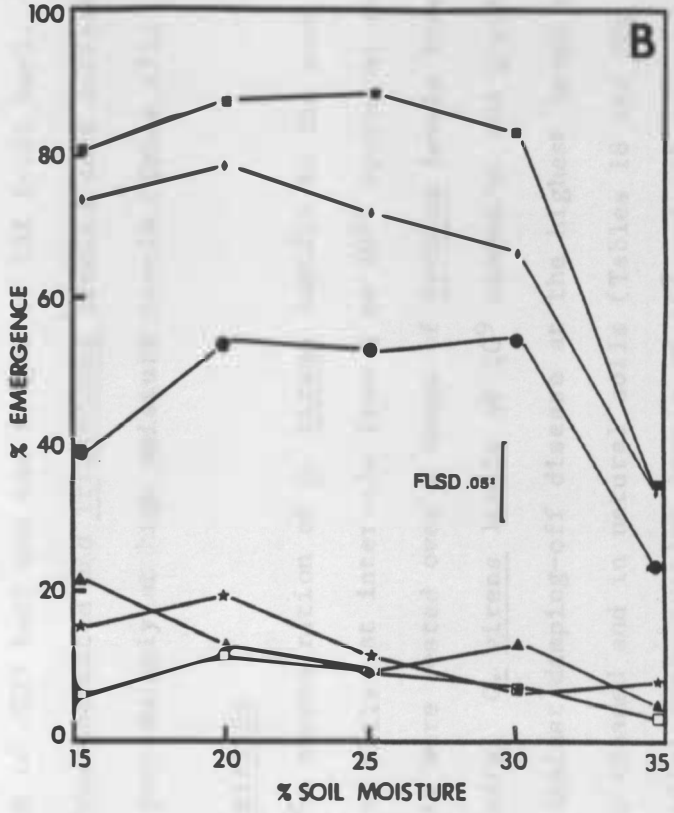
▲——— *T. harzianum*, □——— Untreated.

(B) In natural soil artificially infested with *P. ultimum*. Seed treated with:

■——— metalaxyl, ●——— Captan, ◆——— *G. virens*, ★——— *T. hamatum*,

▲——— *T. harzianum*, □——— Untreated.


A
100
80
60
40
20
0
% EMERGENCE
FLSD .05 =
15
20
25
30
35
% SOIL MOISTURE


B
100
80
60
40
20
0
% EMERGENCE
FLSD .05
15
20
25
30
35
% SOIL MOISTURE

natural soil artificially infested with P. ultimum, emergence was poor in dry soil of 15% moisture (-15 bars), and was worse at higher moistures (Table 16, Fig. 8-B). Metalaxyl seed treatment provided excellent emergence between 15% moisture (-15 bars) and 30% moisture (.03 bar). Gliocladium virens seed treatment was very effective between 15% and 20% moisture (-15 bars to -.35 bars), but was somewhat less effective as moisure was further increased up to 30% (-.03 bars). Captan performed best between 20% and 30% moisture (-.35 bar to -.033 bar). The effectiveness of metalaxyl, G. virens and Captan treatments declined at higher levels of 35% moisture (-.01 bar). No postemergence damping-off was observed in plants from metalaxyl treated seed at any moisture level tested. Negligible postemergence damping-off was recorded for G. virens and Captan between 15% and 30% moisture levels (-15 bars to .033 bar) and was slight at 35% (-.01 bar). The emerged plants from untreated and Trichoderma treated seed suffered heavy losses, particularly at high moisture levels (Table 17).

OTHER VARIABLES

The concentration of G. virens conidia in the seed treatment liquid was varied at intervals from 0 to 10^{10} spores/ml and these treatments were tested over a range of Pythium levels from 0 to 5000 propagules/g. G. virens levels of 10^9 spores/ml and greater protected alfalfa against damping-off disease at the highest level of P. ultimum tested in steamed and in natural soils (Tables 18 and 20; Figs. 8 A and B). Conidial concentration levels of 10^7 and 10^8 spores/ml protected alfalfa at the low level of P. ultimum but were less effective at the

Table 16. Effect of moisture on emergence of Travois alfalfa 3-5 days after planting in natural soil artificially infested with *P. ultimum*.[a]

Treatment and Dosage	Soil Moisture % 15	20	25	30	35	Mean
	-------- Emergence - plants/pot[bc] ----------					
1. Metalaxyl 0.31 g ai/Kg	26.5	29.0	29.3	27.5	10.0	24.5
2. Captan 0.7 g ai/Kg	12.8	18.0	17.5	18.3	7.0	14.7
3. *G. virens* 1.5×10^9 spores/ml	24.3	26.0	23.8	22.0	10.5	21.3
4. *T. hamatum* 1.5×10^9 spores/ml	5.0	6.5	3.8	2.0	2.5	4.0
5. *T. harzianum* 1.5×10^9 spores/ml	7.3	4.3	3.0	4.3	1.3	4.0
6. Untreated	1.8	3.8	3.0	2.3	0.8	2.3
Mean	12.9	14.6	13.4	12.7	5.3	

[a]5000 propagules of *P. ultimum*/g of soil, temperature 30 C, pH 7.4.

[b]$FLSD_{.05}$: Treatment = 1.75, moisture = 1.6, interaction = 3.92.

[c]Perfect stand = 33 plants/pot.

Table 17. Effect of moisture on stand establishment of Travois alfalfa 12 days after planting in natural soil artificially infested with *P. ultimum*.[a]

Treatment and Dosage	Soil Moisture % 15	20	25	30	35	Mean
	Stand - plants/pot[bc]					
1. Metalaxyl 0.31 g ai/Kg	26.5	29.0	29.3	27.5	10.0	24.5
2. Captan 0.7 g ai/Kg	11.8	17.0	16.8	17.5	12.8	15.2
3. *G. virens* 1.5×10^9 spores/ml	23.8	25.5	23.3	21.3	10.5	20.9
4. *T. hamatum* 1.5×10^9 spores/ml	1.8	2.5	1.3	0.5	1.0	1.4
5. *T. harzianum* 1.5×10^9 spores/ml	2.3	1.3	0.5	1.0	1.0	1.2
6. Untreated	0.0	0.3	0.3	0.0	0.8	0.3
Mean	11.0	12.6	11.9	11.3	6.0	

[a]5000 propagules of *P. ultimum*/g of soil, temperature 30 C, pH 7.4.

[b]$FLSD_{.05}$: Treatment = 1.89, moisture = 1.73, interaction = 4.23.

[c]Perfect stand = 33 plants/pot.

Table 18. Effect of G. virens concentration on emergence of Travois alfalfa 3-5 days after planting in steamed soil artificially infested with P. ultimum.[a]

P. ultimum propagules/g of soil	Seed treatment: Concentration of G. virens spores/ml								
	0	10^4	10^5	10^6	10^7	10^8	10^9	10^{10}	Mean
	Emergence - plants/pot[bc]								
1. 5000	0.5	2.0	4.3	4.5	5.3	9.0	24.0	26.3	9.5
2. 2400	6.0	6.0	6.1	6.3	12.0	12.8	25.0	27.3	12.7
3. 1400	6.5	8.3	8.8	10.0	16.3	17.3	26.0	23.8	15.2
Mean	4.3	5.4	6.3	6.9	11.2	13.0	25.0	27.4	

aGrown at 30 C, soil moisture 20% and pH 7.4.

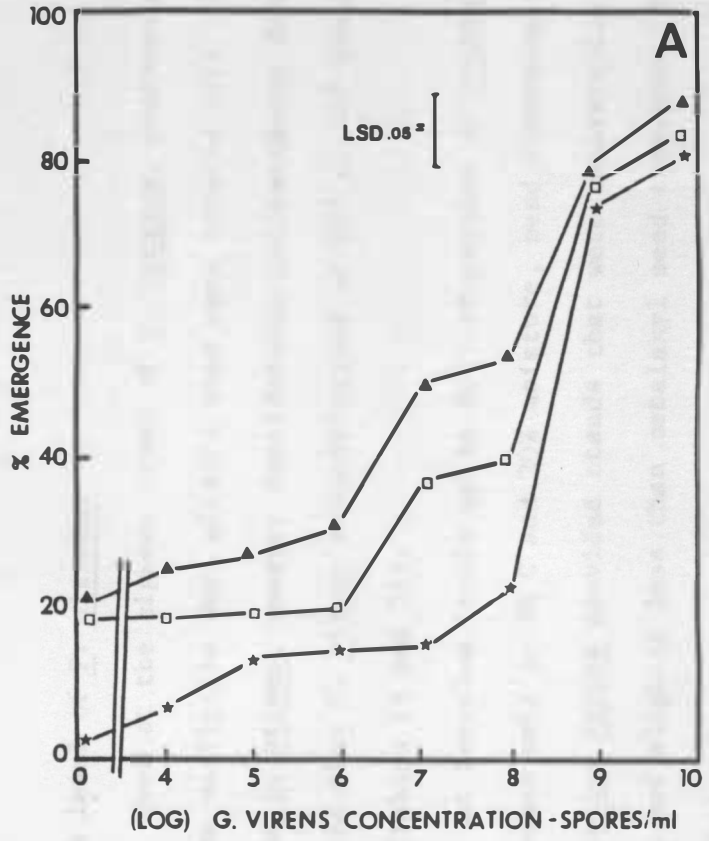
[b]$FLSD_{.05}$: inoculum level = 1.28, G. virens concentration = 2.1, interaction = 3.63.

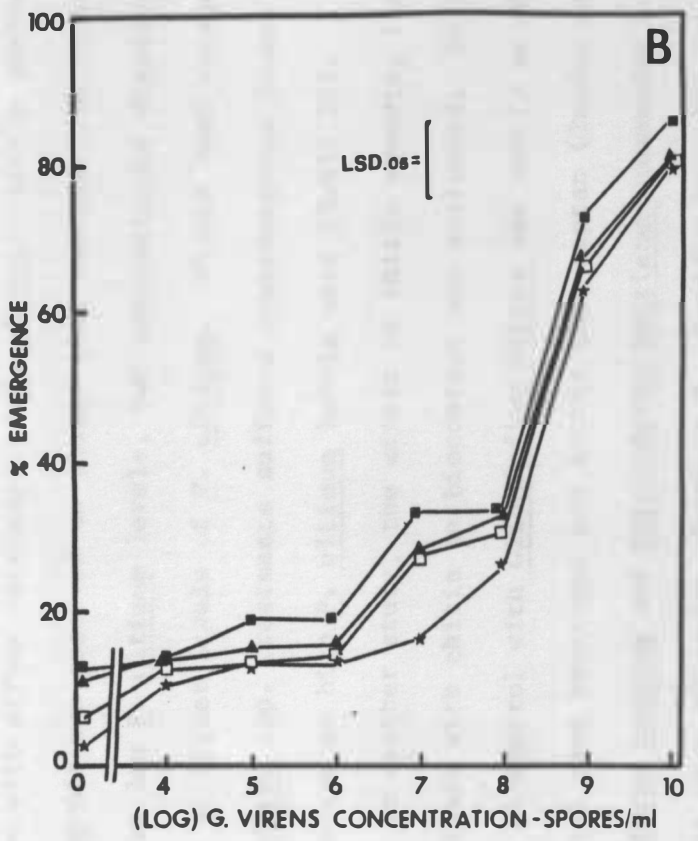
[c]Perfect stand = 33 plants/pot.

Figure 9. Effect of G. virens concentration on emergence of Travois alfalfa at different levels of P. ultimum.

(A) In steamed soil. ▲——— low level, □——— medium level, ★——— high level.

(B) In natural soil. ▲——— low level, □——— medium level, ★——— high level ■——— natural level.


A
LSD.05=
100
80
60
40
20
0
% EMERGENCE
0
4
5
6
7
8
9
10
(LOG) G. VIRENS CONCENTRATION - SPORES/ml


B
LSD.05=
100
80
60
40
20
0
% EMERGENCE
0
4
5
6
7
8
9
10
(LOG) G. VIRENS CONCENTRATION - SPORES/ml

medium level of P. ultimum.

Even at the highest level of P. ultimum, postemergence damping-off was negligible when alfalfa seed were treated with 10^9 spores/ml or more of G. virens; however considerable postemergence damping-off occurred when G. virens concentrations of 10^7 or 10^8 conidia/ml were used (Tables 19 and 21).

At inoculum levels up to 5000 propagules of Pythium ultimum/gm of steamed soil at 30 C and 20% moisture, seed treatment with Gliocladium virens provided stands that were consistently better than captan and slightly less than metalaxyl seed treatment (Table 22, Fig. 10). Trichoderma spp. slightly improved emergence at all but the lowest levels of P. ultimum.

No postemergence damping-off was observed when alfalfa seed were treated with either metalaxyl or G. virens. Little postemergence damping-off was observed on plants that emerged from captan treated seeds at low P. ultimum levels, but postemergence disease was more severe at higher levels of P. ultimum. Plants that emerged from Trichoderma spp. treatments suffered postemergence losses that became more severe at high P. ultimum levels used (Table 23).

In another study, the effect of chitin amending the seed treatment liquid with chitin on biocontrol was evaluated. Without amendments, biocontrol with Gliocladium virens was nearly as effective as metalaxyl seed treatment and better than captan (Tables 24 and 25). Trichoderma hamatum and Trichoderma harzianum unamended seed treatments were not very effective, however T. hamatum provided better control than T. harzianum. Amendment with bleached chitin did not affect the

Table 19. Effect of G. virens concentration on stand establishment of Travois alfalfa 12 days after planting in steamed soil artificially infested with P. ultimum.[a]

P. ultimum propagules/g of soil	Seed Treatment: Concentration of G. virens spores/ml								
	0	10^4	10^5	10^6	10^7	10^8	10^9	10^{10}	Mean
	Stand - plants/pot[bc]								
1. 5000	0.0	0.3	1.0	1.0	2.5	4.3	23.3	26.3	7.3
2. 2400	0.8	0.8	0.8	2.0	4.3	5.3	24.8	26.8	8.2
3. 1400	2.3	2.8	4.0	4.5	10.0	10.5	25.3	28.3	10.9
Mean	1.0	1.3	1.9	2.5	5.6	6.7	24.4	27.1	

[a]Grown at 30 C, soil moisture 20% and pH 7.4.

[b]$FLSD_{.05}$: inoculum level = .65, G. virens concentration = 1.06, interaction = 1.80.

[c]Perfect stand = 33 plants/pot.

Table 20. Effect of G. virens concentration on emergence of Travois alfalfa 3-5 days after planting in natural soil artificially infested with P. ultimum.[a]

P. ultimum propagules/g of soil	Seed Treatment: Concentration of G. virens spores/ml								
	0	10^4	10^5	10^6	10^7	10^8	10^9	10^{10}	Mean
	Emergence - plants/pot[bc]								
1. 5000	0.5	3.3	4.3	4.3	5.3	8.5	20.0	26.0	6.8
2. 2400	1.8	4.0	4.3	4.5	9.0	10.0	21.8	26.5	7.3
3. 1400	3.5	4.5	5.0	5.0	9.3	10.8	22.0	26.5	7.6
4. 600	4.0	5.3	6.3	6.3	11.0	11.0	24.0	28.0	8.9
Mean	2.4	4.3	4.9	5.0	8.6	10.1	22.1	26.8	

[a]Grown at 30 C, soil moisture 20% and pH 7.4.

[b]$FLSD_{.05}$: inoculum level = 1.18, G. virens concentration = 1.67, interaction = 3.35.

[c]Perfect stand = 33 plants/pot.

Table 21. Effect of G. virens concentration on stand establishment of Travois alfalfa 12 days after planting in natural soil artificially infested with P. ultimum.[a]

P. ultimum propagules/g of soil	Seed Treatment: Concentration of G. virens spores/ml								
	0	10^4	10^5	10^6	10^7	10^8	10^9	10^{10}	Mean
	Stand - plants/pot[bc]								
1. 5000	0.0	.5	1.3	1.5	2.0	3.3	20.3	26.0	6.8
2. 2400	0.0	1.0	1.5	1.5	3.0	4.0	21.0	26.5	7.3
3. 1400	0.0	1.5	1.5	1.8	3.8	4.0	22.0	26.5	7.6
4. 600	2.0	2.0	2.3	2.3	2.5	8.0	24.0	28.0	8.9
Mean	.5	1.3	1.6	1.8	2.8	4.8	21.3	26.8	

[a]Grown at 30 C, soil moisture 20% and pH 7.4.

[b]$FLSD_{.05}$: inoculum level = .79, G. virens concentration = 1.12, interaction = 2.24.

[c]Perfect stand = 33 plants/pot.

Table 22. Effect of level of inoculum on emergence of Travois alfalfa 3-5 days after planting in steamed soil artificially infested with *P. ultimum*.[a]

Treatments and Dosages	Propagules of *P. ultimum*/g of soil					
	0	600	1200	2500	5500	Mean
	--------- Emergence - plants/pot[bc] ----------					
1. Metalaxyl 0.31 g ai/Kg	27.0	26.0	27.0	27.8	28.0	27.2
2. Captan 0.7 g ai/Kg	25.0	22.0	20.0	19.0	18.0	20.8
3. *G. virens* 1.5×10^9 spores/ml	26.0	24.3	26.0	23.3	24.0	24.7
4. *T. hamatum* 1.5×10^9 spores/ml	26.8	14.0	12.8	9.0	7.0	13.9
5. *T. harzianum* 1.5×10^9 spores/ml	26.0	10.0	9.5	5.8	4.0	11.1
6. Untreated	27.0	9.8	3.0	3.0	1.8	8.9
Mean	26.3	17.7	16.4	14.6	13.8	

[a]Temperature 30 C, moisture 20%, pH 7.4.

[b]$FLSD_{.05}$: Treatment = 2.20, inoculum level = 2.0, interaction = 4.90.

[c]Perfect stand = 33 plants/pot.

Figure 10. Effect of level of inoculum of *P. ultimum* of emergence of Travois alfalfa in steamed artificially infested soil. Seed treated with: ★——— metalaxyl, ●——— Captan, □——— *G. virens*, ◆——— *T. hamatum*, ■——— *T. harzianum*, ▲——— Untreated.

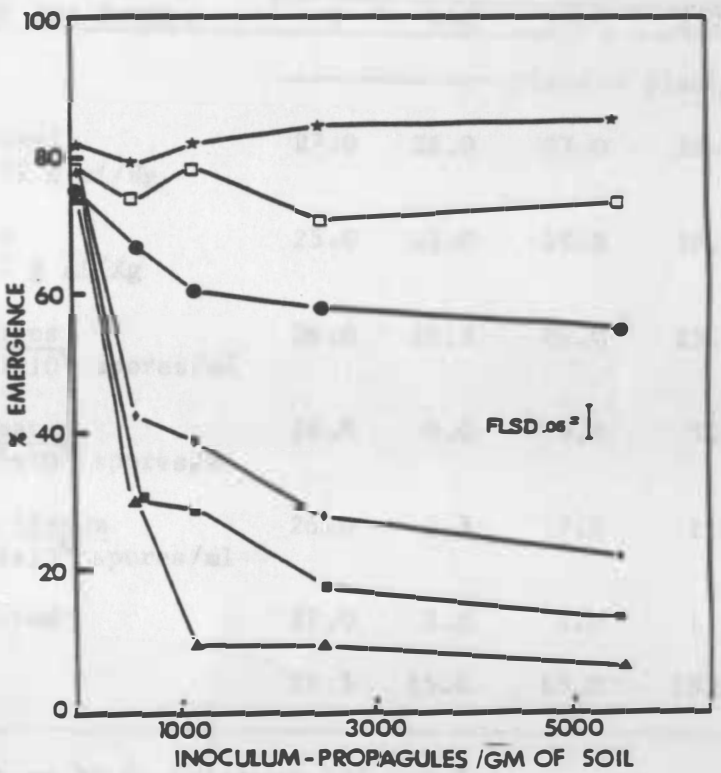

100
80
60
40
20
0
% EMERGENCE
FLSD .05
1000
3000
5000
INOCULUM - PROPAGULES /GM OF SOIL

Table 23. Effect of level of inoculum in stand establishment of Travois alfalfa 12 days after planting in steamed soils artificially infested with *P. ultimum*.[a]

Treatment and Dosage	Propagules of *P. ultimum*/g of soil					
	0	600	1200	2500	5500	Mean
	Stand - plants/pot[b]					
1. Metalaxyl 0.31 g ai/Kg	27.0	26.0	27.0	27.8	28.0	27.2
2. Captan 0.7 g ai/Kg	25.0	22.0	19.8	18.8	16.8	20.5
3. *G. virens* 1.5×10^9 spores/ml	26.0	24.3	26.0	23.3	24.0	24.7
4. *T. hamatum* 1.5×10^9 spores/ml	26.8	9.0	9.0	5.0	2.8	10.5
5. *T. harzianum* 1.5×10^9 spores/ml	26.0	6.3	7.0	2.3	1.3	8.6
6. Untreated	27.0	2.8	1.0	1.3	0.8	6.6
Mean	26.3	15.0	15.0	13.0	12.5	

[a]Temperature 30 C, moisture 20%, pH 7.4.

[b]$FLSD_{.05}$: Treatment = 1.88, inoculum level = 1.72, interaction = 4.20.

[c]Perfect stand = 33 plants/pot.

Table 24. Effect of amended and unamended seed treatments on emergence and stand establishment of Travois alfalfa on steamed soil artificially infested with *P. ultimum*.[a]

Treatment and Dosage	Days from planting 3-5	12
	---- Emergence - plants/pot[b] -----	
1. Metalaxyl 0.31 g ai/Kg	30.3	30.3
2. Captan 0.7 g ai/Kg	23.5	22.3
3. *G. virens* 1.5×10^9 spores/ml	28.3	28.0
4. *G. virens* + bleached chitin	27.8	27.3
5. *G. virens* + unbleached chitin	11.3	9.8
6. T. hamatum 1.5×10^9 spores/ml	11.8	6.3
7. T. hamatum + bleached chitin	10.8	6.3
8. T. hamatum + unbleached chitin	4.0	2.0
9. T. harzianum 1.5×10^9 spores/ml	7.5	3.5
10. T. harzianum + bleached chitin	7.3	2.5
11. T. harzianum + unbleached chitin	3.8	1.5
12. Untreated	2.3	0.0
$FLSD_{.05}$ =	1.6	1.5

[a]5000 propagules of *P. ultimum*/g of soil, temperature 30 C, moisture 20%, pH 7.4.

[b]Perfect stand - 33 plants/pot.

Table 25. Effect of amended and unamended seed treatments on emergence and stand establishment of Travois alfalfa in natural soil artificially infested with *P. ultimum*.[a]

Treatment and Dosage	Days from planting 3-5	12
	----- Emergence - plants/pot[b] -----	
1. Metalaxyl 0.31 g ai/Kg	29.0	29.0
2. Captan 0.7 g ai/Kg	15.3	14.5
3. *G. virens* 1.5×10^9 spores/ml	27.3	26.8
4. *G. virens* + bleached chitin	24.3	24.3
5. *G. virens* + unbleached chitin	3.0	2.0
6. T. hamatum 1.5×10^9 spores/ml	7.5	3.8
7. T. hamatum + bleached chitin	7.0	3.3
8. T. hamatum + unbleached chitin	2.0	1.0
9. T. harzianum 1.5×10^9 spores/ml	2.8	1.3
10. T. harzianum + bleached chitin	2.3	1.5
11. T. harzianum + unbleached chitin	1.8	1.0
12. Untreated	.5	0.0
$FLSD_{.05}$ =	1.7	1.1

[a]5000 propagules of *P. ultimum*/g of soil, temperature 30 C, moisture 20%, pH 7.4.

[b]Perfect stand - 33 plants/pot.

ability of the antagonists to protect seeds against P. ultimum, while unbleached chitin decreased control level.

Postemergence damping-off was not observed in metalaxyl treated pots, and very little was observed on plants emerging from either G. virens on captan treatments. Chitin, whether bleached or unbleached, did not seem to play any role in the control of postemergence damping-off by Gliocladium virens. In the case of Trichoderma treated seeds, unbleached chitin significantly increased postemergence damping-off. Generally postemergence damping-off on plants treated with unamended Trichoderma spp. was appreciable (Tables 24 and 25).

FIELD EXPERIMENTS

An experiment was conducted at Highmore, SD under natural conditions. The soil contained a natural population of 400-600 propagules of P. ultimum/gm of soil when assayed by the method of Stangillini and Hancock (117). The objective was to evaluate G. virens seed treatment against P. ultimum under field conditions, and to compare it with chemical seed treatments. The fungicides metalaxyl and captan as well as G. virens were used as seed treatments; the nematicide carbofuran was applied as a post-plant soil spray. Treatments were arranged in a randomized complete block design with four replications. Ten grams of alfalfa (Medicago sativa c. v. Agate) was machine planted 2 cm deep in four rows, each 10 feet long and stand counts were made three weeks after planting.

All of the seed treatments significantly improved stand over that from untreated seed. There were no significant differences bet-

ween fungicides and G. virens. Carbofuran also improved stand, and the combination of carbofuran soil treatment and metalaxyl seed treatment resulted in the best stand obtained from any treatment (Table 26).

In a field experiment conducted at Brookings, plots were inoculated with Pythium ultimum infested millet prior to planting to ensure that the pathogen was present. Metalaxyl, captan and Gliocladium virens seed treatments protected alfalfa from P. ultimum and Trichoderma spp. gave some control. Addition of unbleached chitin to the seed negated biocontrol with G. virens. Seed treatment with a combination of G. virens and either of the Trichoderma spp. also resulted in poor control (Table 27). Postemergence damping-off was low with metalaxyl, captan and G. virens treatments. Similar results were obtained when soil was infested with vermiculite colonized with P. ultimum (Table 28).

Table 26. Effect of seed treatment and soil treatments on stand establishment of Agate alfalfa in soils naturally infested with P. ultimum in the field at Highmore, 1983.

Seed Treatment and Dose	Soil Treatment	
	Untreated	Carbofuran[a]
	------- plants/2' row[b] ---------	
1. Metalaxyl 0.31 g ai/Kg	55.5	73.5
2. Captan 0.7 g ai/Kg	47.3	.
3. G. virens 1.5×10^9 spores/ml	46.5	.
4. Untreated	18.5	49.5
FLSD	22.1	

[a]Carbofuran at 1.12 kg/ha post-plant incorporated spray.

[b]Perfect stand = 250 plants/2' row.

Table 27. Effect of seed treatment on emergence and stand establishment of Travois alfalfa in soils artificially infested with P. ultimum on millet seed in the field at Brookings, 1983.

Treatment and Dose	Emergence on Day 7	Emergence on Day 21
	-- plants/10' row[a] ---	
1. Metalaxyl 0.31 g ai/Kg	31.0	27.3
2. Captan 0.7 g ai/Kg	28.3	22.3
3. G. virens $1.5x10^9$ spores/ml	24.0	20.0
4. T. hamatum $1.5x10^9$ spores/ml	10.8	7.5
5. T. harzianum $1.5x10^9$ spores/ml	10.3	8.0
6. G. virens + T. hamatum	6.3	5.8
7. G. virens + T. harzianum	8.8	6.3
8. G. virens + unbleached chitin	5.8	5.3
9. T. hamatum + unbleached chitin	6.0	1.3
10. T. harzianum + unbleached chitin	9.8	5.8
11. Untreated	5.8	2.8
$FLSD_{.05}$	7.6	6.3

[a]Perfect stand = 100 plants/10' row.

Table 28. Effect of seed treatment on emergence and stand establishment of Travois alfalfa in soils artificially infested with P. ultimum on vermiculite in the field at Brookings, 1983.

Treatment and Dose	Emergence on Day 7	Emergence on Day 21
	-- plants/10' row[a] ---	
1. Metalaxyl 0.31 g ai/Kg	51.0	49.0
2. Captan 0.7 g ai/Kg	42.0	37.5
3. G. virens $1.5x10^9$ spores/ml	40.5	38.0
4. T. hamatum $1.5x10^9$ spores/ml	22.8	18.5
5. T. harzianum $1.5x10^9$ spores/ml	13.3	11.3
6. G. virens + T. hamatum	17.0	16.5
7. G. virens + T. harzianum	13.8	11.8
8. G. virens + unbleached chitin	13.3	9.8
9. T. hamatum + unbleached chitin	13.0	11.0
10. T. harzianum + unbleached chitin	14.3	12.0
11. Untreated	13.8	9.5
$FLSD_{.05}$	5.2	4.1

[a]Perfect stand = 100 plants/10' row.

DISCUSSION

TEMPERATURE

The optimum temperature range for growth of P. ultimum was between 25 and 30°. This corresponded to most previous reports (1, 58, 70, 84, 100, 124) but was considerably lower than the optimum of 32 C reported by Harter and Whitney (50). The optimum temperature for Trichoderma hamatum and Trichoderma harzianum was 30 C. This value was in accord with results obtained by Danielson and Davey (28) as typical for isolates from geographic regions with moderate temperature. The optimum temperature for G. virens also was 30 C. These relationships would suggest that disease due to P. ultimum would be most severe at relatively warm soil temperatures (25-30°) and also that the biocontrol would be most effective at warm temperatures.

From a practical view, we are interested in relations in relatively cool soil, since relatively low temperatures commonly are encountered when alfalfa is planted in April or May. On PDA plates where the antagonist was introduced with P. ultimum, obvious zones of inhibition occurred around G. virens colonies at all temperature levels between 15 and 35 C regardless of the size of G. virens colonies. These inhibition zones were indicative of antibiotic production at this temperature range. In a similar study Howell (53, 55) reported the appearance of inhibition zones in two membered cultures of P. ultimum and G. virens. My observation suggested that G. virens could perform similarly on the seed coat of alfalfa and one might expect biocontrol

of P. ultimum to occur at these temperatures. However, the 'in vitro' results show that P. ultimum grew relatively fast at low temperature compared to the biocontrols, and we also might predict that biocontrols would be less effective at lower temperature.

Under the relatively competition-free environment of steamed soil, P. ultimum was devastating to alfalfa especially at low temperature levels of 15 to 20°C. Severity of P. ultimum at these low levels of temperature was also reported on other crops but in natural soils (68, 78, 118).

I predicted from the relative growth rate of the pathogen and the biocontrol agent, that disease control would be most difficult at the lower temperatures, and this was the case using G. virens in sterilized soil. However, considerable improvement in biocontrol occurred with increasing temperature levels up to 35 C, where seed treatment with G. virens was better than captan treatment and equal to metalaxyl seed treatment.

In natural soil artificially infested with P. ultimum, the situation was different; Pythium growth apparently was restricted by competition from other soil inhabiting microorganisms, and this probably explains the greater control at low temperature in natural soil. The biocontrols have ready access to the seed coat food base and may have a competitive advantage in such situations.

Some reduction in biocontrol with G. virens as well as with metalaxyl and captan treatments occurred at the high temperature level of 35 C, presumably due to increased susceptibility of alfalfa under

this stressful temperature coupled with the injurious effects of indigenous organisms in the soil.

PH

Pythium ultimum grew well over a wide range of pH levels from 4 to 9 with an optimum pH range of 5 to 7. These results agree with those of Lumsden (78). Rate of growth of P. ultimum also has been measured in the soil (40, 106) with similar findings. In PDA plates where the antagonist was introduced with P. ultimum, obvious zones of inhibition occurred at all pH levels between 4 and 9, regardless of the size of G. virens colonies. These inhibition zones were indicative of antibiotic production over the pH range.

The relationships mentioned above suggested that disease due to P. ultimum would occur at all pH levels from 4 to 9, and also that biocontrol by G. virens might be active through the same pH range if that control was a function of antibiotic production.

T. hamatum, T. harzianum and G. virens grew most rapidly at pH levels between 4 and 5. Above pH 5 growth rate decreased as the pH was increased to pH 9. Optimum pH for growth of Trichoderma spp. as determined by Danielson and Davey (28) was 3.7 to 4.7. They (28) also found that T. hamatum and T. harzianum growth at pH 6.2 was less than 10% of the maximum. The relatively fast growth of P. ultimum and slow growth of the biocontrols suggested that biocontrol would be less effective above pH 5.

From a practical point of view we are interested in soil pH range 6.5 to 7.5 — the optimum for alfalfa growth and a level common

to South Dakota soils. P. ultimum has grown well over a wide range of acidic and alkaline soils (105, 40), but the actual soil reaction that influences the most severe damping-off is controversial. Lumsden (78) reported that alkaline soils favored the disease but Buchholtz (16) reported the acid soils favored the disease. Other studies (6, 27, 43, 116) with reference to the production and activity of pectolytic enzymes, had shown that the greatest enzyme activity occurred in alkaline solution. Pectolytic enzymes are fundamental to the mechanism of damping-off.

My data in steamed soil, and in natural soil infested with P. ultimum, confirmed my prediction that alfalfa damping-off would be severe in both alkaline and acidic soils. Under these conditions, biocontrol with G. virens against alfalfa damping-off as predicted from the observation of inhibition zones, was effective at all soil pH levels from 5 to 8. At pH 5 alfalfa growth was poor and this affected the effectiveness of the treatment.

Trichoderma spp. were very much favored by the acid conditions of the soil and seed treatment with T. hamatum and T. harzianum improved alfalfa emergence at low pH values of 5 and 6 more than in alkaline soils. These results agreed with the other observations that Trichoderma and Gliocladium were strongly favored by acid conditions (109, 125), that suppressiveness with Trichoderma spp. was generated more rapidly in acid soils (75) and that T. harzianum attacked many fungal hosts under acid soil conditions (10, 30).

MOISTURE

In steamed soil, the severity of disease on alfalfa increased with increasing moisture up to saturation. This agreed with most of the literature reports (33, 46, 60, 85, 78). Other reports have indicated that Pythium spp. were capable of germination, vegetative growth and colonization of living or dead substrate over the entire range of soil moisture available to higher plants (approximately 0 to -15 bars) (41, 67, 85, 115, 116). The degree to which these phenomena occurred however, was generally reduced at soil matric potentials lower than -.3 bars. High water potential and accompanying poor aeration conditions indirectly favored Pythium spp. by: (i) decreasing host vigor and increasing host exudation, and (ii) providing a suitable environment for the rapid diffusion and subsequent increased availability of host exudates necessary for spore germination and vegetative growth of the fungus (14, 41, 116).

In steamed soil at high soil moisture levels of 30% to 35% (-.03 bar to -.01 bar), biocontrol with G. virens was not very effective; however, considerable improvement in biocontrol occurred as moisture was decreased to 15% (-15 bars). The best control was obtained at 20% moisture (-.35 bars). Although the effect of soil moisture on G. virens is not known, it appears the effect of moisture on disease control can be explained directly -- P. ultimum is more difficult to control in wet soil. This conclusion was supported by the finding that Trichoderma hamatum and T. harzianum performed better at low moisture levels than at high moistures in spite of the fact that

Trichoderma spp. have a reputation of being highly active at high moistures and of producing high numbers of propagules in moist soil (13, 23, 59, 69, 71, 93, 101, 102).

The effective biocontrol achieved with G. virens against alfalfa damping-off at low and relatively high moisture levels (approximately -15 bars to -.03 bars) in natural soils supports the hypothesis that disease control is easier in natural soils; thus when P. ultimum had to compete with other organisms it was an easier target for biocontrol with G. virens, than in steamed soils. Captan has been shown to decompose rapidly in moist environments (17) and it was surprising that it provided good protection at high moistures.

OTHER VARIABLES

G. virens conidial concentration

According to Baker and Cook, (10) fungi used for biocontrol as seed treatment had greater potential if they were selected on the basis of antibiotic production. One advantage of antibiosis over the other forms of antagonism is that toxic substances diffuse through water films and into water filled pores, thus actual physical contact between antagonist and target organism is not necessary. The distance (10) between the interacting organisms therefore can be greater and activity may occur more rapidly and more effectively than with competitors or hyperparasites.

Not only does G. virens produce the toxin gliovirin (55) which is active against P. ultimum, but also it has other desirable attributes, such as production of resistant chlamydospores that allow survival

during the prolonged dry storage that treated seed would encounter. Furthermore G. virens usually grows rapidly and sporulates profusely. In the progress of my studies I observed G. virens sporulating on the treated alfalfa seed coat within 2 days after planting. Thus it appeared to be an antagonist with many desirable characteristics.

In my results, G. virens conidial concentration of 10^7 or 10^9 protected alfalfa against low and high levels of P. ultimum respectively in steamed soil artificially infested with P. ultimum and in natural soil similarly infested. The results showed that seed treatment with antibiotic producing antagonists have great potential as mentioned by Baker and Cook (10).

Level of inoculum of P. ultimum

Although the natural population of P. ultimum in agricultural clay loam may be as high as 3,800 propagules/gm of soil (117), only low levels are necessary to cause disease. Unlike most of Pythium spp. that produce many zoospores to facilitate infection, P. ultimum has germinated only by production of germ tubes, nevertheless P. ultimum was so aggressive that as few as 5 propagules/gm of soil were sufficient to cause disease (108). Severe disease has occurred with increased level of inoculum (130).

In my study, in steamed soil artificially infested with up to 5000 propagules of P. ultimum/gm of soil, seed treatment with G. virens provided stands that were better than captan and slightly less than metalaxyl seed treatment. This showed how G. virens applied as seed treatment was effective against alfalfa damping-off even under the

heaviest levels of inoculum, and how reliable it would be under normal levels of P. ultimum.

Howell (55) found that the gliovirin produced by G. virens actually reacted with P. ultimum mycelium; thus toxin was necessary for control. Heavy doses of the toxin were required to inactivate large amounts of mycelium.

Amendments

Chitin (N-acetyl-glucosamine polymer) has been an effective amendment for control of soil borne pathogens in several studies (18, 38, 51, 57, 65, 87, 89). Reduction of disease by chitin has been correlated with a decrease in soil population of the pathogen (18, 51, 64), possibly due to antifungal compounds produced in chitin amended soils (107, 112). However with pythiaceous fungi, addition of unbleached chitin compared to bleached chitin has increased disease (38). Increase of disease by unbleached chitin was assumed to be due to glucose and other nitrogenous compounds which were found in the water soluble extracts of unbleached chitin (38). These compounds, and similar ones in seed exudates, stimulated rapid germination of P. ultimum sporangia (25, 26, 64). Such compounds were found as impurities in unbleached chitin sold under various designations (38).

Trichoderma hamatum has been shown to parasitize the hyphae of Pythium spp. generally (48, 49). It produced B-1,3 glucanase, chitinase and cellulose enzymes capable of degrading cell walls of Pythium spp. as well as chitin (20, 22). Trichoderma harzianum has not been described as a mycoparasite of Pythium spp nor has G. virens (48, 121).

The latter antagonized P. ultimum by producing the antibiotic gliovirin (53, 55). Still, T. harzianum and G. virens may utilize chitin and thus have a head start on Pythium.

In this study I applied both unbleached chitin as a powder (Technical grade, Sigma Chemical Co., St. Louis, MO 68178), and bleached chitin prepared according to the method of Lingappa and Lockwood (73). Bleached chitin presumably did not have the above-mentioned impurities and hence should not stimulate P. ultimum in the soil. Chitin (bleached or unbleached) was added to the seed rather than the soil along with the biocontrols to provide a food base for the antagonists and to support their continued activity.

My results in steamed soil artificially infested with P. ultimum and in natural soil artificially infested with P. ultimum showed that amendment with bleached chitin did not affect the ability of the antagonists to protect seeds against P. ultimum, while unbleached chitin decreased the biocontrol level. My results with T. hamatum seed treatment amended with unbleached chitin differed from those of Harman et al. (49) who obtained better biocontrol of unidentified Pythium spp. on peas using chitin amended T. hamatum as a seed treatment.

G. virens and P. ultimum apparently did not have the enzyme system capable of degrading and utilizing bleached chitin and in the case of unbleached chitin, only P. ultimum benefitted from it. However, biocontrol with G. virens alone was so good that if chitin had been beneficial, it would not have been evident.

The use of G. virens as seed treatment for the first time allows its use in relatively small quantitites. In addition, seed treatment seems to be an efficient way to use G. virens, since the toxin theoretically could be produced from energy derived from the seed coat. In that location the toxin would be near the infection courts until the cotyledons were lifted above the soil line. If gliovirin was systemic in the plant, protection could persist for some time after emergence. The fact that the activity of G. virens was retained after planting in steamed soil and in natural soils artificially infested with high levels of P. ultimum makes it an excellent candidate for consideration as a commercial biocontrol agent.

In the light of the facts that G. virens as seed treatment was effective against alfalfa damping-off in alkaline soil, relatively dry, and cold soils that were heavily infested with P. ultimum propagules, we can see how appropriate and practical it would be to apply such control to alfalfa production in South Dakota and that it would be possible to utilize this biocontrol under existing conditions.

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APPENDIX

Table A1. Analysis of variance of effect of temperature on rate of growth of fungi data summerized in Table 2.

Sources of variation	d.f.	MS
Replication	3	0.024$^{n.s.}$
Treatment	3	1063.9160**
Temperature	4	911.5204**
Temperature x Treatment	12	96.1238*
Error	57	.0145

Table A2. Analysis of variance of effect of temperature on sporulation of fungi data summerized in Table 3.

Source of variation	d.f.	MS
Replication	3	.01716$^{n.s.}$
Treatment	2	66.7454**
Temperature	4	70.0356**
Temperature x Treatment	8	6.00903**
Error	42	.0085

Table A3. Analysis of variance of emergence and establishment steamed soil data summarized in Tables 4 and 5.

Sources of variation	d.f.	MS	
		Emergence	Stand establishment
Replication	3	4.0972$^{n.s.}$	2.6083
Treatment	5	1972.6683**	2028.1882**
Temperature	4	955.2833**	919.9875**
Temperature x Treatment	20	150.3683**	147.4925**
Error	87	12.3501	10.4417

Table A4. Analysis of variances of emergence and stand establishment natural soil data summarized in Tables 6 and 7.

Sources of variation	d.f.	MS	
		Emergence	Stand stablishment
Replication	3	5.9889$^{n.s.}$	4.6333$^{n.s.}$
Treatment	5	236.6473**	263.8733**
Temperature	4	342.7917**	271.6792**
Temperature x Treatment	20	47.331666**	47.5442**
Error	87	3.6441	3.8287

Table A5. Analysis of variance of effect of pH on rate of growth of fungi data summarized in Table 8.

Sources of variation	d.f.	MS
Replication	3	.1964$^{n.s.}$
Treatment	3	1611.3829**
Temperature	6	262.4291**
Temperature x Treatment	18	44.7087**
Error	81	.4341

Table A6. Analysis of variance of effect of pH on sporulation of fungi data summarized in Table 9.

Sources of variation	d.f.	MS
Replication	3	0.0004*
Treatment	2	23.286**
Temperature	4	22.3053**
Temperature x Treatment	8	.3588$^{n.s.}$
Error	42	.00164

Table A7. Analysis of variance of emergence and stand establishment in steamed soil data summarized in Tables 10 and 11.

Sources of variation	d.f.	MS	
		Emergence	Stand establishment
Replication	3	13.4853^{ns}	8.6552$^{n.s.}$
Treatment	5	1256.8346**	1521.5857**
pH	3	17.3923$^{n.s.}$	18.1663$^{n.s.}$
pH x Treatment	15	43.2854*	47.4073**
Error	68	23.5668	17.7328

Table A8. Analysis of variances of emergence and stand establishment in natural soils data summarized in Tables 12 and 13.

Sources of variation	d.f.	MS	
		Emergence	Stand establishment
Replication	3	6.3438$^{n.s.}$	2.0000$^{n.s.}$
Treatment	5	1249.1687**	1634.5166*
pH	3	69.1771**	98.5278**
pH x Treatment	15	87.3687**	75.2444**
Error	69	11.8365	9.9565

Table A9. Analysis of variances of emergence and stand establishment in steamed soil data summarized in Tables 14 and 15.

Sources of variation	d.f.	MS	
		Emergence	Stand establishment
Replication	3	40.0306$^{n.s.}$	42.0083$^{n.s.}$
Treatment	5	463.5884**	768.6483**
Temperature	4	1698.7791**	1428.1166**
Temperature x Treatment	20	58.6092**	85.0566**
Error	87	22.1225	16.2037

Table A10. Analysis of variance of emergence and stand establishment in natural soil data summarized in Tables 16 and 17.

Sources of variation	d.f.	MS	
		Emergence	Stand establishment
Replication	3	15.8555$^{n.s.}$	16.8333$^{n.s.}$
Treatment	5	1885.5733**	2390.780**
Temperature	4	324.6958**	164.0708**
Temperature x Treatment	20	45.5608**	55.6508**
Error	87	7.6659	8.9425

Table 11. Analysis of variance of emergence and stand establishment in steamed soil data summarized in Tables 18 and 19.

Sources of variation	d.f.	MS	
		Emergence	Stand establishment
Replication	3	4.8715$^{n.s.}$	.6493$^{n.s.}$
Treatment	2	265.5417**	115.1354**
Temperature	7	971.7099**	1382.2366**
Temperature x Treatment	14	10.3869$^{n.s.}$	5.5018**
Error	69	6.5889	1.6854

Table 12. Analysis of variance of emergence and stand establishment in natural soil data summarized in Tables 20 and 21.

Sources of variation	d.f.	MS	
		Emergence	Stand establishment
Replication	3	5.3411$^{n.s.}$	3.0495$^{n.s.}$
Treatment	3	45.9661**	24.1536**
Temperature	7	1298.6595**	1737.2131**
Temperature x Treatment	21	1.8411$^{n.s.}$	2.4453$^{n.s.}$
Error	93	5.5992	2.5065

Table A13. Analysis of variance of emergence and stand establishment in steamed soil data summarized in Tables 22 and 23.

Sources of variation	d.f.	MS	
		Emergence	Stand establishment
Replication	3	28.3222$^{n.s.}$	14.3861$^{n.s.}$
Treatment	5	1147.760**	1606.6283**
Temperature	4	586.200**	786.00**
Temperature x Treatment	20	87.535**	112.945**
Error	87	12.8279	8.9723

Table A14. Analysis of variance of emergence and stand establishment in steamed soil data summarized in Table 24.

Sources of variation	d.f.	MS	
		Emergence	Stand establishment
Replication	3	.96528$^{n.s.}$	.5764$^{n.s.}$
Treatment	11	436.5663**	551.6572**
Error	33	1.1471	1.0158

Table A15. Analysis of variance of emergence and stand establishment in natural soil data summarized in Table 25.

Sources of variation	d.f.	MS	
		Emergence	Stand establishment
Replication	3	.4422$^{n.s.}$	.3542$^{n.s.}$
Treatment	11	468.3106**	513.748**
Error	33	1.4268	.6269

Table A16. Analysis of variance of stand establishment at Highmore, 1983, data summarized in Table 26.

Sources of variation	d.f.	MS
		Stand establishment
Replication	3	87.2639$^{n.s.}$
Treatment	5	1264.4416**
Error	15	248.9306

Table A17. Analysis of variance of emergence and stand establishment Brookings, 1983 data summarized in Table 27.

Sources of variation	d.f.	MS	
		Emergence	Stand establishment
Replication	3	24.8788$^{n.s.}$	17.5152$^{n.s.}$
Treatment	10	366.9545	303.7545**
Error	30	27.3788	18.9485

Table A18. Analysis of variance of emergence and stand establishment in Brookings, 1983, data summarized in Table 28.

Sources of variation	d.f.	MS	
		Emergence	Stand establishment
Replication	3	15.8409$^{n.s.}$	21.5379$^{n.s.}$
Treatment	10	810.4045	796.3182**
Error	30	12.9742	8.0212