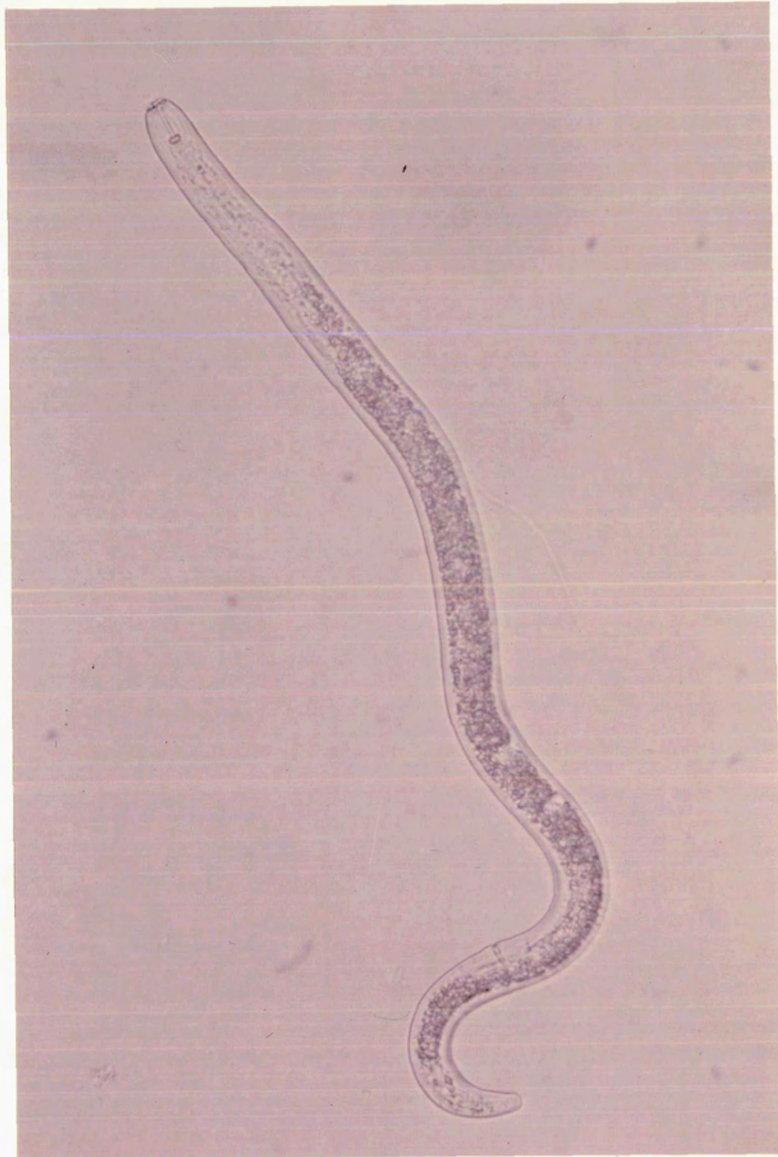
Frontispiece: A female *Pratylenchus penetrans*



THE NEMATODE-FUNGAL INTERACTION

OF PRATYLENCHUS PENETRANS AND

FUSARIUM SPECIES ON ALFALFA

by

Brian Eric Mauza

B.Sc.Ag., University of Alberta, 1972

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in the Department

of Biological Sciences

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Simon Fraser University

July 1976

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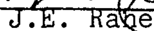

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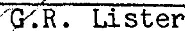

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Abstract

The effects on the growth of alfalfa, *Medicago sativa*, 'Vernal' were compared when seedlings were inoculated with various combinations of either *Pratylenchus penetrans* and *Fusarium solani* or *P. penetrans* and *F. oxysporum* f.sp. *medicaginis*. A synergistic disease interaction occurred when *F. oxysporum* was inoculated one week before or simultaneously with *P. penetrans*. Greater reductions in growth occurred with increasing inoculum levels of *P. penetrans* and *F. oxysporum*, but not with *F. solani*. Seedlings inoculated with the nematode alone consistently gave poorer yields of tops and roots than when inoculated with either *Fusarium* species. *P. penetrans* reduced the root weight of alfalfa more than it did the weight of tops. *F. oxysporum* appeared to be more pathogenic to alfalfa than did *F. solani* under similar soil conditions, although in a sterile sand culture *F. solani* caused death of 20% of the seedlings. *F. oxysporum* did not alter the populations of *P. penetrans* in alfalfa roots, whereas *F. solani* when inoculated onto alfalfa 1 week prior to or simultaneously with *P. penetrans* caused a significant decrease in the number of *P. penetrans* in the roots. The possible roles of the nematode and fungus in this plant disease complex are discussed.

Acknowledgements

I am sincerely greatful to the following people for their inspiration and guidance. To Dr. E.J. Hawn who stimulated my interest in nematology; to my senior supervisor Dr. J.M. Webster for his support and guidance throughout; to Dr. J.E. Rahe who along with Dr. F.D. McElroy made useful suggestions during the study and during the revision of the manuscript; to J.A. McLean for his help with the statistical analysis; to Maija Siekkinen for her assistance in the greenhouse; to Marilyn Chevaldayoff for her patience and direction during the preparation of the manuscript; to Ron Long for his excellent photography throughout the entire study; and to the entire Department of Biological Sciences for providing the facilities.

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INTRODUCTION

The nematode genus *Pratylenchus* has both a wide geographic distribution and a broad host range. *P. penetrans*, one of the more pathogenic species of this genus, is recorded on over 350 hosts in tropical and temperate areas. Alfalfa, an important forage crop in Canada, is one of the hosts for this nematode. The effect of *P. penetrans* on the growth of red clover and alfalfa in the United States was reported by Chapman (1958) who found that this nematode reduces yields of alfalfa grown in the greenhouse by as much as 40%. Since then Willis and Thompson (1969) have shown that *P. penetrans* is responsible for reduction in yields of alfalfa in the Maritime provinces of Canada. In other areas of Canada *P. penetrans* causes serious damage to other crops, but its effect on alfalfa in these areas has never been determined.

Fusarium species are frequently isolated from alfalfa that have root rots or wilts and predominate in alfalfa roots suffering from winter injury, but often the symptoms cannot be reproduced by inoculating healthy plants with the isolates (Weimer, 1927). When conditions are made extremely favorable for these fungi, damping-off of seedlings can be produced with most of the *Fusarium* species isolated. From this Weimer (1927) concluded that these *Fusarium* species are mainly saprophytes. Cormack (1937) described root rot symptoms on alfalfa roots caused by five *Fusarium* species from field soils in Alberta. He found that temperature influences the activity of these *Fusarium* species and that the damage is particularly severe

after winter dormancy. *F. roseum* is one of the fungal pathogens contributing to crown bud rot of alfalfa in southern Alberta (Hawn and Cormack, 1952), particularly in early spring. Willis (1965) reported *Fusarium* species to be the most prevalent fungi associated with root rots of red clover in the Maritimes. This prevalence of *Fusarium* in root rots of legumes attacked by plant parasitic nematodes leads one to speculate that nematode-fungus interactions may be increasing the destruction of forage legumes.

Nematode-fungus associations in plant disease complexes have been reported to occur in a range of agricultural crops. Hastings and Bosher (1938) were the first to report such an association in Canada, where the reduction in growth of potatoes, carrots, red clover, tomatoes, spinach and violet seedlings by *Cylindrocarpon radicicola* and *P. pratensis* as a mixed culture was usually greater than the sum of the reductions caused by either pathogen alone. In the interaction of *Rhizoctonia solani* and *P. minyus* on winter wheat (Benedict and Mountain, 1956) a close association was demonstrated between the number of nematodes and the amount of fungus. The effects of both pathogens are necessary to produce full expression of the disease, and a growth increase of the wheat results from a decrease in either pathogen. Mountain and Patrick (1959) while studying the peach replant problem in Ontario, showed that the activities of *P. penetrans* cause the production of phytotoxic substances through hydrolysis of

the cyanogenic glycoside, amygdalin, which is involved in the formation of lesions on the roots. Necrosis of the root tissue within the lesion occurs rapidly and appears to take place in advance of the invading nematode. The main role of *P. pratensis* in this peach replant failure is to incite root degeneration by providing lesioned areas that are easily infected by soil microorganisms. A *Pratylenchus* infection of any root usually results in secondary invasion by other organisms. The effects of these secondary pathogens on the plant host vary considerably with the species of host, the pathogens involved, and their effects on each other.

P. penetrans is an obligate plant parasite which feeds mainly in the root cortex. In the host, cells in the cortex are broken and cavities formed. It reproduces sexually with the female laying eggs singly in the roots or soil. A life cycle is completed in 30 to 86 days depending on the temperature. *Fusarium* is a common soil fungus which survives in the soil as a resting stage, the chlamydospore. These chlamydospores are stimulated to germinate and produce hyphae by the presence of a nutrient source. If the nutrient source happens to be a plant, the hyphae may penetrate the plant and parasitize it or they may live saprophytically on sloughed off plant debris. In culture media *Fusarium* species produce abundant asexual conidia which can be used to establish a population in plants or soil.

Since *Fusarium* species are frequently isolated from the roots of alfalfa (Weimer, 1927; Cormack, 1937; Willis, 1965) and since *P. penetrans* has been reported to be pathogenic to alfalfa (Chapman, 1958; Willis and Thompson, 1969), I decided to study two interactions on alfalfa: *P. penetrans* and *F. oxysporum* f.sp. *medicaginis* and *P. penetrans* and *F. solani*. *F. oxysporum* is host specific, causing *Fusarium* wilt of alfalfa, whereas *F. solani* is a common root rot parasite with a broad host range.

GENERAL MATERIALS AND METHODS

A. Source and Maintenance of Stock Materials

1. Alfalfa Plants

The host plant used throughout the project was alfalfa, *Medicago sativa* L. 'Vernal', obtained from Buckerfield's Seed Company, Abbotsford, B.C. Alfalfa plants were maintained in a greenhouse at Simon Fraser University, Burnaby, B.C., at 18-25 C with Gro-lux lights supplementing the photoperiod to 14 hours. A 3:1:2 mixture of loam:sand:peat was used as the potting medium. Prior to use the mixed soil was moistened and then heated (80 °C for 30 minutes) in an electric soil pasteurizer. Every 3-4 weeks during the course of an experiment a commercial 20-20-20 water soluble fertilizer was added to each pot. Two-spotted spider mites were a problem in the compartment used and populations were controlled on non-experimental plants only, by treatment with Pentac WP Miticide (Hooker Chemical Corporation).

2. *Pratylenchus penetrans*

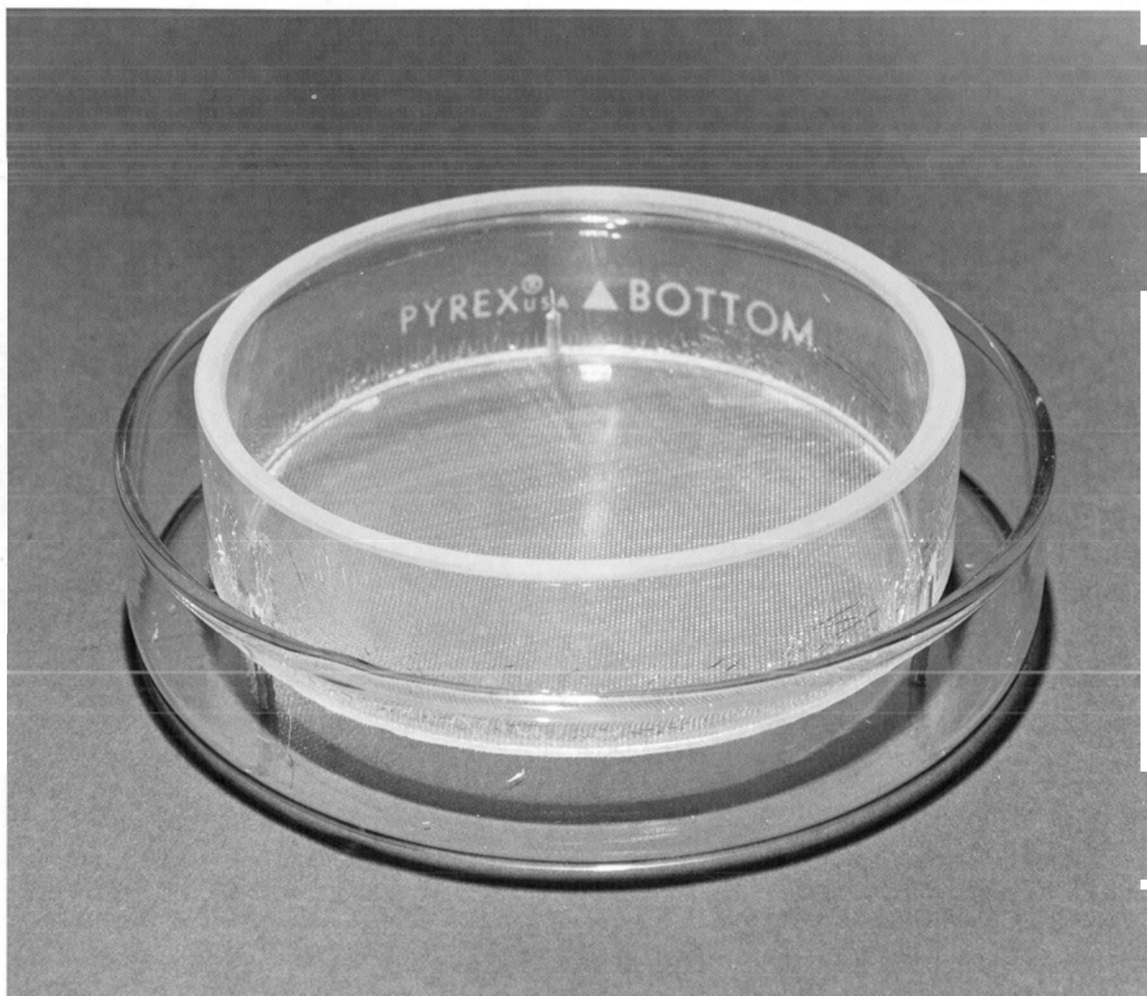
Stocks of *P. penetrans* (Cobb, 1917) Chitwood and Oteifa, 1952, were obtained from two sources. Dr. J.L. Townshend of Canada Agriculture, Vineland Station, Ontario, supplied axenic cultures reared on alfalfa callus tissue and Dr. F.D. McElroy of Canada Agriculture Research Station, Vancouver, B.C., supplied a monoculture of *P. penetrans* in red clover, hairy vetch, and in assorted grass species grown in tubs of soil.

P. penetrans were reared in Trifolium pratense because this plant tolerated and yielded high populations of this nematode. These stock plants were grown in 10 cm diameter pots and in 50 X 60 cm tubs of soil under the same soil and greenhouse conditions used for experimental plants. When nematodes were required, stock plants were removed from their containers and as much soil as possible shaken from the roots, which were then washed gently in tap water to remove adhering soil. Roots were cut into 1-2 cm lengths and the nematodes extracted from them using either a Seinhorst mistifier or a Burrell wrist action shaker. The wrist action shaker was preferred because it used water at room temperature rather than cold water from the tap to extract the nematodes. The warmer water enabled extraction of the majority of nematodes in 4 days, whereas the cold water extraction in the Seinhorst mistifier required 7-14 days for equivalent nematode recovery. Also, the volume of water in which the extracted nematodes were collected was much less with the wrist action shaker than with the mistifier.

The resulting nematode suspension was concentrated using a sieve with 37-micron apertures. The concentrated nematode suspension was poured onto a disc of Whatman No.1 filter paper which was placed over the fine nylon mesh of a perspex sieve standing on three legs in a Petri dish of tap water (see Fig.1). Active P. penetrans migrated through the filter paper and mesh within 24 hours resulting in a

Figure 1. Perspex sieve apparatus used to clean aqueous suspensions of *Pratylenchus penetrans* that had been extracted from alfalfa roots.

9b.



debris-free suspension of active, viable nematodes suitable for experimental use. The soil that had been shaken from the mature plant roots was seeded again to red clover. In this way the initial stock of a single population of P. penetrans was maintained and increased continuously.

3. Fusarium species

Two isolates of F. solani (Mart.) Sacc. were obtained from Dr. C.B. Willis of Canada Agriculture Research Station, Charlottetown, Prince Edward Island. A culture of F. oxysporum f.sp. medicaginis (Weimer) Snyder and Hansen was obtained from the American Type Culture Collection. All fungal stocks were maintained and subcultured on potato dextrose agar slants. Both Fusarium species were maintained at room temperature (approximately 22°C) in 2 litre jars of 10% corn meal soil (Hawn, 1958).

Conidial inoculum of both Fusarium species was produced by inoculating 200 ml of sterile Tochinai broth in 500 ml flasks which were incubated on a rotary shaker at room temperature for 5-7 days. The contents of the flasks were then filtered through gauze, the filtrate centrifuged, decanted and the remaining propagules washed with two changes of deionized water. Suspensions of washed propagules were used as inoculum in the experiments.

B. Inoculation Procedures

1. Pratylenchus penetrans

After extraction from the roots the mean number of *P. penetrans* in a known volume of water was determined from the number of nematodes in three aliquots counted in a Doncaster counting dish. The suspension was then diluted with tap water to achieve the desired concentration of nematodes for a particular experiment. Two holes 3 cm deep were punched with a pencil near the alfalfa seedlings and an aqueous suspension of adult and larval nematodes was pipetted into each hole which was then filled with soil.

2. *Fusarium* species

(a) Inoculation prior to seeding

The number of *Fusarium* propagules contained in a known quantity of the infested 10% corn meal soil was estimated by plating a dilution series on PCNB agar (Nash and Snyder, 1962) and counting the subsequent colonies. The inoculum consisted of an amount of infested soil which gave the required initial density of *Fusarium* propagules. This infested soil was mixed with the pasteurized potting soil, potted and seeded with alfalfa.

(b) Inoculation after seeding

For inoculating 'Vernal' seedlings, the Fusarium inoculum consisted of propagules which were obtained from Tochinai broth. The number of propagules present in the suspension was estimated using a haemocytometer. A volume containing the required number of propagules of Fusarium was pipetted into two 3 cm deep holes punched in the soil near the seedlings with a pencil, and then filled with soil. Attempts were made to produce pure cultures of Fusarium chlamydospores in liquid media for inoculation purposes, following the procedures of Qureshi and Page (1970), but suspensions free of conidia were never obtained. Therefore the inoculum contained chlamydospores, micro and macro conidia and hyphal fragments.

C. Observations during Growth

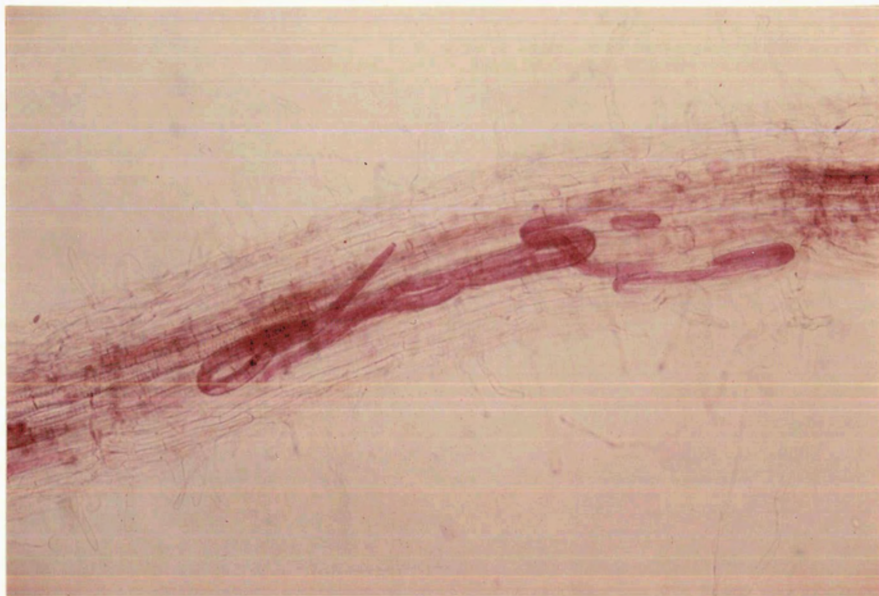
A nondestructive method was used to quantify the effect of fungal and nematode inoculations on alfalfa. The height of each seedling was measured from the cotyledons to the end of the petiole at the base of the youngest open trifoliate leaf. The number of trifoliate leaves on the plant at this time was recorded. The product of the number of leaves and the height of the plant was used as the growth index.

Upon termination of the experiments the fresh weight of tops, dry weight (105 C for 24 hours) of tops, fresh weight of roots (blotted dry with paper towels), number of nematodes in the roots, and number of *Fusarium* propagules in the roots and soil was recorded.

The size of the root system determined the method of counting *P. penetrans* in the roots. Very small root systems which could be spread on a 5 X 8 cm glass slide were stained in a hot 0.1% cotton blue in lactophenol mixture for 3 minutes, cleared in fresh, hot lactophenol and mounted in lactophenol under a coverglass (Southey, 1970). The clearing process took from 3 hours to 3 days and was speeded by autoclaving the roots in the lactophenol for 10 minutes. The number of *P. penetrans* in the roots was counted under the compound microscope. Fig.2 shows an example of *P. penetrans* that have infected a small portion of an alfalfa root. When root systems were too large to be examined by the method described above, a weighed sample of roots was cut into 1-2 cm lengths, placed in a flask of tap water and the nematodes extracted by shaking the flask for 4 days at room temperature on a Burrell wrist action shaker. The nematodes in the resulting suspension were counted in a Doncaster counting dish under a compound microscope.

The number of *Fusarium* propagules in the roots was determined by the PCNB agar technique (Nash and Snyder, 1962). Larger roots were

Figure 2. Young alfalfa root infected with _Pratylenchus penetrans_, showing the brown necrotic lesions. Root was stained with 0.1% acid fuchsin in lactophenol. 80X mag.



surface sterilized by dipping in 70% ethanol followed by 30 seconds in Javex bleach (active ingredient 6% sodium hypochlorite) and then washed in running tap water for one minute. Finer roots were given only a momentary dip in 70% ethanol followed by a water wash. All roots were homogenized in a Waring Blendor in a 0.1% agar solution and samples from a dilution series were plated on PCNB agar. The colonies of *Fusarium* were compared with stock cultures of the same age and were counted 5-7 days after plating (Fig.3).

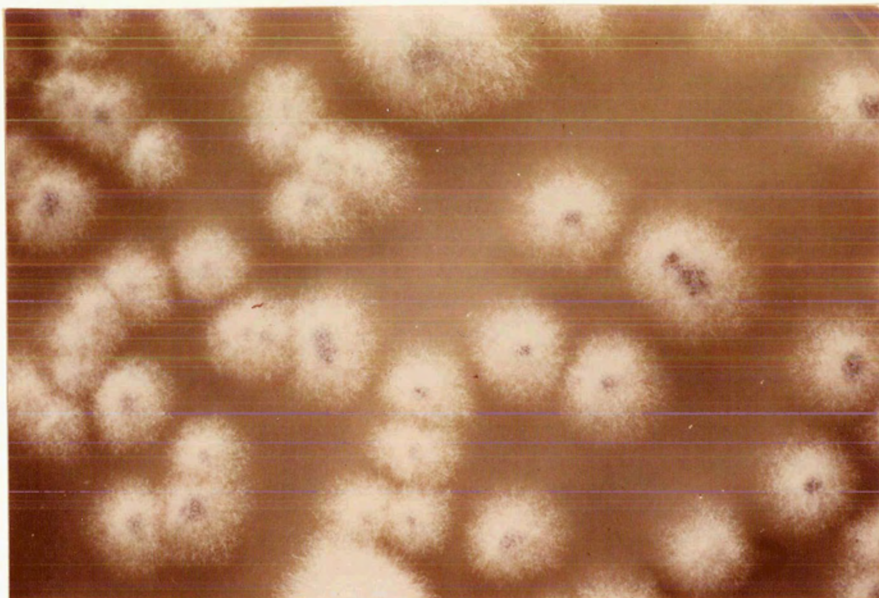
D. Statistical Analysis

All data were analyzed by an analysis of variance and treatments ranked using a Studentized range test or according to Fisher's modified least significant difference (LSD). All tests were carried out for a probability of $P<0.05$ unless otherwise stated.

Figure 3. Examples of *Fusarium* colonies on PCNB agar 7 days after plating.

Top. Colonies of *Fusarium solani*. 10X mag.

Bottom. Colonies of *Fusarium oxysporum* f.sp. *medicaginis*. 10X mag.



EXPERIMENTS AND RESULTS

A. The effect on alfalfa of *Pratylenchus penetrans* and *Fusarium oxysporum* f.sp. *medicaginis*.

1. The inoculum densities of *P. penetrans* and *F. oxysporum* f.sp. *medicaginis* inoculated simultaneously into soil containing alfalfa.

Four inoculum levels of *P. penetrans* 0, 200, 400, and 800 nematodes (designated N1, N2, N3, and N4 respectively), and four levels of *F. oxysporum* 0, $5X10^5$, $5X10^6$, and $5X10^7$ spores (designated F1, F2, F3, and F4 respectively), were combined in sixteen treatment combinations each of which was replicated five times. Each replicate was a 7.5 cm diameter pot of soil containing two alfalfa seedlings. Plants were grown for 85 days from January to April, 1975. Both pathogens were inoculated as aqueous suspensions into the soil when the plants were 15 days old. Growth indices were recorded weekly for each seedling. These measurements were discontinued after 54 days of growth because the plants began to branch, drop older leaves, and produce leaves of varying sizes, thereby contributing inconsistent bases for the growth indices. At termination of the experiment the plants were lifted, the weight of tops and roots determined and the final population of nematodes and fungus estimated.

A commonly observed symptom of *F. oxysporum*-infected alfalfa was a reddish discoloration of the lower leaves followed by chlorosis and necrosis. A *P. penetrans* infection caused severe stunting of the plants but little discoloration of the foliage. The presence of both pathogens caused discoloration and stunting. Root systems infested

with *P. penetrans* were reduced in size with many dark brown to black lesions. Older roots of alfalfa infected with *F. oxysporum* appeared only slightly discolored on their surface, but when cut longitudinally a reddish brown discoloration of the vascular system was revealed (Fig.4). The fungus and nematode significantly reduced the fresh weight of tops either alone or in combination, and in proportion to increasing inoculum densities (Table I; Figs.5b and 6b). The reduction in fresh weight of tops, when expressed as a percentage of the uninoculated control (N1F1) was 31% and 28% for treatments N1F4 and N4F1 respectively (Table I). In treatment N4F4 the reduction in top weight was 69%. This reduction is significantly greater than that resulting from the sum of the reductions caused by either pathogen alone in N1F4 or N4F1. Reductions in dry weight of tops (Table I) were similar to the reductions in fresh weight of tops. Where both pathogens were inoculated simultaneously at each of their highest levels (N4F4), the reduction in dry weight of tops was greater than the sum of the reductions caused by either pathogen alone (Table I).

The fresh weight of roots was also decreased significantly and in proportion to increasing inoculum of *P. penetrans* and *F. oxysporum* (Figs.5a and 6a). The effect on fresh weight of roots was significantly greater than on fresh weight of tops. Furthermore, the nematode inocula caused a significantly greater decrease in yield of roots than did the fungal inocula (Figs. 5a and 6a).

An analysis of variance of the growth indices recorded weekly during the first 54 days (Appendix A) indicated that F. oxysporum (at $P<0.1$) and P. penetrans (at $P<0.05$) significantly decreased seedling growth. The combined inoculum in the N4F4 treatment slowed the growth rate of the plants significantly as compared with controls and with the N1F4 treatment (Table II). Growth rates for plants in the N4F1 and N1F4 treatments were not significantly different from each other or from the controls. The size of the inoculum of one pathogen did not significantly affect the final population of the other pathogen (Table II).

Table I: Weight of alfalfa roots and tops, 85 days after seeding, from pots inoculated simultaneously with *Pratylenchus penetrans* (N) and *Fusarium oxysporum* (F). (Mean of 5)

TREATMENT*		TOPS				ROOTS	
		Fresh Weight (gm)	% Reduction	Dry Weight (gm)	% Reduction	Fresh Weight (gm)	% Reduction
N1	F1	1.18	0	0.217	0	1.73	0
	F2	1.00	15.3	0.180	17.1	1.07	38.2
	F3	0.81	31.4	0.158	27.2	0.77	55.5
	F4	0.81	31.4	0.165	24.0	1.08	37.6
N2	F1	0.71	39.8	0.131	39.6	0.86	50.3
	F2	0.93	21.3	0.172	20.7	1.09	58.9
	F3	0.48	59.3	0.198	8.8	1.43	17.4
	F4	0.45	41.9	0.094	56.7	1.04	39.9
N3	F1	0.87	26.3	0.178	18.0	1.28	26.0
	F2	0.96	18.6	0.173	20.3	1.02	41.1
	F3	1.05	11.0	0.160	26.3	1.62	6.4
	F4	0.44	42.7	0.084	41.3	1.07	38.2
N4	F1	0.85	28.0	0.165	24.0	0.90	48.0
	F2	0.40	66.1	0.078	44.1	1.29	25.4
	F3	0.53	55.1	0.104	32.1	1.44	16.8
	F4	0.37	68.6	0.070	67.7	0.62	64.2
for $P<0.05$		LSD=0.33		LSD=0.06		LSD=0.39	

* Number of nematodes N1=0, N2=200, N3=400, N4=800,
Number of fungal propagules F1=0, F2=$5X10^5$, F3=$5X10^6$, F4=$5X10^7$

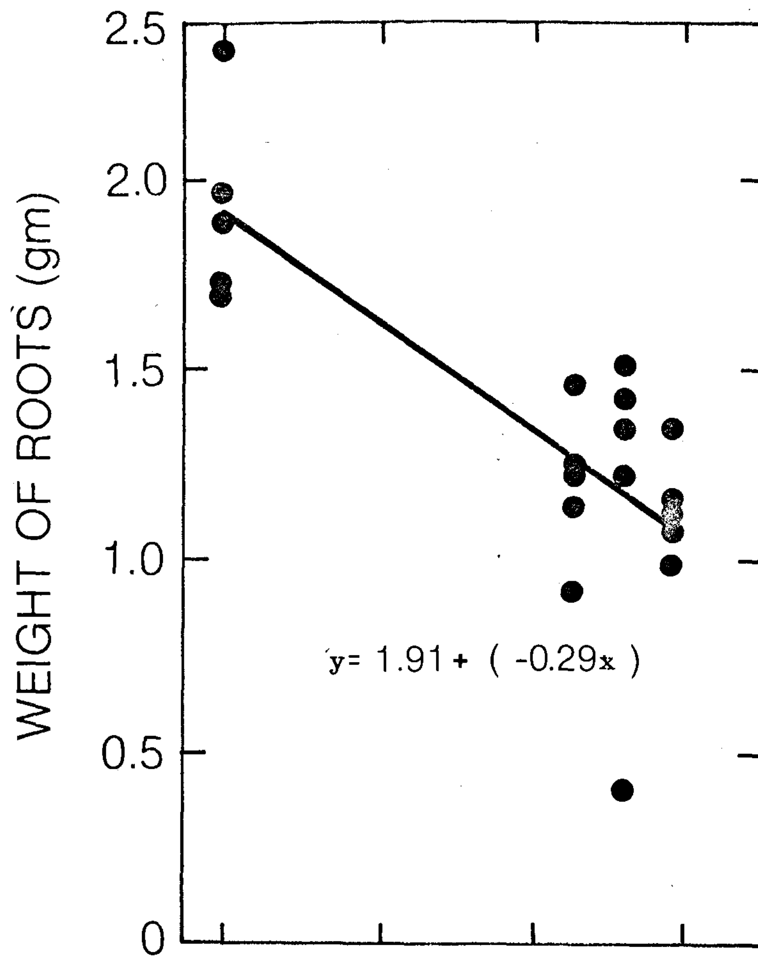
Table II: Effect of initial inoculum levels of Pratylenchus penetrans (N) and Fusarium oxysporum (F) on the growth of alfalfa seedlings and on the final population of nematodes per gram fresh weight of roots and of fungal propagules per gram dry weight of soil, 85 days after seeding.

Treatment*		Growth Rate	Number of P.penetrans (mean of 5)	Propagules of F.oxysporum (mean of 5)
N1	F1	0.045	0	0
	F2	0.043	0	348.5
	F3	0.042	0	772.0
	F4	0.041	0	4098.0
N2	F1	0.040	715.4	37.0
	F2	0.039	469.6	379.0
	F3	0.043	469.8	436.9
	F4	0.034	630.3	4167.0
N3	F1	0.044	1400.4	48.9
	F2	0.039	816.0	378.0
	F3	0.039	1068.4	784.1
	F4	0.035	1998.0	4636.0
N4	F1	0.035	2053.0	32.9
	F2	0.034	2422.0	269.3
	F3	0.039	1950.6	2130.0
	F4	0.033	2002.7	6459.0

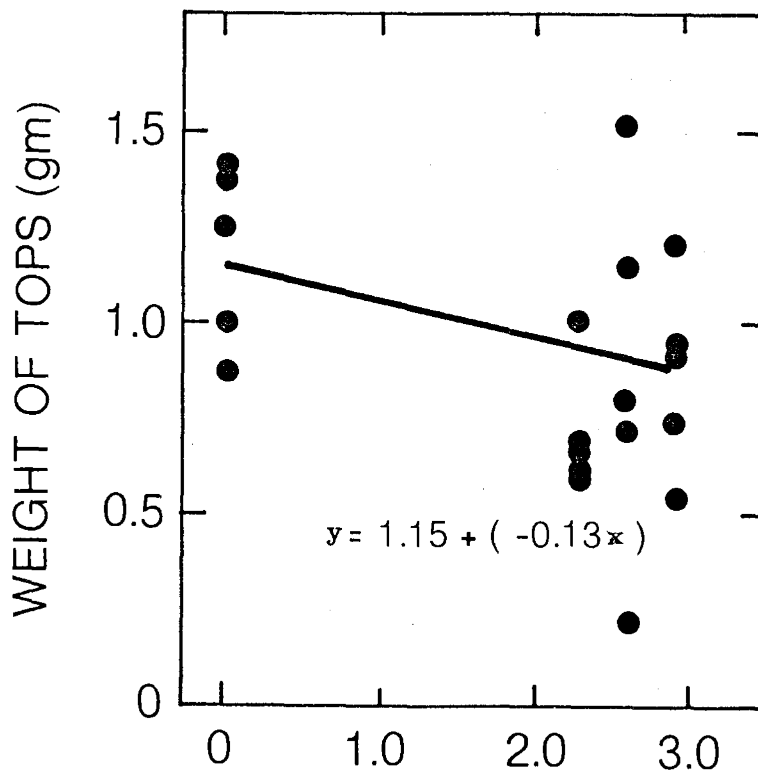
* Number of nematodes N1=0, N2=200, N3=400, N4=800
Number of fungal propagules F1=0, F2=$5X10^5$, F3=$5X10^6$, F4=$5X10^7$

Figure 4. Roots of alfalfa uninfected (left) and infected (right) with *Fusarium oxysporum* f.sp. *medicaginis*. The infected root shows characteristic darkening of the vascular system associated with *Fusarium* wilt.

Figure 5. The regression of fresh weight yields of roots and tops of alfalfa on the *Pratylenchus penetrans* inoculum.

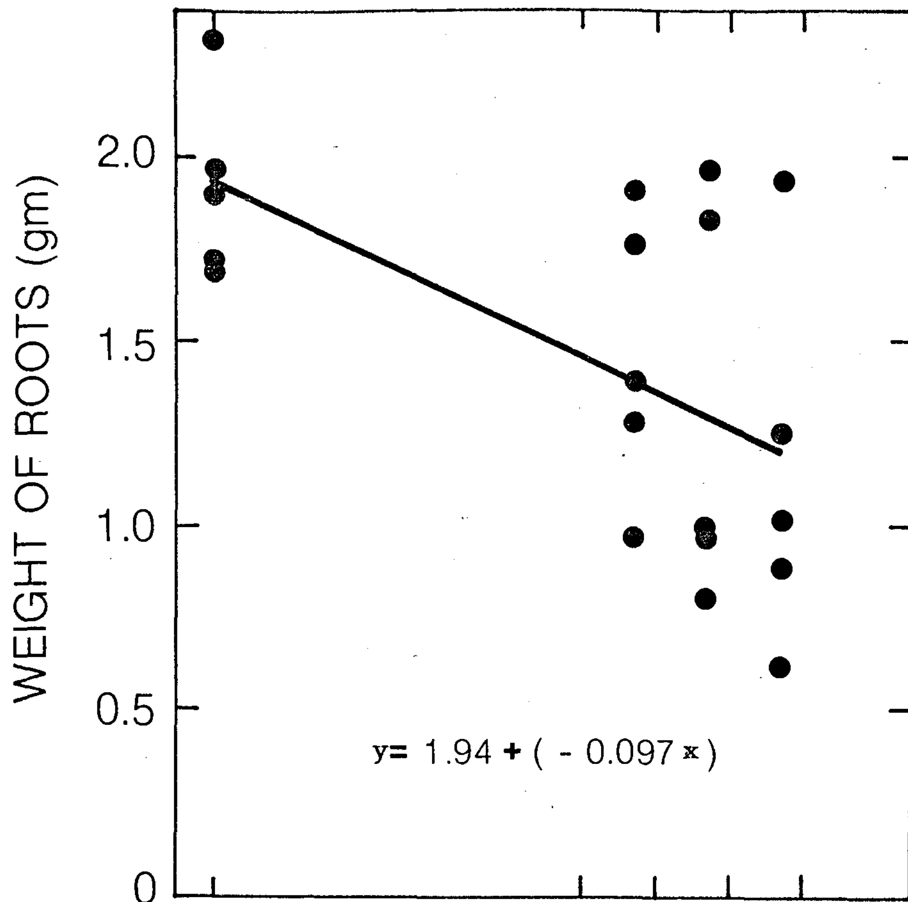


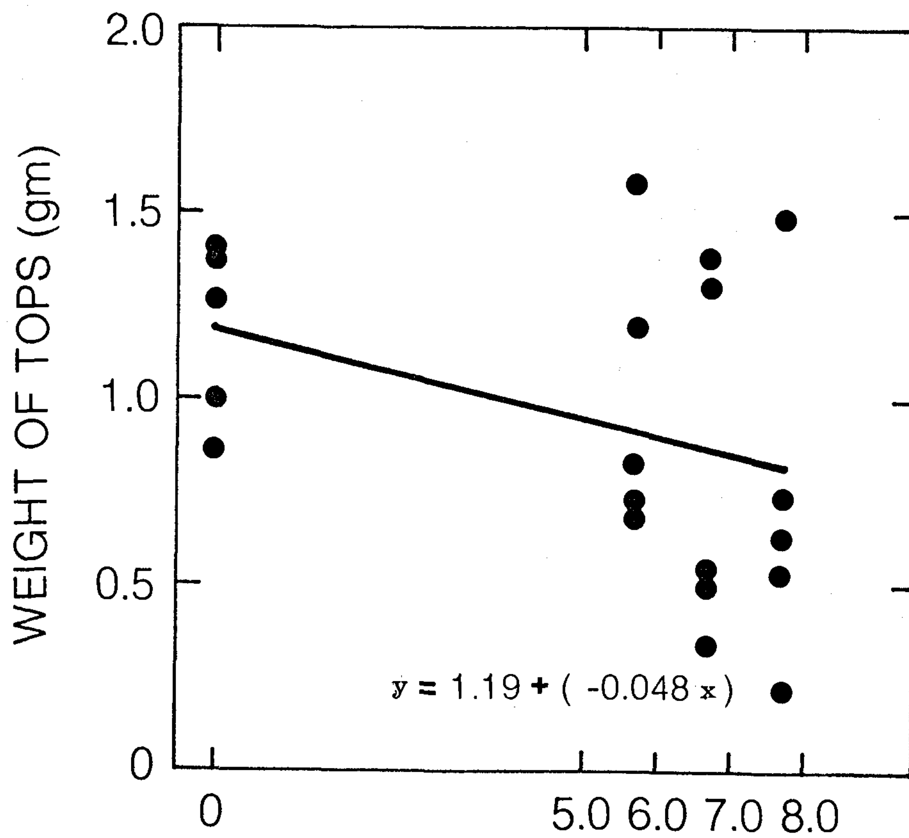
2.5
2.0
1.5
1.0
0.5
0
WEIGHT OF ROOTS (gm)
y= 1.91 + (-0.29x)



1.5
1.0
0.5
0
WEIGHT OF TOPS (gm)
y= 1.15 + (-0.13x)
0
1.0
2.0
3.0
P. penetrans INOCULUM LOG 10 (X+1)

Figure 6. The regression of fresh weight yields of roots and tops of alfalfa on the *Fusarium oxysporum* f.sp. *medicaginis* inoculum.


WEIGHT OF ROOTS (gm)
2.0
1.5
1.0
0.5
0
y= 1.94 + (- 0.097 x)


WEIGHT OF TOPS (gm)
2.0
1.5
1.0
0.5
0
y = 1.19 + (-0.048 x)
0
5.0 6.0 7.0 8.0
F. OXYSPORUM INOCULUM LOG 10 (X+1)

2. Inoculation of alfalfa with P. penetrans prior to F. oxysporum f.sp. medicaginis.

Four treatments were compared, each with ten replicates. Each replicate was a 7.5 cm diameter pot of soil containing two alfalfa seedlings. The four treatments were an uninoculated control, P. penetrans, F. oxysporum, and P. penetrans followed by F. oxysporum. Eleven days after seeding a 4 ml aqueous suspension of P. penetrans containing approximately 1400 nematodes was inoculated into the soil of each pot receiving this treatment. Seven days later 2.25×10^7 propagules of F. oxysporum in 5 ml of water were inoculated into each pot receiving this treatment. Upon termination of the experiment, 80 days after seeding, the plants were lifted, the weight of the tops and roots determined and the final population of the nematodes and fungus estimated.

When compared with controls, each of the inoculated treatments significantly reduced the fresh weight of roots (Table III). P. penetrans caused a significantly greater reduction in root weights than F. oxysporum. Root weights were reduced more by the combined treatments than by F. oxysporum, but not more than by P. penetrans (Table III). F. oxysporum reduced the fresh weight of tops 24% over the controls, but this was not significant at $P<0.05$. P. penetrans significantly reduced the fresh weight of tops by 54%, as compared to controls. The combined treatment reduced the top weight by 66%, more

than either pathogen alone. The percent reduction in dry weight of tops caused by the inoculated treatments was similar to the reduction of fresh weight yields of tops (Table III).

The growth of the plants inoculated with *F. oxysporum* alone began to slow when the plants were about one month old or about 2 weeks after inoculation with the fungus (Table IV). In the combined treatment a decline in growth over and above that caused by *P. penetrans* did not become evident until the plants were 39-46 days old (28-35 days after inoculation). By the time the seedlings were 53 days old each inoculated treatment had a significantly smaller growth index than the uninoculated control. Growth indices were affected significantly only by *P. penetrans*, as indicated by analysis of variance of growth indices obtained throughout the experiment (Table IV), and by regression of growth indices on age of seedlings (Fig.7). The growth rate was not reduced more by the combined treatment than by the *P. penetrans* treatment (Table IV; Fig.7).

The number of *P. penetrans* extracted from the roots of seedlings inoculated with *P. penetrans* and *F. oxysporum* together did not differ from the number extracted from the roots inoculated with the nematode alone (Table V). No significant difference was found in the number of *F. oxysporum* propagules in roots or soil with or without prior *P. penetrans* inoculation.

Table III: Weights of roots and tops, 80 days after seeding, from uninoculated controls and from pots inoculated with *Pratylenchus penetrans* (P) and *Fusarium oxysporum* (F) alone and in sequence. (Mean of 10 replicates)

TREATMENT	TOPS				ROOTS	
	Fresh Weight (gm)	% Reduction	Dry Weight (gm)	% Reduction	Fresh Weight (gm)	% Reduction
Control	0.35	0	0.071	0	0.45	0
P	0.16	54.1	0.035	51.4	0.19	57.1
F	0.27	23.6	0.057	20.4	0.35	23.2
P+F	0.12	66.1	0.026	63.9	0.16	65.8
for P<0.05	LSD=0.12		LSD=0.02		LSD=0.08	

Table IV: Growth indices and growth rates for alfalfa seedlings inoculated with <u>Pratylenchus</u> <u>penetrans</u> (P) and <u>Fusarium</u> <u>oxysporum</u> (F) alone and in sequence. (Mean ± S.E.)

TREATMENT	DAYS AFTER SEEDING						GROWTH RATE
	18	25	32	39	46	53	
CONTROL	3.8±0.2	10.9±0.6	16.9±0.9	21.1±1.5	26.1±1.4	31.7±3.0	0.75 a*
P	2.9±0.4	6.5±1.4	9.3±1.7	11.8±2.0	14.9±2.6	18.7±1.9	0.41 b
F	3.7±0.4	12.1±1.5	17.3±2.6	17.3±3.1	19.6±3.7	21.5±4.1	0.63 a
P+F	2.8±0.3	7.5±0.9	10.7±1.2	13.0±1.6	14.4±1.9	15.6±1.9	0.38 b

* any two treatments with different letter designations are significantly different at $P<0.05$.

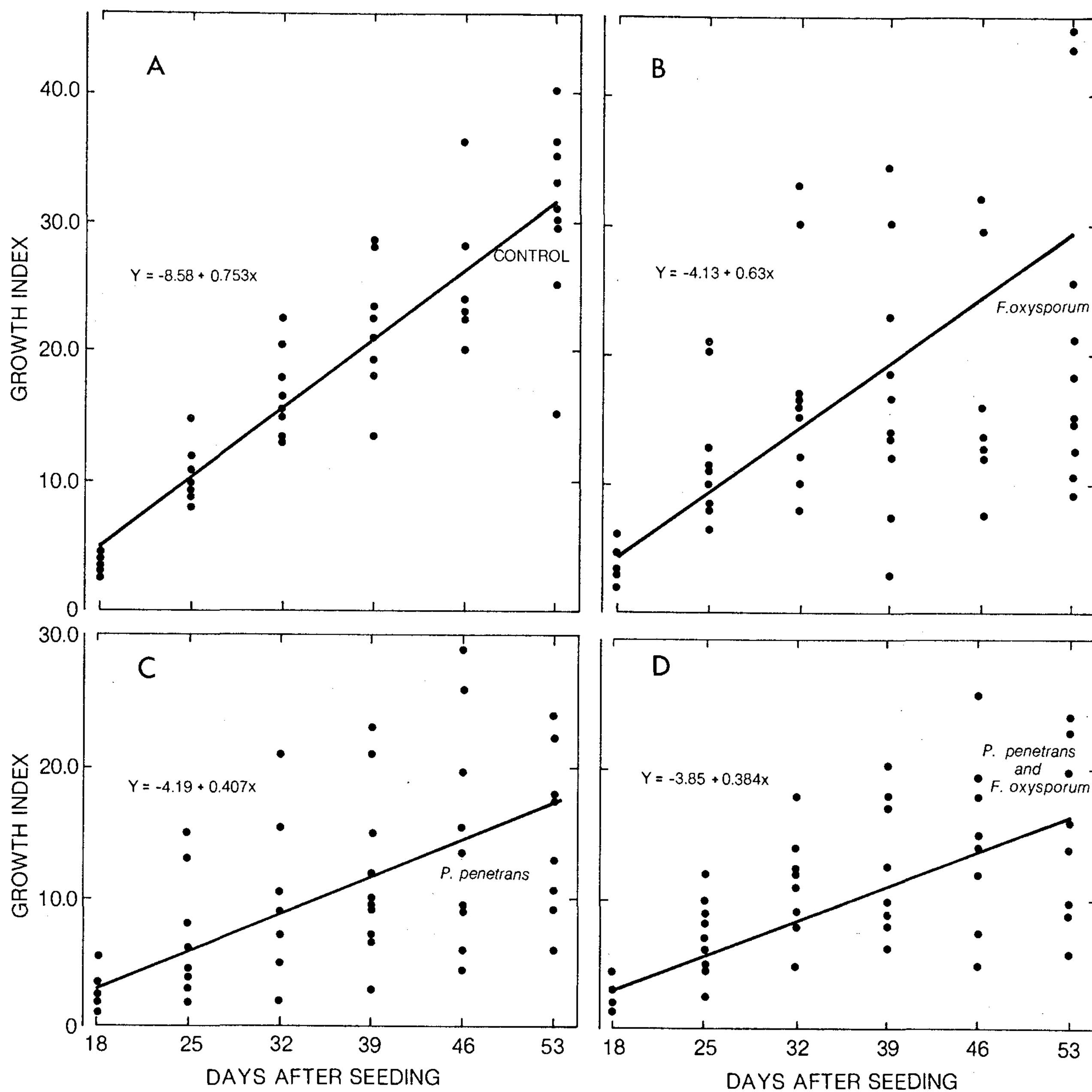
Table V: Number of propagules of *Fusarium oxysporum* recovered per gram fresh weight of root and per gram dry weight of soil and the number of *Pratylenchus penetrans* per gram fresh weight of root after artificial inoculation of alfalfa seedlings. (Mean ± S.E.)

Treatment	Number of *P. penetrans*	Number of *F. oxysporum* Roots	Soil
Control	0	0	0
P.penetrans	1782±326	0	0
F.oxysporum	0	$5.1X10^5 \pm 1.2X10^5$	$1.8X10^4 \pm 1.8X10^2$
P.penetrans prior to *F.oxysporum*	1744±437	$1.2X10^6 \pm 5.6X10^5$	$1.9X10^4 \pm 1.5X10^2$

initial nematode inoculum = 1400 *P. penetrans*
initial fungal inoculum = $2.25X10^7$ propagules

Figure 7. The effect of *Pratylenchus penetrans* and *Fusarium oxysporum* f.sp. *medicaginis* treatments on the growth rate of alfalfa seedlings.

Regression of growth index on days after seeding for seedlings inoculated with (A) Controls, (B) *F. oxysporum*, (C) *P. penetrans*, and (D) *P. penetrans* and *F. oxysporum* combined.



A
B
C
D
GROWTH INDEX
GROWTH INDEX
40.0
30.0
20.0
10.0
0
Y = -8.58 + 0.753x
CONTROL
Y = -4.13 + 0.63x
F.oxysporum
Y = -4.19 + 0.407x
P. penetrans
Y = -3.85 + 0.384x
P. penetrans
and
F. oxysporum
18
25
32
39
46
53
DAYS AFTER SEEDING
DAYS AFTER SEEDING

3. Inoculation of alfalfa with *F. oxysporum* f.sp. *medicaginis* prior to *P. penetrans*.

Four treatments were compared, each with ten replicates. Each replicate consisted of a 7.5 cm diameter pot of soil containing two alfalfa seedlings. The four treatments were an uninoculated control, *P. penetrans*, *F. oxysporum*, and *F. oxysporum* followed by *P. penetrans*. *F. oxysporum* was inoculated by mixing 5 gm of soil which contained 2.5×10^7 propagules, with the soil of each pot to be treated with *Fusarium*. The alfalfa seeds were sown and 18 days later those pots to be treated with nematodes were inoculated with an aqueous suspension containing approximately 1400 *P. penetrans* at various stages of development.

Sixty-eight days after seeding the roots of the plants which were infected with *P. penetrans* weighed significantly less than the roots of uninoculated plants or plants inoculated with *F. oxysporum* alone (Table VI). The plant roots which were inoculated with both the fungus and nematode weighed significantly less ($P<0.1$) than did those of other treatments.

P. penetrans decreased the fresh weight of tops significantly more than controls whereas *F. oxysporum* did not (Table VI). The combined inoculum significantly decreased the fresh weight of tops

more than the sum of the reductions by both P. penetrans and F. oxysporum alone. The percent reduction in dry weight of tops by the inoculated treatments was similar to the reduction in the fresh weight of tops. P. penetrans alone caused a greater percent reduction in the fresh weight of roots than in the fresh weight of tops, but the reductions in root and top weights from the combined treatment were not different (Table VI).

Plant size was significantly reduced by P. penetrans as compared with controls and this was reflected in the mean growth index of the seedlings 52 days after seeding (Table VII). At that time the combined treatment had a significantly lower growth index than all other treatments. The mean growth indices are graphed in Fig.8. During the experiment, death of seedlings occurred only in treatments inoculated with both the fungus and nematode. The growth of alfalfa seedlings infected with F. oxysporum alone was similar to that of control plants (Fig.8). A regression of the treated seedlings on age of seedlings showed that only the treatments containing P. penetrans significantly reduced the growth rate of the alfalfa seedlings over the duration of the experiment (Fig.9 and Table VII). The analysis of variance on the growth of the seedlings during each treatment indicated that both F. oxysporum and P. penetrans contributed significantly to reduce the growth of alfalfa seedlings.

Significantly greater numbers of *F. oxysporum* propagules were found in the roots of plants infected with *P. penetrans* than in the absence of *P. penetrans* (Table VIII). The number of *P. penetrans* extracted from roots infected with *F. oxysporum* was not significantly different from the number extracted when *F. oxysporum* was absent.

Table VI: Weight of roots and tops of alfalfa, 68 days after seeding, from uninoculated controls and from those inoculated with *Pratylenchus penetrans* and *Fusarium oxysporum* alone and in sequence. (Mean of 10 replicates)

TREATMENT	TOPS				ROOTS	
	Fresh Weight (gm)	% Reduction	Dry Weight (gm)	% Reduction	Fresh Weight (gm)	% Reduction
Control	0.17	0	0.042	0	0.35	0
P.penetrans	0.12	30.2	0.026	38.1	0.18	48.6
F.oxysporum	0.19	-10.4	0.045	-7.1	0.39	-9.0
F.oxysporum prior to *P.penetrans*	0.05	70.3	0.013	69.1	0.11	68.8
for P<0.05	LSD=0.05		LSD=0.01		LSD=0.07	

Table VII: The growth indices and growth rates for alfalfa seedlings inoculated with Pratylenchus penetrans (P) and Fusarium oxysporum (F) alone and in sequence. (Mean ± S.E.)

TREATMENT	DAYS AFTER SEEDING					GROWTH RATE
	24	31	38	45	52	
Control	8.4±1.1	16.3±1.2	25.1±1.6	32.9±2.1	42.5±3.3	1.16 a*
P	5.4±0.8	10.5±1.2	14.9±1.0	18.2±1.6	21.9±2.8	0.49 b
F	6.7±1.0	14.5±1.1	23.1±2.0	31.4±2.8	38.1±2.7	1.14 a
F+P	4.4±0.6	8.8±1.2	11.7±1.3	12.2±2.6	12.2±2.7	0.32 b

* any two treatments with different letter designations have significantly different growth rates at $P<0.05$.

Table VIII: The number of *Fusarium oxysporum* propagules per gram fresh weight of root and per gram dry weight of soil, and the number of *Pratylenchus penetrans* per gram fresh weight of root (Mean ± S.E.) from pots inoculated with *P. penetrans* (P) and *F. oxysporum* (F) alone and in sequence.

Treatment	*P. penetrans*	*F. oxysporum* Roots	*F. oxysporum* Soil
Control	0	0	4.5±3.7
P	8390±1153	0	46.6±23.4
F	0	$4.6X10^5 \pm 6.9X10^4$	$2.0X10^4 \pm 8.7X10^3$
F+P	7245±1286	$1.1X10^6 \pm 4.4X10^4$	$1.5X10^4 \pm 4.5X10^3$

Figure 8. The pattern of growth of alfalfa seedlings infected with *Pratylenchus penetrans* and *Fusarium oxysporum* f.sp. *medicaginis* as measured by the growth index.

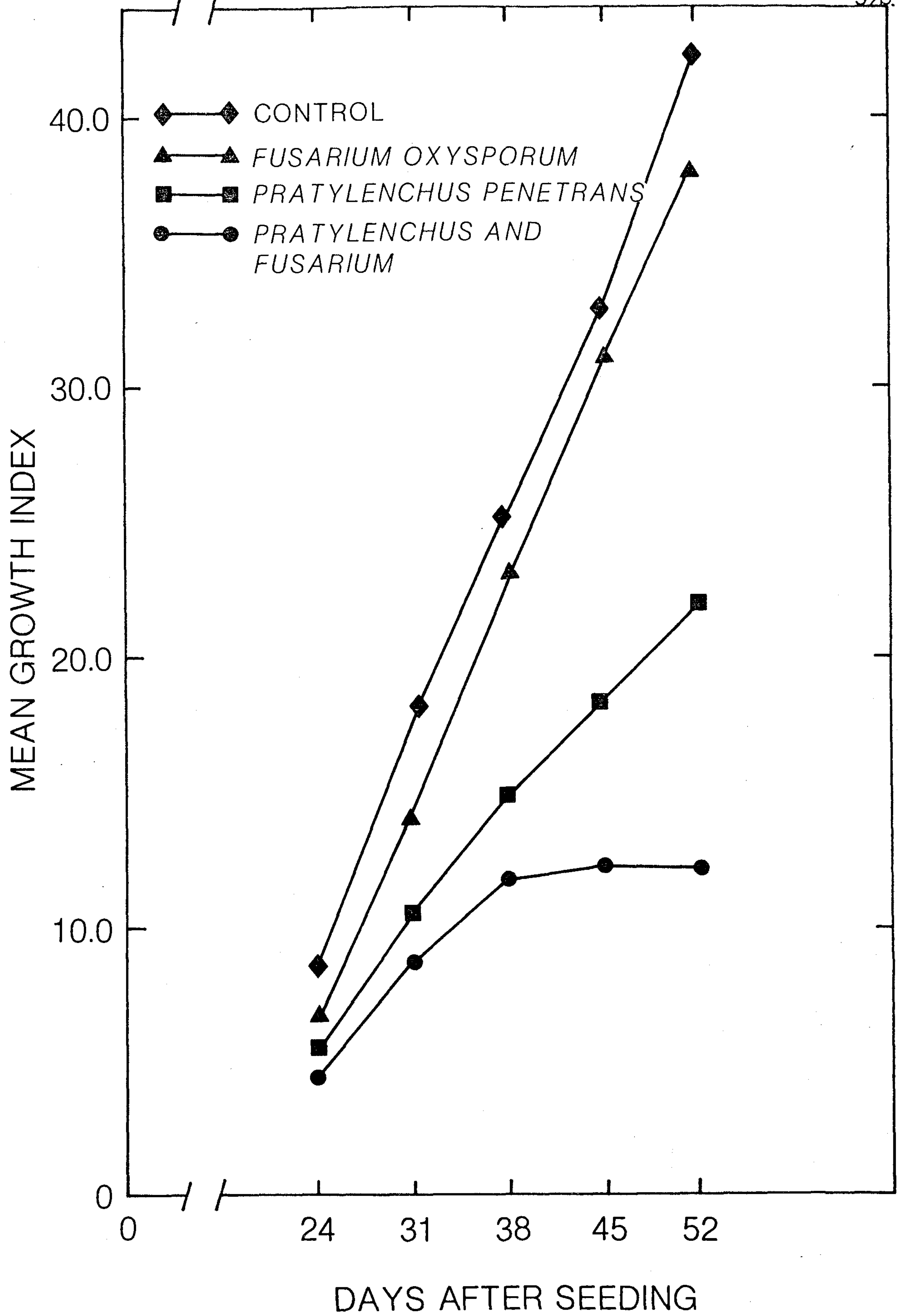
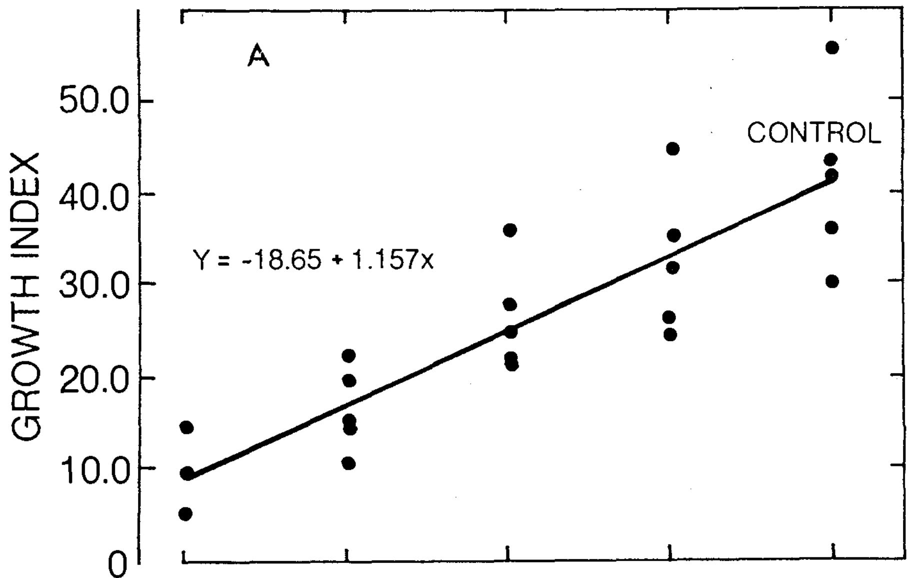
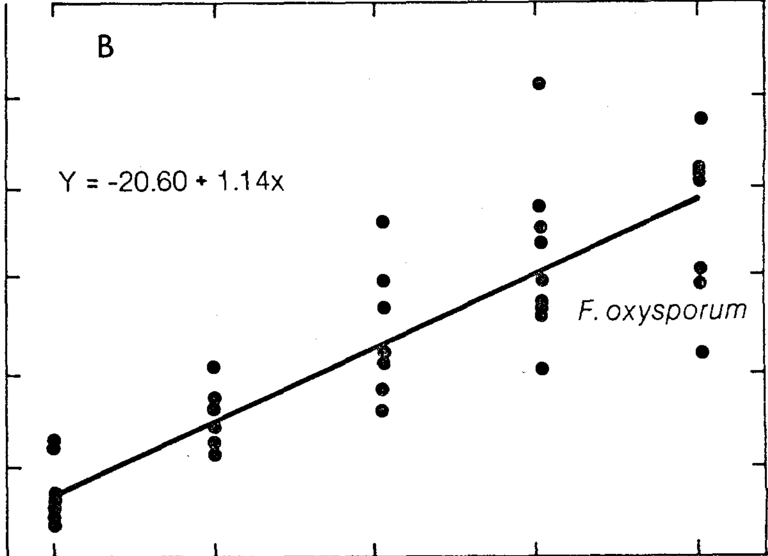
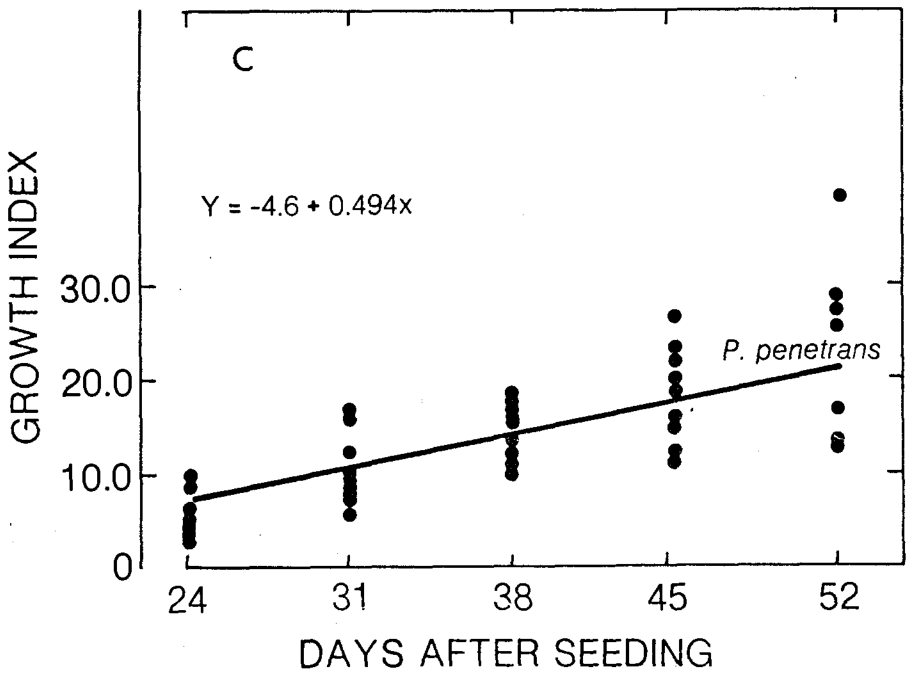
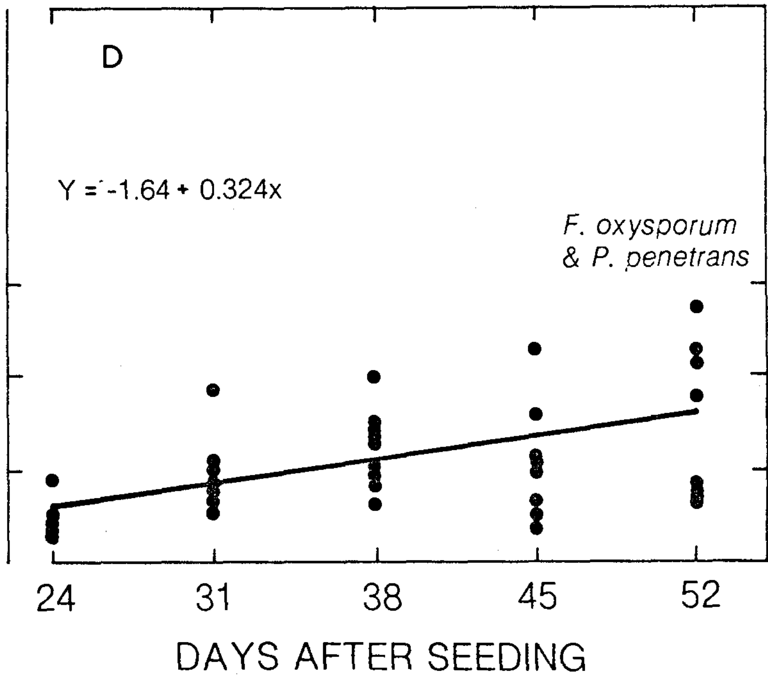

CONTROL
FUSARIUM OXYSPORUM
PRATYLENCHUS PENETRANS
PRATYLENCHUS AND FUSARIUM
MEAN GROWTH INDEX
40.0
30.0
20.0
10.0
0
0
24
31
38
45
52
DAYS AFTER SEEDING

Figure 9. The effect of *Pratylenchus penetrans* and *Fusarium oxysporum* f.sp. *medicaginis* on the growth rate of alfalfa seedlings inoculated alone and in sequence. Regression of growth index on days after seeding for (A) controls, (B) *F. oxysporum*, (C) *P. penetrans*, and (D) *F.oxysporum* prior to *P. penetrans*.


A
Y = -18.65 + 1.157x
CONTROL
GROWTH INDEX
50.0
40.0
30.0
20.0
10.0
0


B
Y = -20.60 + 1.14x
F. oxysporum


C
Y = -4.6 + 0.494x
P. penetrans
GROWTH INDEX
30.0
20.0
10.0
0
24
31
38
45
52
DAYS AFTER SEEDING


D
Y = -1.64 + 0.324x
F. oxysporum
& P. penetrans
24
31
38
45
52
DAYS AFTER SEEDING

B. The effects of *Pratylenchus penetrans* and *Fusarium solani* on alfalfa.

1. The inoculum densities of *P. penetrans* and *F. solani* inoculated simultaneously into soil containing alfalfa.

Four inoculum levels of *P. penetrans* 0, 200, 400, and 800 nematodes (designated N1, N2, N3 and N4 respectively), and four inoculum levels of *F. solani*, 0, $1X10^5$, $1X10^6$, and $1X10^7$ propagules (designated F1, F2, F3 and F4 respectively), were combined in sixteen treatment combinations and replicated five times. Each replicate was a 7.5 cm diameter pot of soil containing two alfalfa seedlings. Pots were inoculated when seedlings were 10 days old. Growth indices were recorded weekly from the time of inoculation until the plants were 70 days old. The experiment ran for 84 days from November 1974 to January 1975. At termination the plants were lifted, the weight of the tops and roots determined, and the final population of nematodes and the fungus estimated. The symptoms of *P. penetrans* infection were the same as for other experiments. No symptoms of an infection by *F. solani* were observed. Growth typical of each treatment at termination of the experiment is illustrated in Fig.10.

The fresh weight of roots was reduced to 50% of the control (N1F1) root weight by treatments N2F1 and N3F1, and 73% by N4F1 (Table IX). *F. solani* alone at any inoculum level did not cause a significant reduction in the fresh weight of roots when compared with controls, and this was also indicated by the regression of fresh

weight of roots on the inoculum levels of *F. solani* used. As the inoculum of *P. penetrans* increased, the fresh weight of the roots decreased (Fig.11a). Reductions in root weights by the N1F1, N2F1, N3F1, and N4F1 treatments were 0, 50, 50 and 80% of controls respectively.

The fresh and dry weight of tops was significantly reduced by *P. penetrans* but not by *F. solani*. Increasing the inoculum of *P. penetrans* from 0 to 800 nematodes significantly reduced the fresh weight of tops from 0 to 73% of controls (Table IX; Fig.11b). Fresh weight of tops were not reduced more by simultaneous inoculation with *F. solani* and *P. penetrans* than by inoculation with *P. penetrans* alone (Table IX).

An analysis of variance of the growth indices recorded throughout the experiment indicated that *P. penetrans* contributed to a significant ($P<0.01$) decrease in the growth of seedlings (Appendix B), whereas *F. solani* did not. Growth rates for treatments N4F1, N1F4, and N4F4, were compared with the growth rate of control seedlings (Fig.12). Only those plants that had *P. penetrans* in their treatment had growth rates significantly reduced (Table X).

In all treatments where *P. penetrans* was inoculated with *F. solani* the number of *P. penetrans* extracted from the roots was less than when *P. penetrans* was inoculated alone, but only in the N4F4

treatment was this decrease significant at $P<0.05$ (Table X). The number of propagules of *F. solani* recovered from the soil was not significantly changed by the presence of *P. penetrans* in the roots (Table X).

Table IX: Weight of roots and tops, 84 days after seeding, from pots inoculated simultaneously with <u>Pratylenchus penetrans</u> (N) and <u>Fusarium solani</u> (F). (Mean of 5 replicates)

TREATMENT*		TOPS				ROOTS	
		Fresh Weight (gm)	% Reduction	Dry Weight (gm)	% Reduction	Fresh Weight (gm)	% Reduction
N1	F1	0.44	0	0.055	0	0.26	0
	F2	0.51	-15.9	0.067	-21.8	0.35	-34.6
	F3	0.35	20.5	0.051	7.3	0.23	11.6
	F4	0.50	-13.6	0.071	-29.0	0.26	0
N2	F1	0.23	47.7	0.040	27.4	0.13	50.0
	F2	0.29	34.1	0.049	10.9	0.19	26.9
	F3	0.27	38.6	0.048	12.7	0.17	34.6
	F4	0.29	34.1	0.055	0	0.23	11.5
N3	F1	0.23	47.7	0.045	18.1	0.13	50.0
	F2	0.16	63.6	0.029	52.7	0.09	65.4
	F3	0.24	45.5	0.039	29.1	0.12	58.6
	F4	0.20	54.5	0.037	32.7	0.14	46.2
N4	F1	0.12	72.7	0.029	47.3	0.07	73.1
	F2	0.14	68.2	0.024	56.4	0.07	73.1
	F3	0.12	72.7	0.020	63.5	0.07	73.1
	F4	0.09	79.5	0.016	70.9	0.05	80.8
for $P<0.05$		LSD=0.14		LSD=0.019		LSD=0.12	

* number of nematodes N1=0, N2=200, N3=400, N4=800
number of fungal propagules F1=0, F2=$1X10^5$, F3=$1X10^6$, F4=$1X10^7$

Table X: Comparison of the final number of propagules of _Fusarium solani_ (F) in the soil, the number of _Pratylenchus penetrans_ (N) in the roots, and the plant growth rate, as a result of various treatments.

Treatment*		Growth Rate	_P.penetrans_ per gram FWR (mean of 3)**	_F.solani_ per gram ODWS (mean of 5)***
N1	F1	0.694	0	23.8
	F2	0.774	0	146.7
	F3	0.554	0	2820.0
	F4	0.596	0	3010.0
N2	F1	0.472	2812.7	0
	F2	0.675	1522.7	161.0
	F3	0.408	1280.3	1789.0
	F4	0.557	1929.3	12800.0
N3	F1	0.497	8577.3	5.0
	F2	0.390	5519.3	965.0
	F3	0.404	2941.7	2028.0
	F4	0.418	5804.3	4903.0
N4	F1	0.267	14830.0	131.9
	F2	0.316	4700.0	213.5
	F3	0.225	4685.7	2694.0
	F4	0.208	4041.3	4526.0

* Number of nematodes N1=0, N2=200, N3=400, N4=800
Number of fungal propagules F1=0, F2=$1X10^5$, F3=$1X10^6$, F4=$1X10^7$

** FWR- Fresh weight of roots

*** ODWS- Oven dry weight of soil

Figure 10. A comparison of alfalfa seedlings inoculated with four levels of *Pratylenchus penetrans* and four levels of *Fusarium solani* alone and in combination.

P. penetrans inoculum(left to right)

0, 200, 400, 800 nematodes

F. solani inoculum(top to bottom)

0, $1X10^5$, $1X10^6$, $1X10^7$ propagules.

1.

2.

3.

4.

Figure 11. The regression of fresh weight yields of roots and tops of alfalfa on the *Pratylenchus penetrans* inoculum.

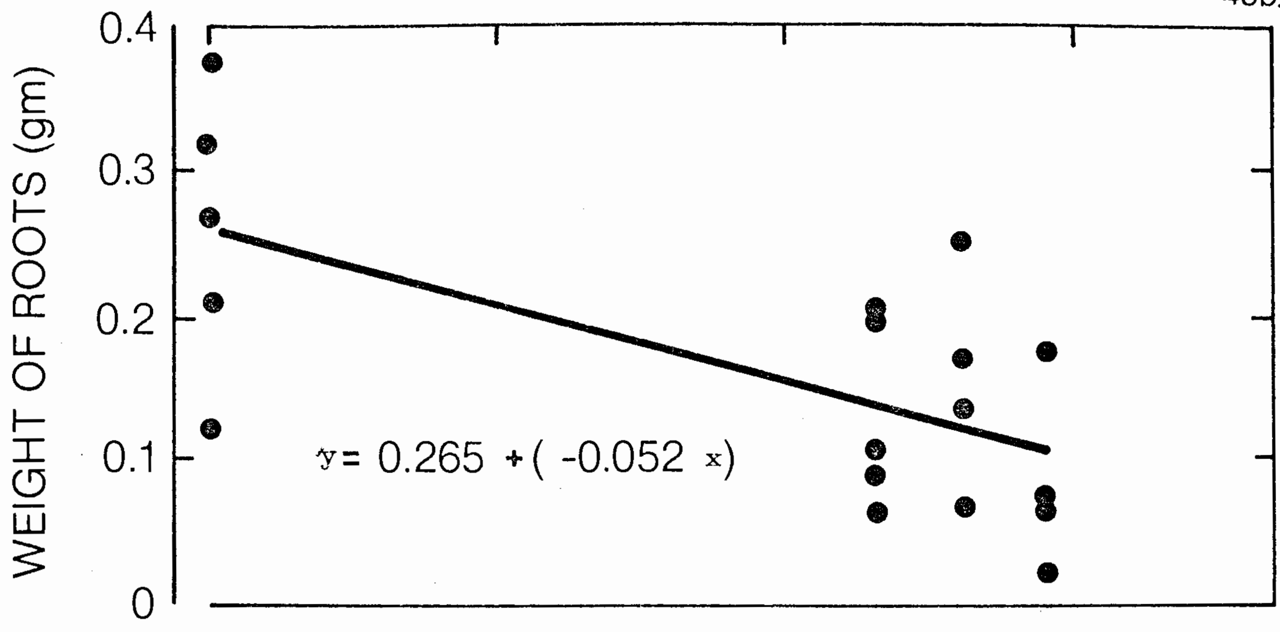
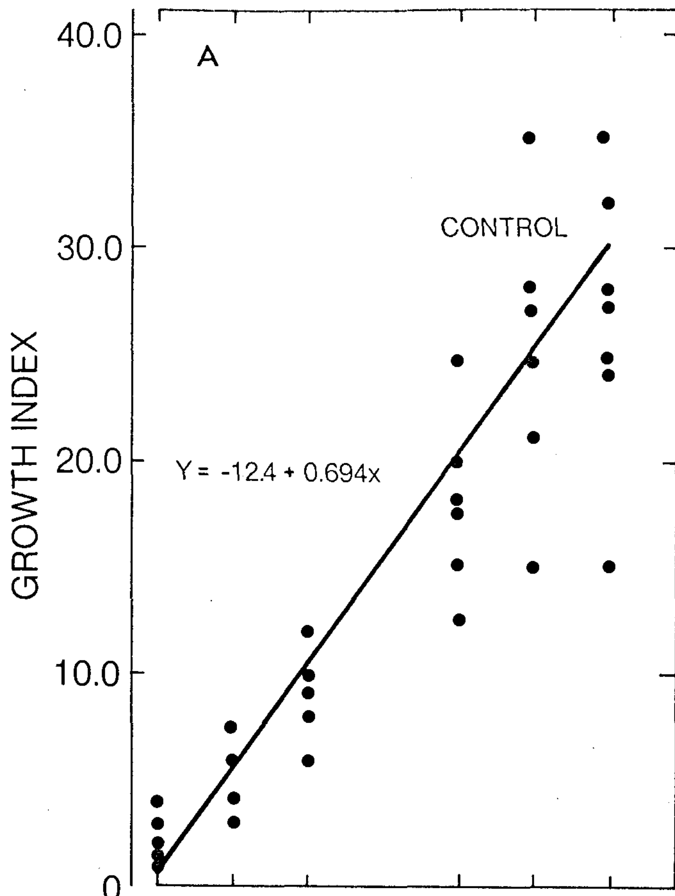
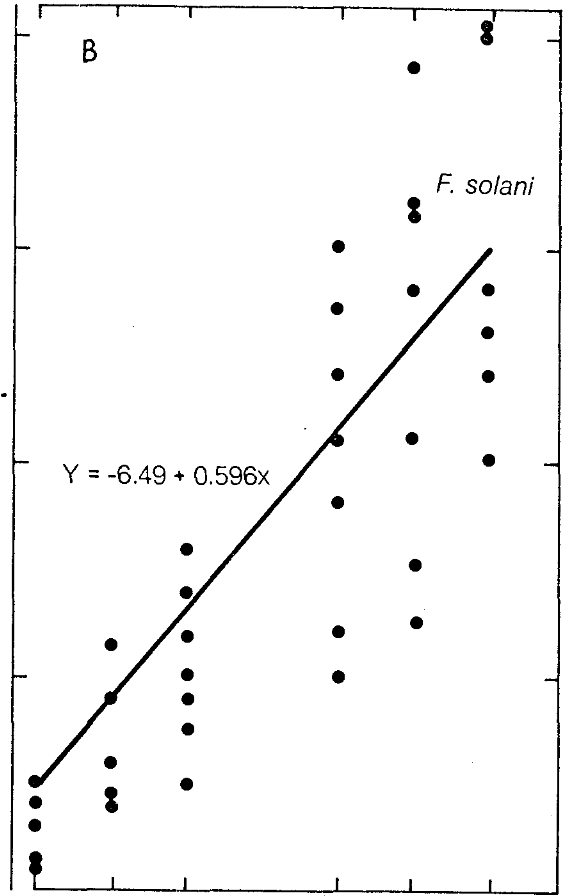
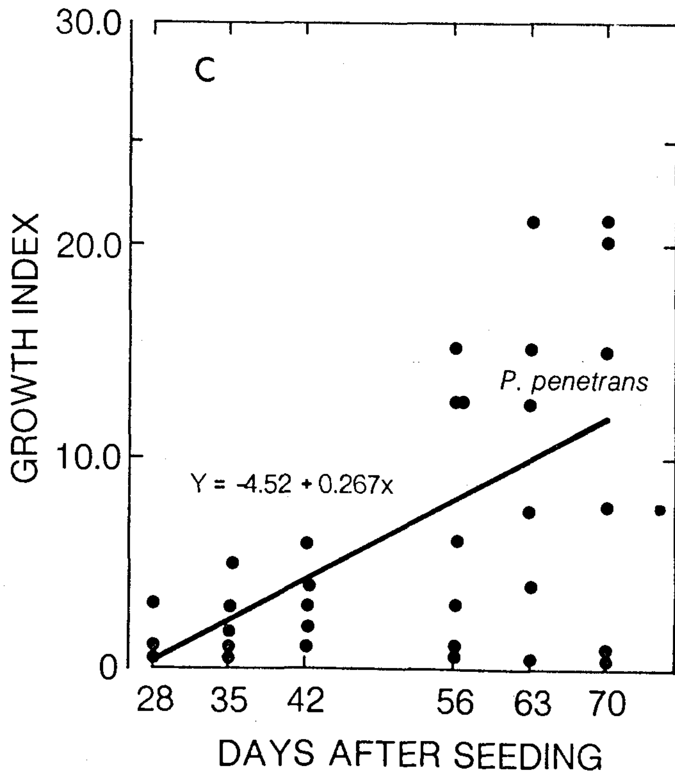

WEIGHT OF ROOTS (gm)
0.4
0.3
0.2
0.1
0
y= 0.265 +(-0.052 x)
WEIGHT OF TOPS (gm)
0.6
0.5
0.4
0.3
0.2
0.1
0
y= 0.44 + (-0.085 x)
0
1.0
2.0
3.0
P. PENETRANS INOCULUM LOG 10 (X + 1)

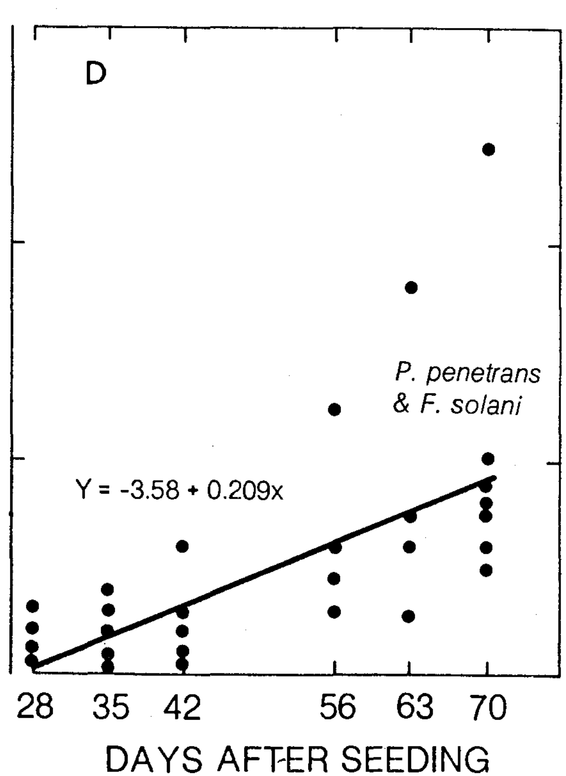
Figure 12. The growth rate of alfalfa seedlings inoculated with 800 *Pratylenchus penetrans* and $1X10^7$ propagules of *Fusarium solani* alone and in sequence.

The regression of growth index on days after seeding for seedlings inoculated with (A) controls, (B) *F. solani*, (C) *P. penetrans*, and (D) *P. penetrans* and *F. solani* combined.


A
CONTROL
Y = -12.4 + 0.694x
GROWTH INDEX
40.0
30.0
20.0
10.0
0


B
F. solani
Y = -6.49 + 0.596x


C
P. penetrans
Y = -4.52 + 0.267x
GROWTH INDEX
30.0
20.0
10.0
0
28 35 42 56 63 70
DAYS AFTER SEEDING


D
P. penetrans
& F. solani
Y = -3.58 + 0.209x
28 35 42 56 63 70
DAYS AFTER SEEDING

2. The sequence of inoculation of *P. penetrans* and *F. solani* into soil containing alfalfa.

Seven treatments were compared and each treatment was replicated nine times. A replicate consisted of two alfalfa seedlings grown in a 7.5 cm diameter pot of soil. The inoculum consisted of aqueous suspensions of 1600 *P. penetrans* and $2.5X10^7$ propagules of *F. solani* The seven treatments were:

(a) Uninoculated control

(b) 8-day-old seedlings inoculated with *P. penetrans* (P1)

(c) 8-day-old seedlings inoculated with *F. solani* (F1)

(d) 8-day-old seedlings inoculated with *P. penetrans* and 7 days later with *F. solani* (P1F2)

(e) 8-day-old seedlings inoculated with *F. solani* and 7 days later with *P. penetrans* (F1P2)

(f) 15-day-old seedlings inoculated with *P. penetrans* (P2)

(g) 15-day-Old seedlings inoculated with *F. solani* (F2)

The plants were grown through July and early August, 1975. During the experiment the growth indices for each plant were recorded and at termination the yields of roots and tops, the number of *P. penetrans* in the roots and the population of *F. solani* in the soil and roots were determined.

The fresh and dry weight of tops were significantly reduced in only those treatments where *P. penetrans* was inoculated onto 8-day-old

alfalfa seedlings (P1 and P1F2) (Table XI). The P1F2 treatment reduced them significantly ($P<0.13$) more than did the F1P2 treatment (Table XI).

The fresh weight of roots was reduced significantly by both P1 and P2 treatments but not by either F1 or F2 treatments. The nematode inoculation of 8-day-old alfalfa seedlings caused a 20% greater reduction in the fresh weight of roots than did the nematode inoculation of the 15-day-old seedlings, but this was not significant at $P<0.05$. The P1F2 treatment caused a 31% greater reduction in fresh weight of roots than did the F1P2 treatment (Table XI). Examples of root systems from these treatments are shown in Figs.13 and 14.

A regression of the growth indices on the age of seedlings for each treatment shows no significant differences between the growth rates for any pair of treatments (Table XII).

The number of *P. penetrans* extracted from roots (Table XII) was significantly smaller when *F. solani* was inoculated prior to *P. penetrans* than when the fungus was added after the nematode. The number of nematodes extracted from the roots of seedlings in the P1 treatment was not significantly different from the number recovered from the P2 treatment. No fungal counts were obtained due to contamination of the plating medium.

Table XI: Weight of roots and tops, 43 days after seeding, of alfalfa inoculated with *Pratylenchus penetrans* at 8 days after seeding (P1) and 15 days after seeding (P2), and with *Fusarium solani* at 8 days after seeding (F1) and 15 days after seeding (F2) (Mean of 9 replicates).

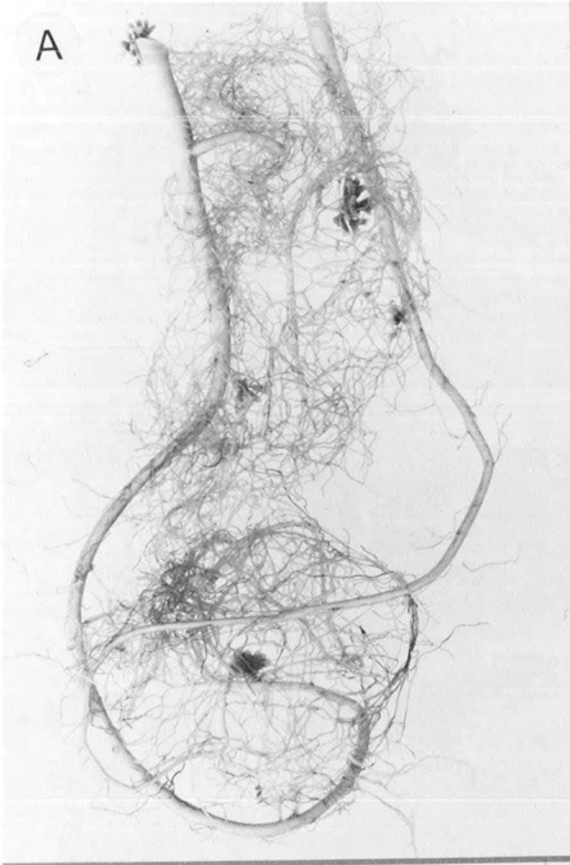
TREATMENT	TOPS				ROOTS	
	Fresh Weight (gm)	% Reduction	Dry Weight (gm)	% Reduction	Fresh Weight (gm)	% Reduction
Control	1.22	0	0.195	0	0.63	0
P1	0.69	43.2	0.120	38.5	0.31	50.0
P2	0.79	34.5	0.154	21.0	0.44	30.5
F1	1.42	-11.7	0.245	-11.5	0.66	-6.0
F2	1.03	15.6	0.179	8.2	0.57	10.0
P1F2	0.56	54.0	0.105	46.2	0.27	56.6
F1P2	0.93	23.2	0.168	12.8	0.47	25.2
for $P<0.05$	LSD=0.46		LSD=0.07		LSD=0.20	

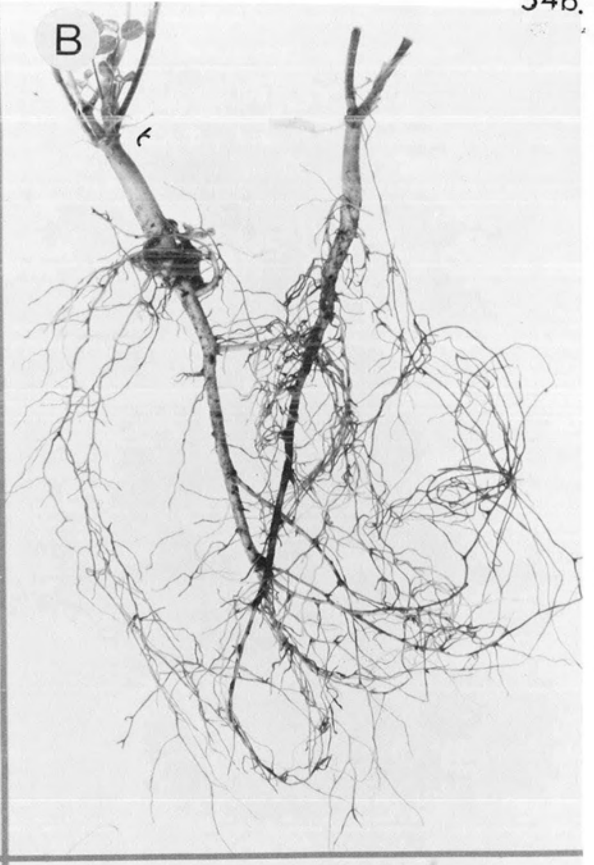
Table XII: Number of *Pratylenchus penetrans* extracted from roots of 43 day-old alfalfa seedlings inoculated with *Pratylenchus penetrans* at 8 (P1) and 15 (P2) days of age, and with *Fusarium solani* at 8 (F1) and 15 (F2) days of age (mean of 9 replicates), and growth rates for those seedlings.

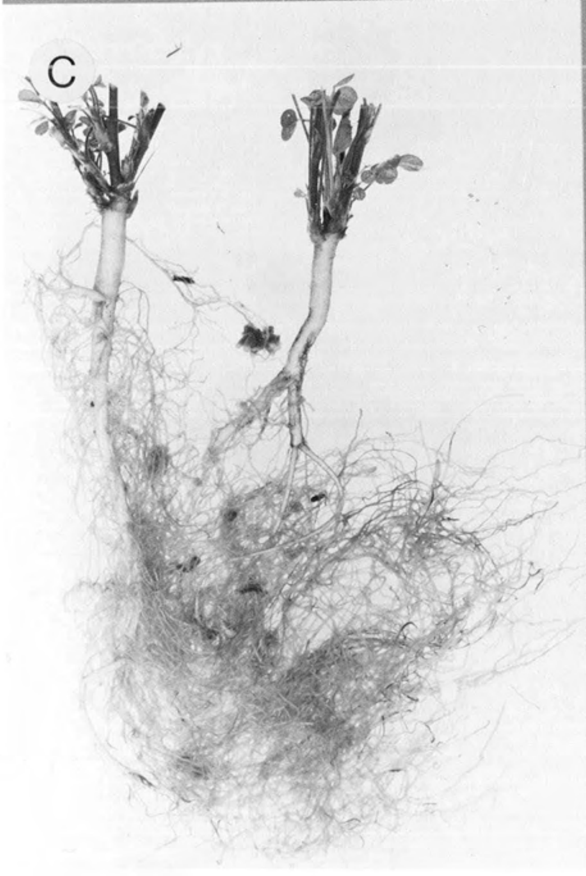
Treatment	*P. penetrans* per gram root	Growth Rate
Control	0	0.057
P1	7776.3	0.052
P2	6630.3	0.047
F1	0	0.056
F2	0	0.060
P1F2	10335.0	0.051
F1P2	5306.4*	0.048

* Significantly different from P1F2 at $P<0.01$

Figure 13. The root growth of alfalfa infected with two pathogens. (A) Control (B) *Pratylenchus penetrans* (C) *F. solani* (D) *F. solani* followed seven days later by *P. penetrans*.
Note the lack of smaller roots and the presence lesioned areas in (B).


A


B


C

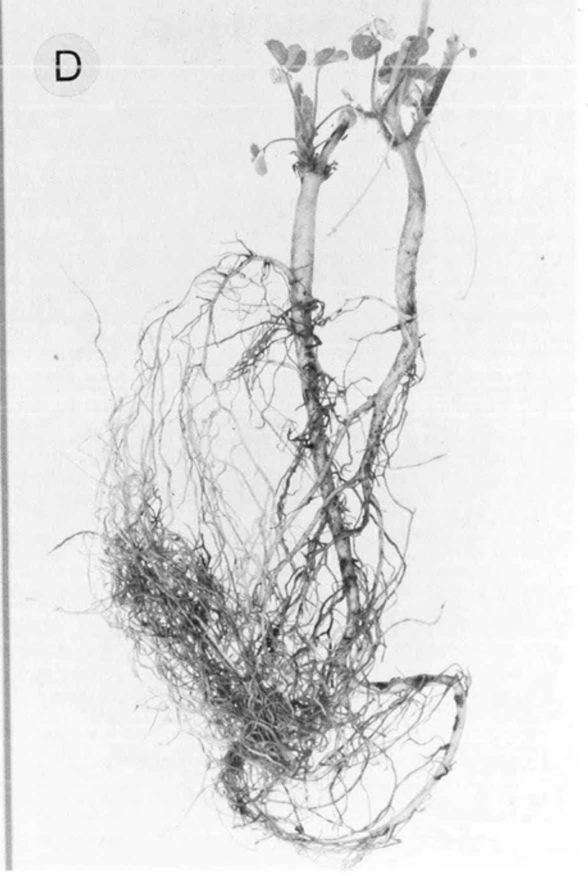
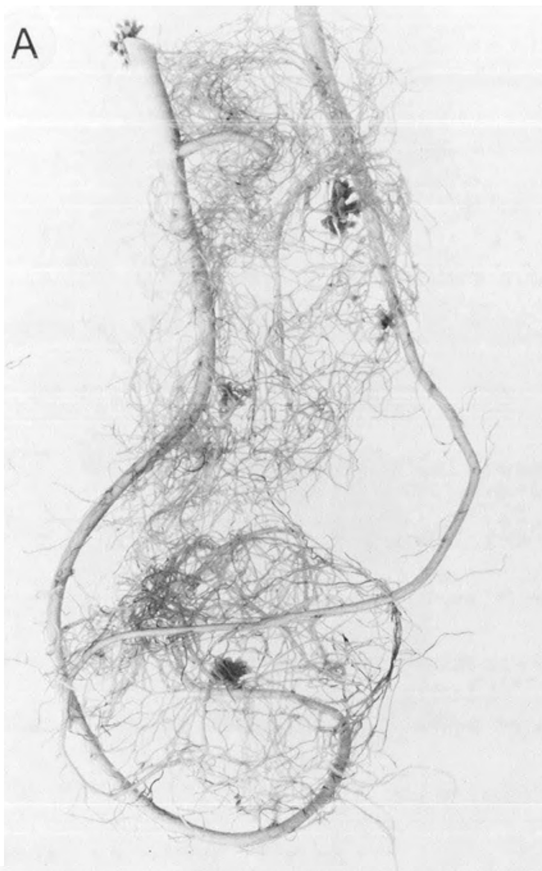
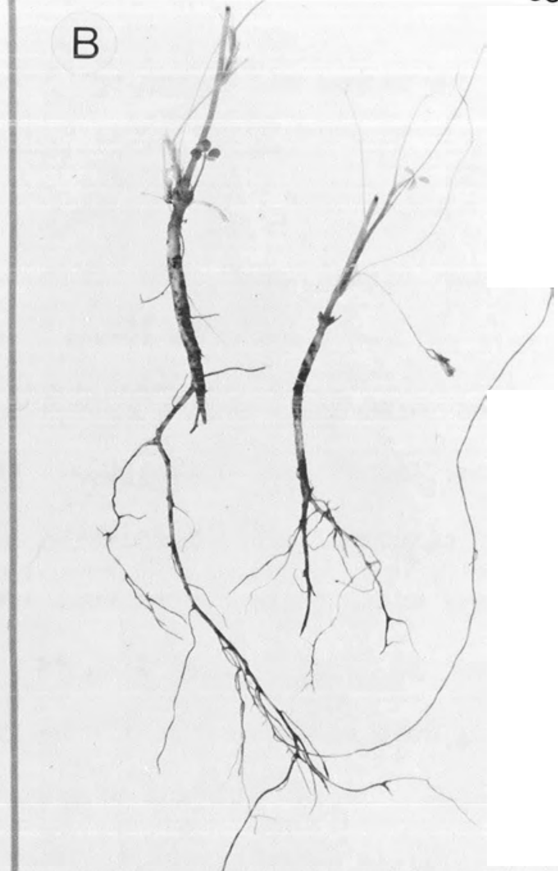

D

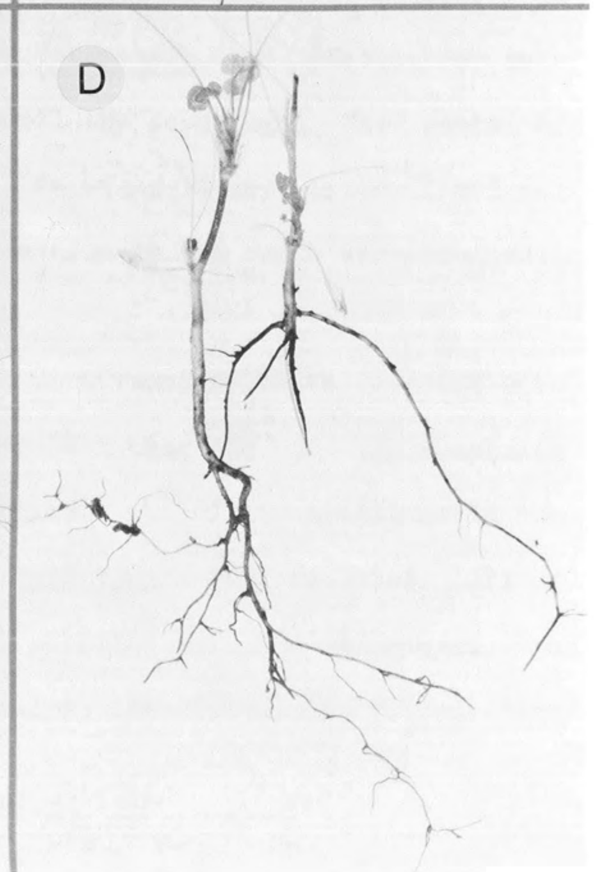
Figure 14. The root growth of alfalfa infected with two pathogens. (A) Control (B) *Pratylenchus penetrans* (C) *Fusarium solani* (D) *P. penetrans* followed 7 days later by *F. solani*.

Note the damage caused by *P. penetrans*.


A


B

C


D

3. Effect of an infection of *F. solani* on the number of *P. penetrans* entering alfalfa roots.

Two treatments were compared, each with ten single seedling replicates. They were grown in sterilized sand under Gro-lux lights at 25 C with a 14 hour photoperiod and watered every 2 days with a half strength Hoagland's nutrient solution. The fungal inoculum consisted of 6 gm of infested soil containing $2X10^5$ propagules of *F. solani* per gram, mixed with the sand in the vials to be treated. Acid scarafied seeds were germinated at 23°C in the dark on sterile moist filter paper and when they were 4 days old they were planted into vials containing *F. solani*-infested or sterile sand. When the seedlings were 11 days old a drop of deionized water containing 50 fourth stage larvae and adult *P. penetrans* was inoculated into depressions made with a pencil near all the seedlings. The roots of the seedlings were washed from the vials 14 days later, fixed and stained (Southey, 1970), and the nematodes in the roots were counted.

A mean of 19.3 ± 5.2 nematodes were found in roots infected with *F. solani* and this was significantly less than the 32 ± 4.2 nematodes found in roots uninfected by the fungus. 20% of the seedlings in the *F. solani* treatment died before *P. penetrans* were inoculated. 37% of those remaining died before the termination of the experiment. No deaths were recorded for those seedlings inoculated with *P. penetrans* alone.

DISCUSSION

This study has demonstrated that an interaction occurred between P. penetrans and F. oxysporum which reduced the weight of roots and tops of alfalfa more than did either pathogen alone. This interaction was more pronounced when F. oxysporum was inoculated before or simultaneously with P. penetrans. F. solani interacted with P. penetrans by decreasing the number of nematodes in the roots, whereas F. oxysporum appeared to have no effect on the number of P. penetrans. A nematode infection of the roots did not affect the population of Fusarium propagules in the soil. The growth of alfalfa seedlings in pots of soil was not significantly reduced by F. solani, but in sand culture this fungus caused death of 20% of the alfalfa seedlings. The nematode consistently reduced the growth of alfalfa and in most cases this reduction was substantially more than that caused by either Fusarium species.

It is necessary to discuss the index that was used as a measure of seedling growth before continuing with the implications of the results. The simple formula used to calculate the growth index was a means of comparing plant responses to treatments without sacrifice of the plant, rather than a measure of the size of the assimilatory system. In young alfalfa seedlings most leaves were of a similar size and only one stem was present. Once secondary branches emerged leaf size as well as number varied considerably and so the measurements were discontinued. In some cases this occurred as early as 40 days

after the start of an experiment that ran 85 days. The two measurements which could be obtained regularly, simply and without sacrifice of the seedlings were height of the plant and the number of leaves on the plant at the time of the height measurement (See Materials and Methods). These two measurements were multiplied rather than added together to enable the separation of the growth responses to various treatments when seedlings were very young. Often the increases in the mean growth index measured in this manner were found to be linear.

There was considerable interexperimental variation in the growth of the seedlings because of uncontrollable factors. The largest variations were due to the amount and intensity of light available, which varied with the time of year. Although experiments received the same fertilizer treatments the mineral loam used to formulate the potting mix varied in type, pH and probably in nutrient content. These and other factors affecting the host and pathogens were not controlled absolutely but any variations which occurred should have randomly affected all replicates within any individual experiment.

A review by Bergeson (1972) on nematode-fungus associations in plant disease complexes indicates that, in many cases, the nematode appears to be the primary pathogen. Bergeson organized his review according to possible roles of the nematode in such disease complexes. The role of nematodes as wounding agents, host modifiers, rhizosphere

modifiers, and resistance breakers as well as the effects of fungi on nematodes discussed therein probably apply to the *Pratylenchus*-*Fusarium* interactions in alfalfa described here. These roles are discussed below.

Of major importance to our understanding of what occurs in these interactions is the nature of invasion by *Pratylenchus*. This nematode invades roots by penetrating directly into or between epidermal cells, and then it tunnels extensively through the cortex. On severely infested root systems, nearly all the feeder roots are damaged or destroyed (Fig.14B). Injured cells leak their contents into the cavities produced by tunneling thereby increasing the concentrations of amino acids, sugars, and other organic compounds in the rhizosphere.

Both *Fusarium* species used in this study penetrate roots directly without the aid of mechanical injury such as that provided by feeding nematodes (Weimer, 1928; Chi, *et*.*al*., 1964). Hence, mechanical wounding of the roots by the nematode is likely of little significance for fungal entry, and certainly of lesser importance than the nematode's role as a rhizosphere or as a host modifier.

When alfalfa was inoculated with *P*. *penetrans* after the *F*. *oxysporum*, there was a 69% reduction in yield (Table VI) similar to the reduction when *P*. *penetrans* was inoculated prior to

F. oxysporum (Table III). When alfalfa was seeded to soil which had been inoculated previously with F. oxysporum (see Experiments and Results A.3.) no decrease in weight of roots or tops occurred (Table VI), but when 11-day-old seedlings were inoculated with F. oxysporum (see Experiments and Results A.2.) a significant decrease in root weights occurred (Table III). This may have been due to a more concentrated inoculum applied adjacent to seedlings in experiment A.2., compared to A.3. where the inoculum was thoroughly mixed with the soil prior to seeding. The results of these sequences of inoculation suggest that P. penetrans may be acting as a rhizosphere modifier by damaging root cells during its entry and subsequent feeding thereby increasing root exudates. The increased exudations may have stimulated germination of the chlamydospores of F. oxysporum and aided its pathogenesis. Schroth and Hildebrand (1964) stated that establishment of a pathogen on a host is partly a function of the nutrient environment as influenced by root exudates and the interactions between associated microorganisms whose activities are also mediated by the exudation of energy sources. In this study (Tables VI and XI) high levels of propagules of Fusarium often were not sufficient to cause a decrease in the root and top weights of alfalfa except in the presence of P. penetrans. In the soil Fusarium survives in its resting stage, as chlamydospores. Certain carbon sources common to root exudates are known to stimulate chlamydospore germination and multiplication in the soil (Papavizas, et.al., 1968) and this effect is enhanced by nitrogen. The synergistic reduction in growth of alfalfa evident in Table VI may be due to the increased root

exudates stimulating F. oxysporum development. This indicates that F. oxysporum may require the higher levels of nutrients from root exudates released by penetration and feeding by P. penetrans, to provide the energy source necessary for penetration.

Schroth and Hildebrand (1964) suggested that soil microorganisms can influence the saprophytic and pathogenic activities of fungi. It has been reported that certain species of Streptomyces and Trichoderma are antagonistic to Fusarium (Chi, 1960). During this study colonies of Streptomyces occurred occasionally on agar plates that had been inoculated with soil dilutions, but they were never identified to species. Lockwood (1964) states that conidia of Fusarium species seem to be less sensitive to mycostasis than hyphae or chlamydospores and that mycostasis can be annulled by nutrients. Soil mycostasis may account for the lack of damage by both Fusarium species when inoculated alone (Experiments and Results Sections A.2., B.1.and B.2.). Mycostasis may be overcome by the increased root exudates released by penetration and feeding of the nematode on alfalfa roots in soil, thereby enabling a significant interaction when P. penetrans is present. This contention is supported by the fact that F. solani caused death of 20% of the seedlings in a sterile sand medium (see Experiments and Result Section B.3.) where mycostasis would be minimal.

Inhibition of Fusarium may have occurred indirectly by exudates stimulating antagonistic microorganisms or directly by the substances contained in the exudates. M. sativa produces the antifungal compound medicarpin in response to infection by two non-pathogens, Helminthosporium turcicum and Colletotrichum phomoides (Higgins and Millar, 1968), but no medicarpin was detected when the pathogen Stemyphyllium botryosum was infected. P. penetrans has also been reported to elicit accumulation of the antifungal compound phaseollin in Phaseolus vulgaris seedlings (Abawi, et.al., 1971). Phytoalexins produced and exuded from the roots may play a role in rhizosphere mycostasis and protection of root systems. Such an effect would vary with the species of plant infected, since the phytoalexin response is known to be species specific (Kuć, 1972). The possibility exists that the alfalfa produces antifungal compounds in response to wounding or lesion formation caused by P. penetrans. Specific biochemical responses of a host to a parasite play a large part in necrosis caused by Pratylenchus, because one Pratylenchus species may cause necrosis on roots and another not (Pitcher, 1965). Nematodes functioning as host modifiers can thus influence potential fungal pathogens in ways other than by provision of entry sites or by enhanced nutrient levels in the host rhizosphere.

Infection of Fusarium-resistant peas with P. penetrans induces their susceptibility to Fusarium wilt, whereas severe root pruning and wounding does not (Oyekan and Mitchell, 1971). These authors suggested that P. penetrans may act as a host modifier by inducing

some biochemical or physiological changes which favor wilt development. Although the level of resistance of 'Vernal' alfalfa seedlings to *Fusarium* wilt was not determined in this study it is likely that changes induced by the nematode decrease the normal defenses of the seedlings to *F. oxysporum*, thereby contributing to decreased growth of the plants (see Experiments and Results Section A.). Earlier studies of root rots and wilts of alfalfa and other legumes caused by *Fusarium* species demonstrated that any factors involved in reducing the vigor of the host increased the incidence and severity of the diseases (Fulton and Hanson, 1960; O'Rourke and Millar, 1966). Where plants were predisposed by removing top growth there was a decrease in the carbohydrate levels in the roots (Lukezic, et.al., 1969). Feltner and Massengale (1965) found a correlation between a decrease in carbohydrate levels in the roots and an increase in the susceptibility of the plants. Rhizosphere populations of *Fusarium* species increase with increased cutting frequency of alfalfa (O'Rourke and Millar, 1966). In a field situation a reduction in vigor of alfalfa plants by *P. penetrans* might be a major factor in the development of *Fusarium* infections which would decrease the longevity and productivity of the stand.

Since both fungal pathogens can penetrate alfalfa directly without the aid of mechanical injury, I suggested that the role of *P. penetrans* in the interaction as a wounding agent was of secondary importance to its roles as a host and rhizosphere modifier, which have

been discussed. Wounding may nevertheless contribute, because the site of action of a wilt-causing pathogen is usually in the xylem tissue, and to reach the vascular cylinder F. oxysporum would first have to traverse the cortex. The prominent infection courts caused by penetration by P. penetrans may allow greater and earlier infection of the vascular cylinder. Seinhorst and Kuniyasu (1971) suggested that P. penetrans enhances the extent of penetration by F. oxysporum f.sp. pisi Race 2 into the vascular tissue of peas rather than the degree of attack on the cortex.

Pratylenchus populations are frequently affected by fungal pathogens in the same host. Edmunds and Mai (1966) found that F. oxysporum-infected roots were more attractive to P. penetrans than uninfected roots. Further, at higher spore concentrations in the fungus inoculum significantly more nematodes entered the roots of fungal infected alfalfa than those of control plants. They also found P. penetrans was attracted to CO_2, higher levels of which were given off by Fusarium-infected roots than by uninfected roots. Seinhorst and Kuniyasu (1971), however, reported that the presence of F. oxysporum f.sp. pisi Race 2 in roots of peas probably reduces the rate of multiplication of P. penetrans. They suggested that the fungal attack made the pea root cortex less suitable host tissue for P. penetrans. Results from the present study indicate that the number of P. penetrans recovered from roots inoculated with F. oxysporum (Tables II, V, and VIII) was not significantly decreased as compared

with the controls. My form of inoculation of F. oxysporum differed from that of Edmunds and Mai (1966), which could account for the different results with respect to number of P. penetrans in the roots. In my experiments when F. solani was present in the soil prior to inoculation with P. penetrans or when F. solani was added to the soil simultaneously with the nematode, there occurred a significant decrease in the number of nematodes in the roots (Tables X and XII). The number was not affected when F. solani was inoculated after P. penetrans. Therefore, it would appear that the fungus inhibits penetration by the nematode or its development once in the root, since counts were made 14 days after infection with P. penetrans. F. solani may be altering the roots biochemically in such a way as to make them less attractive to the nematodes. It is known that phenolic substances are inhibitory to P. penetrans (Pitcher, et.al., 1960). The Fusarium infection may stimulate the production of phenolics in the roots which, in turn, may retard nematode entry. Rich and Keen (1975) reported that P. scribneri induced the production of coumestrol and that this substance reduced the mobility of the nematode in lima bean roots, which in turn may be linked with its reduced reproduction. An infection by F. solani may induce a similar reaction in alfalfa and could account for the decreased numbers of P. penetrans in the roots (Table XII; Experiments and Results B.3.).

The number of Fusarium propagules recovered from an aliquot of soil from each pot did not increase significantly when P. penetrans

was present in the roots. One might have expected an increase, if increases in root exudations were stimulating germination and development of the fungus as was suggested previously in the discussion. It seems reasonable that the effect of exudations may be mostly in the rhizoplane immediately adjacent to nematode infection sites. Since P. penetrans infection is not evenly distributed over the entire root system during the term of these experiments, the effect could have occurred but escaped detection, since all soil in each pot was thoroughly mixed together before the fungus population was estimated.

The number of Fusarium propagules in root samples was determined in the same way as it was for soil samples. Since propagules in roots would be a mixture of hyphae, conidia and chlamydospores, the number varies with the length of time the roots are macerated. In soil the fungus persists mainly in its resting stage, chlamydospores, therefore soil counts of Fusarium propagules are more consistent than root counts when using this method. In one experiment (Table VIII) the number of F. oxysporum propagules in roots was significantly higher where P. penetrans was also present in the roots than where the nematode was absent. In corresponding experiments using F. solani (see Experiments and Results B.2.) heavy contamination by bacteria and yeasts on the plates made counting and subsequent comparisons of the numbers of propagules in the roots impossible.

Two disease interactions on alfalfa were studied: *P. penetrans* and *F. oxysporum* and *P. penetrans* and *F. solani*. It became evident during the short terms of the experiments that *P. penetrans* consistently caused a greater decrease in yields of alfalfa than either *Fusarium* species alone, and that *F. oxysporum* decreased yields more than *F. solani*. *Fusarium* species could cause greater damage to alfalfa over a longer period of time than what was demonstrated in these short term experiments. The results are interpreted on the basis that more severe disease development is stimulated when increased nutrient availability in the rhizosphere resulting from invasion of roots by *P. penetrans*, aids *Fusarium* species to overcome soil mycostasis.

Appendix A: Analysis of variance of growth indices for alfalfa inoculated with _Pratylenchus penetrans_ (N) and _Fusarium oxysporum_ (F), over 54 days after seeding.

TREATMENT	ERROR TERM	F	SS	DF	MS
N	P(NF)	2.84*	2241.6	3	747.2
F	P(NF)	2.42**	1906.8	3	635.6
TIME (W)	WP(NF)	387.56	126612.5	4	31653.1
NF	P(NF)	0.92	2176.5	9	241.8
NW	WP(NF)	2.59	2534.3	12	211.2
FW	WP(NF)	2.36	2312.5	12	192.7
P(NF)			16811.7	64	262.7
NFW	WP(NF)	0.89	2613.9	36	72.6
WP(NF)			20908.3	256	81.7

* significant at P<0.05
** significant at P<0.10

Appendix B: Analysis of variance of growth indices for alfalfa inoculated with _Pratylenchus penetrans_ (N) and _Fusarium solani_ (F), over 70 days.

TREATMENT	ERROR TERM	F	SS	DF	MS
N	P(NF)	24.53*	34861.5	3	11620.5
F	P(NF)	1.13	1611.8	3	537.3
TIME (W)	WP(NF)	251.68	88000.0	5	17600.0
NF	P(NF)	0.51	2166.4	9	240.7
NW	WP(NF)	15.99	16783.0	15	1118.9
FW	WP(NF)	0.94	989.6	15	65.9
P(NF)			30317.5	64	473.7
NFW	WP(NF)	0.44	1394.1	45	30.9
WP(NF)			22377.6	320	69.7

* significant at P<0.01

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