Effect of Dinitramine and Trifluralin on Growth, Reproduction, and Infectivity of Aphanomyces euteiches

C. R. Grau

Department of Plant Pathology, University of Minnesota, St. Paul, MN 55108. Present address: Department of Plant Pathology, University of Wisconsin, Madison, WI 53706.

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ABSTRACT

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Aphanomyces euteiches produced less hyphal growth, fewer zoospores, and had a lower zoospore germination percentage when grown in dinitramine and trifluralin solutions than in the absence of these herbicides. Root disease severity was less and fewer plants died when zoospore inoculum (10,000 zoospores/ml) was amended with dinitramine and trifluralin (0.12 μg/ml), than when inoculum was not amended. Dinitramine was more effective than trifluralin in altering growth and reproduction of *A. euteiches* and in lowering disease severity.

Additional key words: herbicides, *Pisum sativum,* root rot, soil-borne plant pathogen.

Aphanomyces euteiches Drechs. causes severe root rot of processing pea (*Pisum sativum* L.) and annually reduces yield and quality of peas in Minnesota. Though weed control is the primary function of trifluralin, dinoseb, and dinitramine, these three herbicides also reduce severity of root rot in pea caused by *A. euteiches* in the greenhouse and field and they increase yields of pea in the field (4, 6, 7). Trifluralin also reduces clubroot of cabbage caused by *Plasmodiophora brassicae* (1) and trifluralin + dinoseb reduces root rot of snap bean caused by *Pythium irregulare* (10). Harvey et al. (6) have shown trifluralin to be inhibitory to hyphal growth and zoospore formation of *A. euteiches*.

The objective of this study was to ascertain whether trifluralin and dinitramine are inhibitory to *A. euteiches* and whether these herbicides affect development of pea root rot caused by *A. euteiches*.

MATERIALS AND METHODS

Herbicides used in in vitro experiments.—Commercial and technical grades of the herbicides N^3, N^3-diethyl-2, 4-dinitro-6-trifluoromethyl-*m*-phenylene diamine (dinitramine) (U. S. Borax & Chemical Corp., Anaheim, CA 92805) and α, α, α-trifluoro-2, 6-dinitro-N, N-dipropyl-*p*-toluidine (trifluralin) (Eli Lilly & Co., Greenfield, IN 46140) both were assayed for their inhibitory effects on *A. euteiches*. Different concentrations of commercial and technical grade of dinitramine or trifluralin were dissolved in 95% ethanol. These stock solutions were made so that the final concentration of ethanol in assay media was 1%. The stock solutions were stored in screw-cap bottles at 10 C.

Isolate of Aphanomyces euteiches.—Isolates C and M of *A. euteiches* were from infected plants grown in a commercial field near Le Sueur, Minnesota, and a pea disease nursery at St. Paul, respectively. Isolate P was provided by J. A. Lewis, USDA Soil-Borne Disease Laboratory, Beltsville, Maryland.

Effect of herbicides on hyphal growth.—Difco cornmeal agar (CMA) was used as an assay medium to test the effect of dinitramine or trifluralin on growth and development of three isolates of *A. euteiches*. The autoclaved medium was allowed to cool before herbicides were added. One ml of the stock solution for each herbicide concentration was added to 99 ml of medium in a 250-ml Erlenmeyer flask. Flasks were shaken by hand to mix the herbicide into the medium. The media then were poured into petri dishes and used immediately. An inoculum plug (5 mm in diameter) cut from the margin of an actively growing colony of *A. euteiches* was placed on the CMA medium. All isolates assayed for linear growth were incubated at 25 C in darkness. Colony diameter was measured when the colony of the control treatment (no herbicide) had reached the edge of the petri dish. A 1% ethanol-CMA control was also included.

Effect of herbicides on zoospore formation and motility.—Zoospore inoculum of *A. euteiches* was produced in the following manner. Five-mm-diameter plugs from the margin of a colony of *A. euteiches* were transferred to screw-cap tubes (2.4 × 15 cm) containing 15 ml of peptone-maltose broth (2). The cultures were incubated for 4 days at 25 C. Zoospore formation was achieved using a salt solution and procedures described by Mitchell and Yang (8).

To study the effect of dinitramine and trifluralin on

zoospore formation, motility, and germination, the above method of zoospore production was altered as follows: mycelial mats were washed for the final time with salt solutions that contained different concentrations of dinitramine or trifluralin. Control treatments were the salt solution alone or a 1% ethanol-salt solution. The treated mycelial mats were incubated in a water bath with a reciprocal shaker at 27 C and shaken at 150 strokes per minute for 16 hr. Mycelial mats were stored at 4 C to stop sporulation and induce encystment of zoospores to facilitate counting. Each treatment was replicated three times with two subsamples per replicate.

Motile zoospores were not observed each time zoospore production of *A. euteiches* was evaluated in the presence of dinitramine or trifluralin. In contrast, abundant motile zoospores appeared in the absence of the herbicides. The following experiments were performed: (i) to quantify the observation that motile zoospores were not observed in the presence of dinitramine or trifluralin using the procedure outlined above; and (ii) to evaluate the effect of dinitramine or trifluralin on zoospore motility by introducing motile zoospores produced in an herbicide-free environment into a salt solution containing the herbicides. Twelve hours after the final mycelial wash, zoospores in suspension were added to equal volumes of salt solution amended with herbicides; final concentrations of herbicides were 0.03 and 0.06 μg/ml for

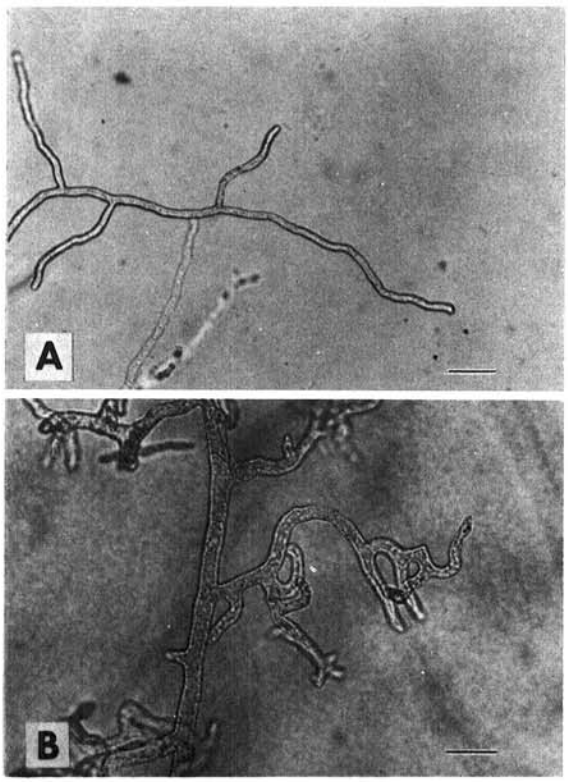

Fig. 1-(A, B). Comparison of hyphae of *Aphanomyces euteiches* grown on cornmeal agar amended with: **A)** no herbicide (Bar = 21 μm); **B)** dinitramine at 1.0 μg/ml (Bar = 8 μm).

TABLE 1. The effect of commercial and technical grades of dinitramine or trifluralin on linear hyphal growth of three isolates of *Aphanomyces euteiches* grown on Difco cornmeal agar after 96 hr at 25 C

Herbicide	Concentration[a] (μg/ml)	Colony diameter (mm) per isolate[b] Isolate C	Isolate M	Isolate P
None (Ck)		79	78	76
1% ethanol (Ck)		76	75	78
Dinitramine				
Technical	0.25	64	61	71
	0.50	54	53	53
	1.00	36	42	37
Commercial	0.25	70	65	72
	0.50	53	57	53
	1.00	43	40	39
Trifluralin				
Technical	0.25	71	72	78
	0.50	69	69	78
	1.00	67	62	72
Commercial	0.25	71	72	77
	0.50	69	68	78
	1.00	68	62	73
Tukey's test $w_{.05}$ =		4	4	4

[a]One ml of the stock solution (herbicide solubilized in 95% ethanol) for each herbicide concentration was added to 99 ml of cornmeal agar.

[b]Values are the means of four replications per treatment.

TABLE 2. The effect of commercial and technical grades of dinitramine or trifluralin on zoospore production by three isolates of *Aphanomyces euteiches* incubated in a salt solution (8) at 25 C for 16 hr

Herbicide	Concentration[a] (μg/ml)	Zoospores/ml per isolate (× 10^3) Isolate C	Isolate M	Isolate P
None (Ck)		101[b]	88	122
1% ethanol (Ck)		92	62	152
Dinitramine				
Technical	.06	0	2	47
	.12	4	3	45
	.25	2	0	42
Commercial	.06	40	7	60
	.12	15	3	48
	.25	5	6	42
Trifluralin				
Technical	.25	80	6	63
	.50	40	4	58
Commercial	.12	80	33	103
	.25	75	8	70
	.50	33	8	40
Tukey's test $w_{.05}$ =		53	17	44

[a]One ml of a stock solution (herbicide solubilized in 95% ethanol) for each herbicide concentration was added to 99 ml of a salt solution (8).

[b]Each value is the mean of three replicates with two subsamples per replicate for each treatment.

dinitramine, and 0.12 and 0.25 μg/ml for trifluralin. Initial concentration of zoospores was determined; the zoospores were incubated in herbicide-salt solutions in a water bath at 25 C and shaken at 150 strokes per minute for 1 hr.

Effect of herbicides on root infection.—The effect of commercial grades of dinitramine or trifluralin on infection of pea roots by *A. euteiches* and resulting disease severity was estimated by counting the number of dead pea plants 10-14 days after inoculation with zoospores with or without the respective herbicides in the suspension. Ten-day-old pea plants, cultivar Green Giant (GG) 549 grown in autoclaved vermiculite, were inoculated with 10,000 and 100,000 zoospores/ml of sterile distilled water alone or distilled water amended as follows: ethanol at 1%, trifluralin at 0.12 and 0.25 μg/ml, or dinitramine at 0.06 and 0.12 μg/ml. Plants were grown in treatment solutions for 8 hr after which they were transplanted into 360-ml paper cups (five plants per cup and five replicate cups per treatment). Plants were transplanted into individual cups which contained autoclaved vermiculite; these were watered until the vermiculite was saturated. Plants were incubated in a growth chamber at 25 C with a 16-hr photoperiod (18,000 lx). Plants were counted as dead when the foliage at the terminal node became necrotic.

Statistics.—Analysis of variance and Tukey's test, $P = 0.05$, was used to compare treatment means (9).

RESULTS

Effects of trifluralin and dinitramine on hyphal growth.—The technical and commercial grades of dinitramine both provided progressively greater inhibition of hyphal growth of the three isolates of *A. euteiches* as the concentration was raised from 0.25 to 1.0 μg/ml of CMA. Both grades of dinitramine were more inhibitory to *A. euteiches* than either technical or commercial grades of trifluralin. Isolates of *A. euteiches* did not differ in their reaction to both grades of each herbicide (Table 1). The hyphae of *A. euteiches* grown in the presence of either dinitramine or trifluralin branched abundantly, and the hyphal tips were curved or they became enlarged and globose (Fig. 1-B) compared to hyphae grown on CMA not amended with an herbicide (Fig. 1-A).

Effects of dinitramine and trifluralin on production and motility of zoospores.—All three isolates of *A. euteiches* produced fewer zoospores at all concentrations of commercial or technical grade dinitramine tested than in salt solutions with no herbicide (Table 2). Only isolate M produced fewer zoospores in all concentrations of

TABLE 3. The effect of commercial grade dinitramine or trifluralin on the production of zoospores and the motility of produced zoospores of *Aphanomyces euteiches* (isolate C) incubated at 25 C for 16 hr

Herbicide	Concentration[a] (μg/ml)	Zoospores/ml (× 10^3) Motile	Zoospores/ml (× 10^3) Nonmotile	Tukey's test $w_{.05}$ =
None (Ck)		58[b]	190	38
1% ethanol (Ck)		43	221	47
Dinitramine	0.03	0	140	35
	0.06	0	20	18
Trifluralin	0.12	0	205	32
	0.25	0	106	20
Tukey's test $w_{.05}$ =		22	49	

[a]One ml of a stock solution (herbicide solubilized in 95% ethanol) for each herbicide concentration was added to 99 ml of a salt solution (8).
[b]Each value is the mean of three replications with two subsamples per replicate for each treatment.

TABLE 4. The effect of commercial grade dinitramine and trifluralin on the motility of zoospores of *Aphanomyces euteiches* (isolate C) first produced in the absence of an herbicide and then incubated in the presence or absence of an herbicide at 25 C for 1 hr

		Zoospore concentration 1 hr after herbicide treatment		
		Zoospores/ml (× 10^3)		Tukey's test
		Motile	Nonmotile	$w_{.05}$ =
Herbicides	Concentration[a] (μg/ml)	Initial zoospore concentration		
		91[b]	99	25
None (Ck)		33	169	54
1% ethanol (Ck)		34	168	27
Dinitramine	0.03	13	98	34
	0.06	13	78	19
Trifluralin	0.12	14	126	25
	0.25	12	101	32
Tukey's test $w_{.05}$ =		16	61	

[a]Equal volumes of zoospores suspended in a salt solution (8) and dinitramine- or trifluralin-amended salt solutions were mixed to obtain the herbicide concentrations used in this study.
[b]Each value is the mean of three replicates with two subsamples per replicate for each treatment.

trifluralin tested. Trifluralin at 0.50 μg/ml was needed to reduce zoospore production by isolates C and P. The 1% ethanol treatment reduced zoospore production somewhat in isolate M, but not in C or P. Technical and commercial grades of dinitramine or trifluralin did not differ significantly in effects on zoospore production. The greatest differences were between isolates of *A. euteiches* and not between the specific activities of technical and commercial grades of the herbicide (Table 2).

No motile zoospores were produced by *A. euteiches* at any of the concentrations of dinitramine or trifluralin tested. The 1% ethanol-salt solution had some effect on zoospore production and motility, but was not statistically significant ($P = 0.05$). In concentrations of 0.03 and 0.06 μg of dinitramine/ml, fewer nonmotile zoospores were produced than in controls, but 0.25 μg of trifluralin/ml was needed to significantly reduce zoospore (nonmotile) production by *A. euteiches* (Table 3).

The effect of dinitramine and trifluralin on zoospore motility was determined also by introducing motile zoospores produced in an herbicide-free environment into a salt solution containing herbicides. After 1 hr of incubation, the number of motile zoospores decreased significantly and nonmotile zoospores increased in control treatments; there was no change in total zoospore number (motile + nonmotile) (Table 4). However, the number of motile zoospores decreased significantly for each herbicide and each concentration, but no significant differences were observed between herbicide concentrations. The number of nonmotile zoospores also decreased except with trifluralin at 0.12 μg/ml (Table 4).

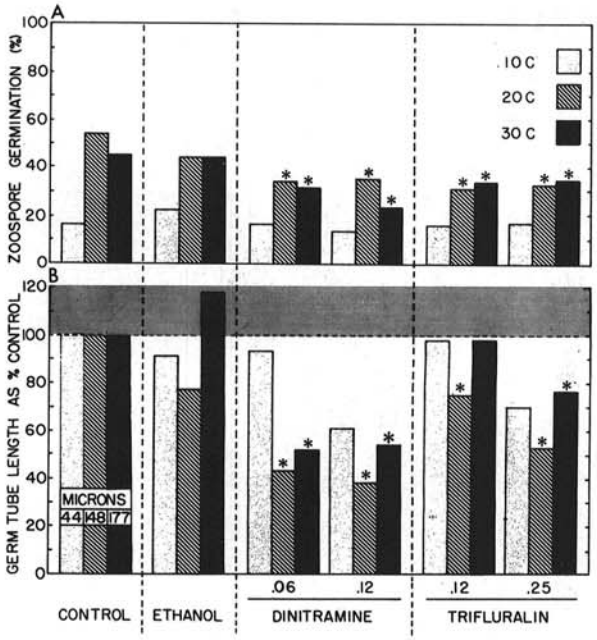

Fig. 2-(A, B). The effect of dinitramine at 0.06 and 0.12 μg/ml of peptone-maltose broth (PMB), trifluralin at 0.12 and 0.25 μg/ml of PMB, PMB + 1% ethanol, and PMB alone on **A)** zoospore germination and **B)** length of germ tubes as percentage of control all at 10, 20, or 30 C. Values are averages of 10 replicates of 30-50 zoospores per replicate (A) and five replicates of six germ tubes per replicate (B). Asterisks indicate significance at $P = 0.05$ using Tukey's test.

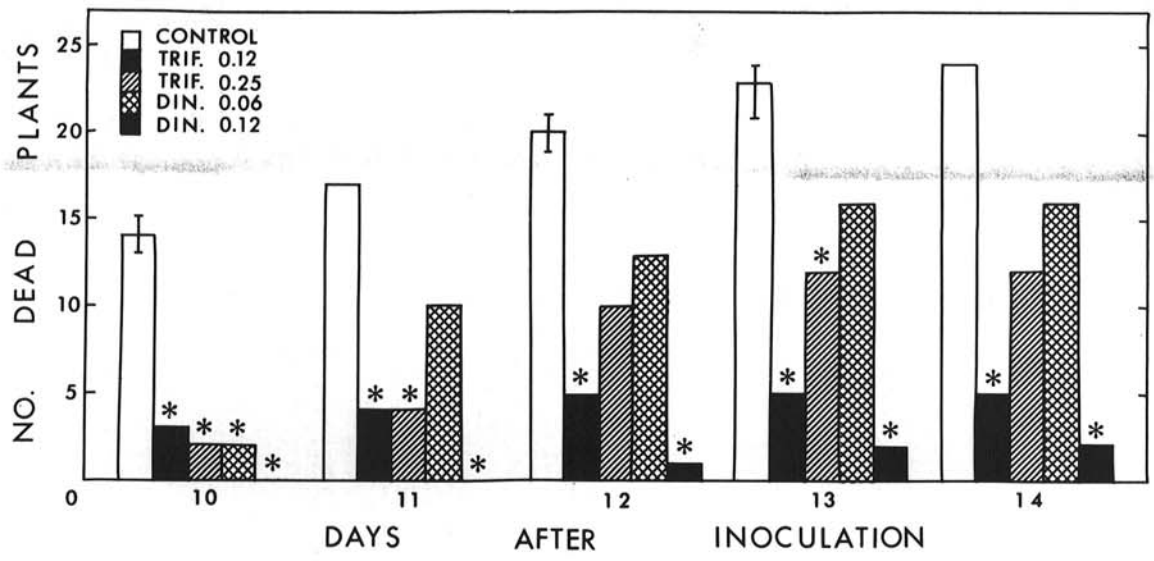

Fig. 3. Number of dead plants 10-14 days after inoculation with 10,000 zoospores of *Aphanomyces euteiches*/ml amended with: No herbicide; trifluralin at 0.12 and 0.25 μg/ml; and dinitramine at 0.06 and 0.12 μg/ml. Control values are a combined mean of the number of dead plants for the control (no herbicide) with and without 1% ethanol. The means of the two controls were not statistically different and vertical bars indicate the range of dead plants observed for the two control treatments. Values presented are the total number of dead plants observed for five replicates and five plants per replicate. Asterisks indicate a statistically significant difference from the control using analysis of variance and Tukey's test for comparisons of means at $P = 0.05$. There were no dead plants in the noninoculated control.

Lysis of zoospores by the herbicides may account for fewer, total zoospores (motile + nonmotile) after 1 hr of incubation compared to the controls.

Effect of trifluralin and dinitramine on germination and germ tube development of zoospores.—Zoospores were produced in the salt solution system without herbicides. A peptone-maltose broth was amended with dinitramine and trifluralin and then equal volumes of zoospore suspensions and herbicide-amended, peptone-maltose broth were mixed in petri dishes and incubated at 25 C for isolate C and 10, 20, and 30 C for isolate M. Controls consisted of zoospores in peptone-maltose broth with and without 1% ethanol. The suspensions were moved to a 4 C environment after 5 hr and examined for the percentage of germination in 10 microscope fields (× 100). Germ tubes of the first six germinated zoospores observed in five microscope fields (× 100) were measured, a total of 30 germ tubes for each treatment.

Neither dinitramine nor trifluralin reduced zoospore germination at 10 C, but both had a slight effect at herbicide concentrations tested at 20 and 30 C (Fig. 2-A). Ethanol did not affect zoospore germination.

Similarly with germ tube elongation, neither dinitramine nor trifluralin inhibited germ tube growth at 10 C. Trifluralin inhibited germ tube growth for 0.12 μg/ml at 20 C and 0.25 μg/ml at 20 and 30 C. Dinitramine was inhibitory at all concentrations at 20 and 30 C (Fig. 2-B). Ethanol inhibited germ tube growth slightly at 20 C.

Effect of trifluralin and dinitramine on infection of pea roots by zoospores.—Statistically significant differences were not observed for the total number of dead plants and the daily rate of plant death for plants inoculated with zoospores at both inoculum densities in the ethanol solution or sterile water. Dead plants were not observed in the noninoculated control.

Dinitramine or trifluralin had no effect on infection of pea roots by *A. euteiches* at an inoculum density of 100,000 zoospores/ml. However, when 10,000 zoospores/ml was amended with dinitramine or trifluralin, both at 0.12 μg/ml, fewer dead plants were observed compared to the inoculated control (no herbicide) for all days observed after inoculation (Fig. 3). Beginning at 10 days after inoculation, fewer dead plants were observed for all herbicides and concentrations compared to the inoculated control (no herbicide). However, the number of dead plants increased with time for dinitramine at 0.06 μg/ml and trifluralin at 0.25 μg/ml. Figure 4 compares the amount of root rot that occurred in plants inoculated with 10,000 zoospores/ml with and without dinitramine or trifluralin 14 days after inoculation.

DISCUSSION

Technical and commercial grades of dinitramine and trifluralin were inhibitory to growth and sporulation of *Aphanomyces euteiches*. Because no difference in inhibition of *A. euteiches* was observed between grades, dinitramine and trifluralin were concluded to be the inhibitors and not inert ingredients in the formulated commercial materials. Root rot was lower in severity, and subsequently, the number of dead plants were fewer when zoospore inoculum of *A. euteiches* was amended with commercial grades of dinitramine and trifluralin. The actual mode of action for this observation is unknown. The number of propagules might have been reduced by the herbicides and/or fewer zoospores germinated, and subsequently, fewer invasion sites occurred. The absorption of herbicides into the cortical cells may have reduced colonization of pea roots by *A. euteiches*. However, alteration of host physiology by the herbicides (such as the induction of phytoalexins, which could have resulted in lower root rot severity) cannot be discounted.

The observation that dinitramine was more effective than trifluralin in reducing root rot of pea caused by *A. euteiches* (4) could be explained by: (i) trifluralin being absorbed to soil particles more readily than dinitramine,

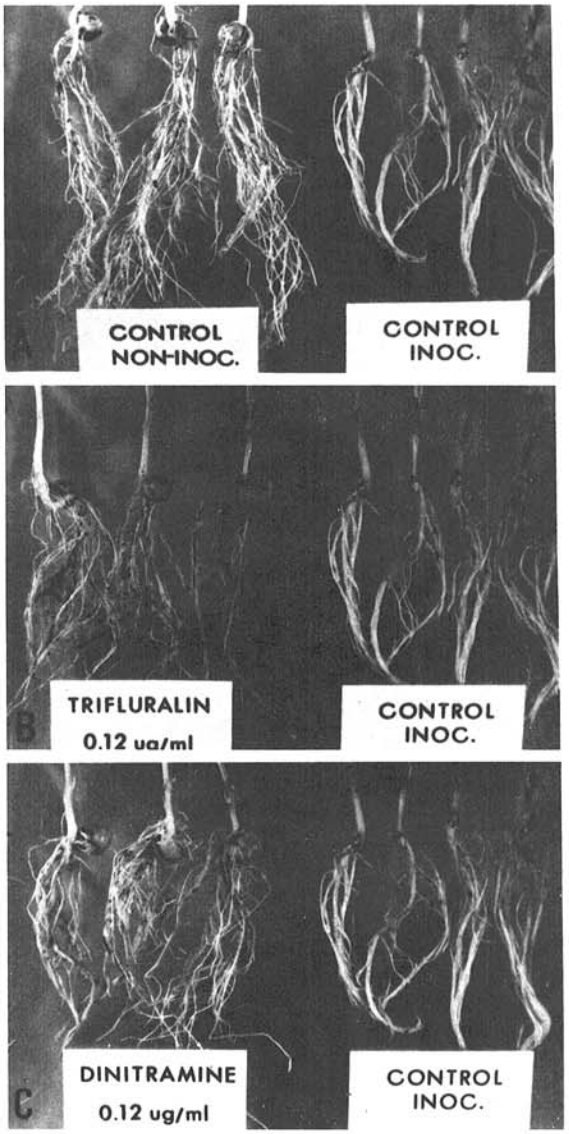

Fig. 4-(A to C). Comparison of root disease severity of pea inoculated with: **A)** no zoospores; **B)** 10,000 zoospores + 0.12 μg/ml trifluralin; and **C)** 10,000 zoospores + 0.12 μg/ml dinitramine.

resulting in a lower concentration of trifluralin in the soil solution (5); and (ii) dinitramine being more inhibitory than trifluralin to growth and sporulation of *A. euteiches*. Harvey (5) estimated the concentration of dinitramine or trifluralin to be approximately 0.02 and 0.01 μg/ml, respectively, in the soil solution if 0.32 kg of herbicide was applied per hectare. Dinitramine or trifluralin often are applied at 0.56-0.84 kg/ha raising the concentration of each herbicide in the soil solution to approximately 0.025-0.05 μg/ml. In my study dinitramine was inhibitory at 0.03 μg/ml but trifluralin was not. However, trifluralin is absorbed by carrot roots at concentrations up to 0.86 μg/ml in the outer cell layers (3). This concentration is well above the concentration of trifluralin found to be inhibitory in my study. Thus, it is possible that *A. euteiches* could encounter inhibitory concentrations of dinitramine or trifluralin in the soil solution and/or the root surface. Lower root rot severity of pea grown in the presence of dinitramine or trifluralin may be due in part to a direct effect of the herbicides on *A. euteiches* (4, 6, 7).

Harvey et al. (6) did not find trifluralin to be inhibitory to *A. euteiches* at low concentrations. Differences in isolates of *A. euteiches* and/or temperatures at which experiments were performed could account for this difference. Whether dinitramine or trifluralin affect oospore viability or germination of oospores of *A. euteiches* or the attraction of zoospores to pea roots is not known (2).

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