PESTICIDE EFFECT ON NITROGEN FIXATION AND NODULATION BY SOYBEAN AND LIMA BEAN

by

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CHAPTER I

INTRODUCTION

For centries farmers have recognized that the yield of cereal crops is higher when they are grown after certain legumes, such as clover or alfalfa. This beneficial effect is due not to the legume crop itself, but to associated microorganisms that are able to reduce atmospheric nitrogen gas to combined forms, which are utilized by the host plant. The enzyme complex involved, nitrogenase, requires Fe and Mo to be active.

Bacteria of the genus Rhizobium, in symbiosis with legumes, provide a major biological source of fixed nitrogen in soil. These bacteria invade plant's root hairs and cortical cells, ultimately inducing the formation of nodules that serve as a "home" for the organisms. The host plant supplies the bacteria with carbohydrates for energy, and the bacteria reciprocate by supplying the plant with fixed nitrogen compounds. The fixed nitrogen in the nodules can enrich the soil when plants die. There is considerable specificity between Rhizobium sp. and host plants.

Any factor, environmental or non-environmental, which reduces the efficiency of the Rhizobium-legume symbiotic process will, in turn, reduce the nitrogen supply, decrease the yields and/or require increased production input. Pesticides are an important part of modern agricultural production and can improve crop yield and quality. A variety of pesticides are currently registered for use on legume crops. Information available for their effects on rhizobia, and subsequent growth and nodulation of plants is incomplete and inconsistent.

Soybean (Glycine max L.) and lima bean (Phaseolus lunatus L.) are two of many widely grown legumes in the world. In 1987, 5.4 million acreas of soybeans were planted in the United States (Kansas Crop and Livestock Reporting Service, 1988). As the chief source of plant protein, it is perhaps the most important legume crop. It is also an important oil crop. Lima beans are cultivated mainly in tropics and it is a significant garden vegetable for American families. The seeds are high in protein and phosphorus (Yamaguchi, 1983). These two legumes were chosen as the test species due to their importance and widespread cultivation.

The objectives of the present study were: 1) determine the effects of 12 pesticides on N_2 fixation and nodulation of soybean and lima bean; and 2) study the mode of action of pesticides which affect N_2 fixation and nodulation of these plants.

CHAPTER II

EFFECTS OF TWELVE PESTICIDES ON NITROGEN FIXATION & NODULATION OF SOYBEAN & LIMA BEAN

LITERATURE REVIEW

The litreature concerning the effect of pesticides on N_2 fixation and nodulation by legume-rhizobia symbiosis is somewhat contradictory. Variations in test methods, soil type, pesticide application rate and method, the strain of Rhizobium used, and the technique of inoculation may have contributed to these conflicting reports (Goring & Laskowski, 1982).

Trifluralin is a widely used herbicide in soybean. A large volume of literature has developed around it. Parker et al. (1976) and Kust et al. (1971) found soybean nodulation was suppressed by trifluralin, whereas Bollich et al. (1985) reported trifluralin did not affect soybean C_2H_4 production, nodule number, or dry weight in four soils. A study by Alaa-Eldin et al. (1981) showed that dinoseb at label rate and trifluralin, especially at 5 times the rate, stimulated nodulation of soybean. Mallik & Tesfai (1985), however, reported that trifluralin at the recommended and 5x rates adversely affected nodulation and N_2 fixation. Baltazar & Brotonegro (1979) reported that trifluralin had no effect on N_2 fixation or nodulation of soybean when applied 10 days before sowing but was reductive when applied 5 days before seeding and inhibitory to both processes when applied at sowing.

Five herbicides (trifluralin, prometryne, alachlor, methabenzthiazuron and metribuzin) proved to decrease nodule number and nodule weight of faba bean (Vicia faba L.) (Islam & Afendi, 1980). Trifluralin and bentazon were the only two of several herbicides which did not depress nodulation of dry bean (Phaseolus vulgaris L.) (Fischer & Tasistro, 1981), but N_2 fixation was rapidly depressed after treatment with bentazon (Bethlenfalvay et al., 1979; Schnelle, 1986). Nodulation of red clover (Trifolium pratense L.) was also reduced by bentazon (Ljunggren & Martensson, 1980). Sethoxydim, another postemergent herbicide, did not affect N_2 fixation or nodule dry weight of dry bean, soybean, and alfalfa (Medicago sativa L.) (Hensley, 1987).

Likewise, inconsistency exists over the effect of fungicides on legumes. Curley & Burton (1975) found PCNB reduced Rhizobium survival on soybean seed and reduced taproot nodulation, whereas Mallik & Tesfai (1985) reported that PCNB was innocuous, even at 10 times recommended level. Same situation was reported for thiram (Abd El-Monem & El-Sawah, 1984; Chamber & Montes, 1982; Rennie & Dubetz, 1984; Singh & Srivastara, 1984; Tu, 1981) and captan (Abd

El-Monem & El-Sawah, 1984; Chember & Montes, 1982; Rennie & Dubetz, 1984) in soybean. Captan has been shown to reduce nodulation and nitrogenase activity in pea (Pisum sativm L.), lentil (Lens culinaris L.), and fababean (Rennie et al., 1985). Nodulation of cowpea (Vigna unguiculata L.) was not harmed by thiram, PCNB, and PCNB+terrazole, but was decreased by maneb, dicloran+capafol (Staphorst & Strijdom, 1976), and by benomyl, carbendazim, and pyracarbolid (Narayana et al., 1981). Thiram also had no inhibitory effect on nodulation of V. catjang (Vigna unguiculata Subsp. cylindrica (L.) Van Eselt. ex Verde.) (Murthy & Raghu, 1976).

Rodell et al. (1977) found no significant decrease in acetylene reduction of excised soybean nodules due to 4 organophosphate and 5 carbamate insecticides, but methomyl, propoxur, and carbofuran among the insecticides induced phytotoxic effects. Mallik & Tesfai (1985) reported carbaryl and malathion at recommended level had no adverse effect, but severely reduced N_2 fixation of soybean at 10 times recommended level. Acephate, diazinon, and toxaphene reduced N_2 fixation and total N content but not growth and nodulation of soybean at both levels. Carbaryl, diazinon, dicofol, and malathion did not cause any injury to dry bean in Schnelle's study (1986). Aggarwal et al. (1986) reported that low concentrations of six carbamate pesticides

(1-naphthol, sevin, dimetilan, trematan, NaDDC, and dymid) had little effect on N_2 fixation and nodulation of Pisum sativum L. and Vigna sinensis (Vigna unguiculata Subsp. sesquipedalis (L.) Verdc), but higher concentrations adversely affected both processes.

MATERIALS & METHODS

Nitrogen fixation study

Seeds of soybean (Glycine max L. Merrill cv. Williams 82)[1] and lima bean (Phaseolus lunatus L. cv. Geneva)[2] were inoculated with prepared inoculum of Rhizobium japonicum[3] and Rhizobium sp.[4], respectively, before planting. Seeds were germinated and grown in plastic cell packs (7.6 x 7.6 x 7.6 cm). Seedlings, thinned to one plant per cell, were

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[1]Soybeans were purchased from Kansas Crop Improvement Association, Manhattan, Kansas.

[2]Lima beans were purchased from Johnny's Selected Seeds, Albion, Maine.

[3]Rhizobium japonicum peat-based inoculum was purchased from Nitragin Company, Milwaukee, WS. The Rhizobium strains incorporated in the product were 61A101c and 61A118b.

[4]Rhizobium sp. strain 127E15 was kindly provided by Dr. Peter Wong, Division of Biology, Kansas State University.

grown under greenhouse conditions (16-hr, 30°C day; 8-hr, 25°C night) in a sand/loam media (5:1, v/v). The plants were periodically fertilized with nitrogen-free Hoagland solution (Hoagland & Arnon, 1950).

Plants were grown to three trifoliate leaf stage before exposure to postermergent fungicides, insecticides, or herbicides. Preemergent materials were applied the day of planting. All pesticides were applied at triple the manufacturer's recommended rate (3x) (Table 1) to the point of runoff with an aerosol sprayer, whereas bentazon, PCNB, and trifluralin were applied over the top of plants or soil in measured amounts of water. All control plants were sprayed with water only.

Nodules were harvested from treated and control plants 2 and 7 days after application of postemergent pesticides or 6 weeks after application of preemergent pesticides. To determine N_2 fixation ability of excised nodules, an acetylene reduction assay was performed utilizing modified techniques described by Hensley & Carpenter (1979).

Excised nodules were cleaned, blotted dried and placed in 22 ml stopped test tubes. Two ml of air were replaced with commercial grade acetylene. After one hour's incubation at 28°C, 0.5 ml of gas samples were withdrawn using 1 cc syringes and injected into a Varian 6000 automated gas chromatograph, fitted with a stainless steel

Table 1. Names[z] and rates (3x manufacturer's recommended rate) of pesticides evaluated for their influence on nitrogen fixation and nodulation of soybean and lima bean.

Common Name	Trade Name	Rate (3x)
Fungicides		
Postemergent		
captan	Captan	106.5 g/l
maneb	Maneb	8.1 g/l
zineb	Zineb	7.2 g/l
Preemergent		
PCNB	Terraclor	1.35 g/m^2
Insecticides		
Postemergent		
carbaryl	Sevin	15.62 ml/l
diazinon	Spectracide	115.20 ml/l
malathion	Malathion	11.72 ml/l
methomyl	Lannate	18.75 ml/l
permethrin	Ambush	30.00 ml/l
Herbicides		
Postemergent		
bentazon	Basagran	0.28 ml/m^2
sethoxydim	Poast	29.4 ml/l[y]
Preemergent		
trifluralin	Treflan	0.28 ml/m^2

[z] The chemical names of each pesticide are listed in Appendix 1.

[y] Appropriate amount of nonphytotoxic oil concentrate was added as surfactant.

Porapak R column. Temperatures of column, injector, and ionization were 50°C, 90°C, and 105°C respectively. Nodules were oven dried for 24 hours at 80°C before weighing.

Acetylene reduction data were expressed in nmoles ethylene per gram nodule dry weight per hour. Log transformations were conducted on data before statistical analysis using one-way analysis of variance (Ott, 1984).

Nodulation study

Seeds of soybean and lima bean were inoculated and planted as in the previous study. Pesticides and means of application were the same as previously described. Postemergent chemicals were applied when seedlings had one set of true leaves. Preemergent materials were applied the day of planting. All pesticides were applied at 3x label rate. Numbers and weights of oven-dried nodules were determined 4 weeks after treatment of postemergent chemicals or 6 weeks after treatment of preemergent chemicals. Data were statistically analyzed by means of one-way analysis of variance.

All treatments and controls were replicated 5 times and each study repeated. Chemicals which showed an effect on N_2 fixation (acetylene reduction) or nodulation were studied at recommended label rate (1x) in seperate evaluation using the same techniques.

RESULT & DISCUSSION

The response of N_2 fixation (acetylene reduction) by soybean to fungicides, insecticides, and herbicides are presented in Table 2, 3, and 4, respectively. Diazinon was the only pesticide investigated that decreased N_2 fixation within 2 days. However, this decrease in nitrogenase activity was not evident 7 days after application (Table 3). Diazinon also proved to be harmless within 2 days when applied at label rate (Table 3). None of the pesticides examined at 3x rate reduced the N_2 fixation ability of lima bean (Table 5, 6, and 7).

The effects of fungicides, insecticides, and herbicides on soybean nodulation are presented in Table 8, 9, and 10. Soybean nodule numbers were significantly less than control when methomyl (Table 9) and trifluralin (Table 10) were applied at 3x rate. Table 11, 12, and 13 are the results of examined pesticides on nodulation of lima bean. Trifluralin was the only pesticide that decreased lima bean nodule number (Table 13). However, the average dry weights of nodules were not affected in both plants. In seperate studies using label rates of these two suspect chemicals, trifluralin still depressed soybean nodulation (Table 10), while methomyl (Table 9) and trifluralin (Table 13) did not affect nodulation of soybean and lima bean, respectively.

Table 2. Effect of fungicides (3x rate) on nitrogen fixation (acetylene reduction) of soybean 2 and 7 days (for postemergent materials) or 6 weeks (for preemergent material) after application.

Fungicide	Time after Application	Nitrogen Fixation[z] (nmoles C_2H_4/g/hr)	Prob>F
<u>Postemergent</u>			
captan	2d	7272.3	0.34
control		4689.1	
captan	7d	1471.6	0.24
control		2248.3	
maneb	2d	2398.8	0.76
control		2702.0	
maneb	7d	2216.0	0.92
control		2257.1	
zineb	2d	7658.0	0.76
control		6697.3	
zineb	7d	3885.2	0.39
control		3322.4	
<u>Preemergent</u>			
PCNB	6wk	4309.2	0.97
control		4362.7	

[z] There were no statistical differences (F test, 0.05) when treatments were compared with appropriate controls.

Table 3. Effect of insecticides (3x rate or 1x rate if especially noted) on nitrogen fixation (acetylene reduction) of soybean 2 and 7 days after application.

Insecticide	Time after Application	Nitrogen Fixation (nmoles C_2H_4/g/hr)	Prob>F
carbaryl	2d	3430.8	0.88
control		3268.5	
carbaryl	7d	3953.2	0.27
control		2914.8	
diazinon	2d	3467.0**	0.00
control		15199.1	
diazinon	7d	4109.6	0.79
control		3290.3	
diazinon (1x)	2d	5160.0	0.32
control		3785.5	
diazinon (1x)	7d	4641.6	0.51
control		5997.7	
malathion	2d	4908.6	0.81
control		5370.9	
malathion	7d	3805.9	0.94
control		3683.2	
methomyl	2d	3004.4	0.36
control		1903.3	
methomyl	7d	1654.7	0.33
control		2199.0	
permethrin	2d	3547.3	0.28
control		5526.3	
permethrin	7d	12144.9	0.70
control		13307.7	

** Treatment means were significantly different (F test, 0.01) when compared with appropriate controls.

Table 4. Effect of herbicides (3x rate) on nitrogen fixation (acetylene reduction) of soybean 2 and 7 days (for postemergent materials) or 6 weeks (for preemergent material) after application.

Herbicide	Time after Application	Nitrogen Fixation[z] (nmoles C_2H_4/g/hr)	Prob>F
Postemergent			
bentazon	2d	8743.3	0.51
control		7472.4	
bentazon	7d	4669.6	0.20
control		7142.2	
sethoxydim	2d	6563.2	0.45
control		7293.4	
sethoxydim	7d	2997.5	0.59
control		3439.0	
Preemergent			
trifluralin	6wk	8268.9	0.18
control		4506.2	

[z] There were no statistical differences (F test, 0.05) when treatments were compared with appropriate controls.

Table 5. Effect of fungicides (3x rate) on nitrogen fixation (acetylene reduction) of lima bean 2 and 7 days (for postemergent materials) or 6 weeks (for preemergent material) after application.

Fungicide	Time after Application	Nitrogen Fixation[z] (nmoles C_2H_4/g/hr)	Prob>F
<u>Postemergent</u>			
captan	2d	6226.2	0.76
control		6687.3	
captan	7d	4488.3	0.87
control		4286.4	
maneb	2d	4859.4	0.55
control		5469.2	
maneb	7d	4264.0	0.76
control		3818.3	
zineb	2d	6161.5	0.10
control		2010.4	
zineb	7d	4066.2	0.22
control		1890.8	
<u>Preemergent</u>			
PCNB	6wk	931.6	0.27
control		1731.4	

[z] There were no statistical differences (F test, 0.05) when treatments were compared with appropriate controls.

Table 6. Effect of insecticides (3x rate) on nitrogen fixation (acetylene reduction) of lima bean 2 and 7 days after application.

Insecticide	Time after Application	Nitrogen Fixation[z] (nmoles C_2H_4/g/hr)	Prob>F
carbaryl	2d	2649.3	0.36
control		4404.3	
carbaryl	7d	2301.3	0.90
control		2148.1	
diazinon	2d	2860.9	0.65
control		3385.5	
diazinon	7d	2704.5	0.96
control		2835.3	
malathion	2d	3520.5	0.15
control		5552.9	
malathion	7d	3346.9	0.96
control		3466.4	
methomyl	2d	7538.4	0.14
control		5051.2	
methomyl	7d	4030.1	0.71
control		4655.2	
permethrin	2d	5127.5	0.32
control		6352.4	
permethrin	7d	4381.5	0.74
control		4919.5	

[z] There were no statistical differences (F test, 0.05) when treatments were compared with appropriate controls.

Table 7. Effect of herbicides (3x rate) on nitrogen fixation (acetylene reduction) of lima bean 2 and 7 days (for postemergent materials) or 6 weeks (for preemergent material) after application.

Herbicide	Time after Application	Nitrogen Fixation[z] (nmoles C_2H_4/g/hr)	Prob>F
Postemergent			
bentazon	2d	4750.9	0.20
control		7000.8	
bentazon	7d	1030.6	0.85
control		1202.8	
sethoxydim	2d	1519.4	0.56
control		2290.1	
sethoxydim	7d	2596.6	0.37
control		1737.7	
Preemergent			
trifluralin	6wk	5647.5	0.71
control		5227.0	

[z] There were no statistical differences (F test, 0.05) when treatments were compared with appropriate controls.

Table 8. Effect of fungicides (3x rate) on nodulation of soybean 4 weeks (for postemergent materials) or 6 weeks (for preemergent material) after application.

	Nodulation[z]			
Fungicide	avg. no. nod./ plant	Prob>F	avg. dry wt./ nodule(x10^{-3} g)	Prob>F
Postemergent				
captan	38.0	0.82	1.698	0.17
control	36.4		2.117	
maneb	60.0	0.90	1.206	0.24
control	61.8		0.994	
zineb	44.0	0.32	1.643	0.86
control	40.0		1.612	
Preemergent				
PCNB	28.6	0.50	1.532	0.46
control	33.0		1.322	

[z] There were no statistical differences (F-test, 0.05) when treatments were compared with appropriate controls.

Table 9. Effect of insecticides (3x rate or 1x rate if especially noted) on nodulation of soybean 4 weeks after application.

	Nodulation			
Insecticide	avg. no. nod./ plant	Prob>F	avg. dry wt./ nodule($\times 10^{-3}$ g)	Prob>F
carbaryl	31.2	0.41	2.060	0.31
control	25.4		2.809	
diazinon	36.8	0.39	1.705	0.35
control	44.2		2.120	
malathion	36.2	0.55	1.937	0.69
control	42.0		1.829	
methomyl	25.8*	0.04	1.880	0.78
control	41.0		1.806	
methomyl (1x)	41.8	0.61	1.953	0.46
control	37.2		1.737	
permethrin	34.2	0.61	1.842	0.14
control	37.8		1.546	

* Treatment means were significantly different (F test, 0.05) when compared with appropriate controls.

Table 10. Effect of herbicides (3x rate or 1x rate if especially noted) on nodulation of soybean 4 weeks (for postemergent materials) or 6 weeks (for preemergent material) after applicatioin.

Herbicide	Nodulation			
	avg. no. nod./ plant	Prob>F	avg. dry wt./ nodule($x10^{-3}$ g)	Prob>F
Postemergent				
bentazon	23.4	0.26	1.323	0.53
control	33.2		1.184	
sethoxydim	71.2	0.76	1.182	0.66
control	74.2		1.231	
Preemergent				
trifluralin	3.6**	0.00	1.863	0.60
control	18.0		1.448	
trifluralin (1x)	12.0**	0.01	0.842**	0.01
control	22.6		1.662	

** Treatment were significantly different (F test, 0.01) when compared with appropriate controls.

Table 11. Effect of fungicides (3x rate) on nodulation of lima bean 4 weeks (for postemergent materials) or 6 weeks (for preemergent material) after application.

	Nodulation[z]			
Fungicide	avg. no. nod./ plant	Prob>F	avg. dry wt./ nodule($x10^{-3}$ g)	Prob>F
Postemergent				
captan	146.0	0.30	0.684	0.77
control	108.4		0.637	
maneb	132.2	0.85	0.510	0.75
control	127.2		0.542	
zineb	144.0	0.63	0.439	0.46
control	125.0		0.648	
Preemergent				
PCNB	90.6	0.67	0.463	0.31
control	102.2		0.366	

[z] There were no statistical differences (F test, 0.05) when treatments werer compared with appropriate controls.

Table 12. Effect of insecticides (3x rate) on nodulation of lima bean 4 weeks after application.

Insecticide	Nodulation^z^ avg. no. nod./plant	Prob>F	avg. dry wt./nodule ($x10^{-3}$ g)	Prob>F
carbaryl	122.8	0.79	0.499	0.63
control	114.0		0.439	
diazinon	111.2	0.96	0.540	0.84
control	109.5		0.571	
malathion	185.2	0.21	0.390	0.28
control	123.0		0.500	
methomyl	99.0	0.77	0.498	0.80
control	110.0		0.459	
permethrin	136.6	0.44	0.471	0.99
control	114.0		0.470	

[z] There were no statistical differences (F test, 0.05) when treatments were compared with appropriate controls.

Table 13. Effect of herbicides (3x rate or 1x rate if especially noticed) on nodulation of lima bean 4 weeks (for postemergent materials) or 6 weeks (for preemergent material) after application.

	Nodulation			
Herbicide	avg. no. nod./ plant	Prob>F	avg. dry wt./ nodule($\times 10^{-3}$ g)	Prob>F
Postemergent				
betazon	108.8	0.71	0.443	0.82
control	94.4		0.422	
sethoxydim	66.2	0.90	0.785	0.45
control	69.0		0.624	
Preemergent				
trifluralin	34.2**	0.01	0.708	0.21
control	70.4		0.442	
trifluralin (1x)	55.7	0.74	0.893	0.44
control	60.5		o.701	

** Treatment means were significantly different (F test, 0.01) when compared with appropriate controls.

The data indicated that four fungicides (captan, maneb, PCNB, and zineb), three insecticides (carbaryl, malathion, and permethrin), and two postemergent herbicides (bentazon, and sethoxydim) investigated were innocuous to N_2 fixation and nodulation of soybean and lima bean. Comparing these results with earlier investigations mentioned in literature review, some disagreements are found to exist. These are acceptable, as suggested by Wax et al. (1974) and Mallik & Tesfai (1983 & 1985), because of variations of plant cultivars and rhizobial strains used in the studies. Some researchers used pesticides at extreme rates (5 or 10 times the recommended level) in their studies (Alaa-Eldin et al., 1981; Mallik & Tesfai, 1985). These excessive rates are not likely to be present in a production situation and were not evaluated in the present study. However, the 3x label rate used here is more realistic after several application of the pesticides under intensive cultivation.

Compared the results between soybean and lima bean, it is found that differences exist between plants' response to the same pesticide. Summarizing, lima bean appeared to be less sensitive to pesticides. For example, soybeans treated with 3x rate of sethoxydim at the first trifoliate leaf stage displayed "bronzing" of exposed leaves 1 day after application, but no such injury was noticed in lima bean.

This may be due to a more waxy leaf surface of lima bean, which is an effective protective barrier against chemicals. The surface of soybean is hairy.

Trifluralin, a preemergent herbicide, had dramatic effect on plants when applied at the 3x rate. Soybean root growth was obviously limited, especially the development of lateral or secondary roots (Fig. 1). The roots were somewhat thickened, stubby, and devoid or had only a limited number of secondary roots. Shoot growth was also restricted (Fig. 2). This was probably the result by limited root growth. The anatomical study by Kust & Struckmeyer (1971) noticed xylem occlusion in the roots and internode of trifluralin-treated soybean. They suggested a direct relationship between root effects and topgrowth effects because reduction in water transport may decrease mineral nutrient transport to the tops. In lima bean there were few morphological differences between control and treated plants.

Nodulation data showed trifluralin to be detrimental to the nodulation of tested legumes, especially in soybean. Average nodule number per herbicide treated soybean was 5 times less than that of per control plant (3.6 vs. 18.0). It was about twice less (34.2 vs. 70.4) in the case of lima bean. Even recommended rate of trifluralin had adverse effect on nodulation of soybean. This result agrees with

Fig. 1. Soybean root growth 6 weeks after 3x rate of trifluralin treatment (right) and control (left).

Fig. 2. Soybean shoot growth 20 days after 3x rate of trifluralin treatment (left) and control (right).

the study by Mallik & Tesfai (1985). Since trifluralin is a major herbicide for weed control in soybean, its application rate should be carefully evaluated. Label rate of trifluralin was safe to lima bean. It may be concluded that soybeans are more sensitive to trifluralin than lima beans.

Once nodules were successfully formed on the roots after trifluralin treatment, N_2 fixation ability per gram nodule was not significantly different from controls (Table 4, and 7). Although the data were not statistically different, N_2 fixation ability of soybean nodules grown in trifluralin-treated soil were about 1.8 times higher than that of nodules grown in untreated soil (8,268.9 vs. 4,506.2, prob>F: 0.18). Whether this was a means of treated soybean plants to compensate for poor nodulation needs further study.

Bentazon, a Hill reaction inhibitor, has proven to inhibit N_2 fixation capacity of dry bean (Phaseolus vulgaris L.) shortly after treatment (Bethlenfalvay et al., 1979; Schnelle, 1986). The inhibition was not caused by bentazon directly, but indirectly through limiting the availability of photosynthate to support root nodule activity (Bethlenfalvay et al., 1979). However, the present data showed that betazon had no effect on N_2 fixation of soybean or lima bean. Penner (1972) related selectivity of bentazon between tolerant and susceptible plants to less spray retention on the leaf and a higher rate of bentazon metabolism in tolerant plants.

Application of 3x rate of diazinon on young plants seemed to promote branching because of the growth of lateral buds. This may be due to the damage of apical buds by high concentration of diazinon, which in turn reduced apical dominance of terminal buds. However, nodulation of plants were not affected. Diazinon at 3x rate exhibited short-term depressive effect on N_2 fixation in soybean (Table 3), but not in lima bean (Table 6) and dry bean by Schnelle's study (1986). This is another example exhibiting differential pesticidal response among species. Diazinon was safe to use when applied as labeled.

CHAPTER III

MODE OF ACTION OF DIAZINON, METHOMYL, AND TRIFLURALIN IN DEPRESSING N_2 FIXATION AND NODULATION OF SOYBEAN & LIMA BEAN

INTRODUCTION

Previous studies showed that three chemicals, diazinon, methomyl and trifluralin, among 12 pesticides had inhibitory effects on N_2 fixation or nodulation of tested plants. Diazinon depressed N_2 fixation ability of excised nodules in soybean, but not lima bean. Trifluralin reduced nodulation of soybean and lima bean, while methomyl depressed nodulation only in soybean.

LITERATURE REVIEW

The cause of reduced nodulation and N_2 fixation by pesticides has not been extensively studied. Some decline has been associated with impaired foliage or root growth by the host plants (Fischer & Tasistro, 1981; Kumar et al., 1981) or reduction of energy supply to the nodules (Bethlenfalvay et al., 1979; Ljunggren & Martensson, 1980). Some researches have studied the effect of chemical directly on <u>Rhizobium</u>. A major difficulty encountered in assessing the influence of pesticides on rhizobia is the interdependency of the bacterium and the host plant. To circumvent this dilemma, researchers have resorted to pure culture studies. In the study of Kapusta & Rouwenhorst (1973), commercial formulations of 24 pesticides were screened for bacteriostatic activity on R. japonicum using a gradient plate technique. Chlorpropham and disulfoton were the only two pesticides that inhibited growth. They found there was a marked difference in resistance of R. japonicum strains to various pesticides. This was also observed by Staphorst et al. (1976) as well as Tu (1980) and Mallik et al. (1983).

Staphorst et al. (1976) evaluated 13 fungicides for toxicity to two rhizobia strains of the "cowpea" group. The least toxic chemicals were PCNB, benomyl, and terraclor+terrazole. Thiram, mancozeb, and maneb+$ZnSO_4$ appeared to be the most toxic. Tu (1980) used 3 strains of R. japonicum and 14 fungicides and found strain 3I1b110 was the most resistant strain. Thiram was the most toxic chemical, followed by captan, zineb, and then by chlorothalonil, dodine, folpet, and benomyl. Mallik & Tasfai (1983) evaluated 10 strains of R. japonicum and 13 pesticides. The herbicides alachlor and trifluralin inhibited growth of all strains. Diazinon was non-inhibitory among the three insecticides tested. Carbaryl produced inhibition only at 250 μg/ml level, but malathion proved inhibitory at all concentrations. In fungicides, captan, thiram, mancozeb, and carboxin were toxic, but fenaminsulf and PCNB proved non-toxic even at the highest concentration tested.

Smith et al. (1978) reported that seven organophosphate and carbamate insecticides did not inhibit growth of R. meliloti or R. trifolii in broth culture.

MATERIALS & METHODS

Nitrogenase study

This study was conducted to determine direct effect of diazinon on the function of nitrogenase in soybean nodules. Seeds of soybean were inoculated, sown, and seedlings grown as described earlier. Nodules of untreated soybean were removed from the roots when the plants were at trifoliate leaf stage. Detached nodules were placed in 22 ml test tubes containing distilled water or 3x, 1x, 0.5x, or 0.25x label rates of diazinon (Table 14). Nodules were vacuum infiltrated by faucet aspiration for 1 hr to allow for the penetration of chemical. Nodules were then removed, blotted, and placed in 22 ml test tubes. After one hour acetylene reduction assay was performed. Data were comapred with untreated controls. Five replications were used and

Table 14. Pesticides and rates used in the study of mode of action.

Pesticide	Rates	
diazinon	0.25x	9.6 ml/l
	0.5x	19.2 ml/l
	1x	38.4 ml/l
	2x	76.8 ml/l
	3x	115.2 ml/l
methomyl	1x	6.25 ml/l
	2x	12.50 ml/l
	3x	18.75 ml/l
trifluralin	1x	1 ppm
	2x	2 ppm
	3x	3 ppm

the study was repeated. Log transformations were conducted on data before statistical analysis using one-way analysis of variance.

Rhizobium study

In order to determine if suspect chemicals affected rhizobia populations directly, a modified microbial "disc inhibition test" method similar to that described by Tu (1980) and Mallik & Tesfai (1983) was used.

Rhizobium japonium strain 61A118 and R. sp. strain 127E15, kindly provided by Dr. Peter Wong, were grown on yeast extract mannitol agar (YEMA) (Vincent, 1970) (Appendix 2). The cell suspensions of Rhizobium cultures were obtained by inbucation in YEM broth for 1-2 days at 28^{o}C on a shaker. Pesticides, methomyl and trifluralin, that showed nodulation inhibition in soybean and lima bean were tested. Filter paper discs (8 mm diameter) were soaked in prepared solutions of methomyl and trifluralin at 1, 2, and 3x the recommended rates (Table 14) for 45 min. Control discs were soaked in distilled water.

Plates of yeast extract mannitol agar were seeded by 0.1 ml of the inoculum (about 10^7 cells/ml) with 5 ml of unsolidified YEMA (0.75% agar) and spread uniformly. After air drying, four filter paper discs containing different concentrations of a pesticide were placed on the plate, equidistant from each other. Plates were incubated at 28°C for 7 days and the zones of growth inhibition surrounding the discs were measured and compared to controls. The presence of inhibition zone indicated the growth of Rhizobium was adversely affected by the pesticide at that concentration. There were five replications of each plate and each study was repeated.

RESULTS & DISCUSSION

Nitrogenase Study

Statistical analysis showed that nitrogenase activities of excised nodules were severely affected by diazinon at all concentrations tested (Table 15). This strongly indicated that diazinon may have harmed the function of nitrogenase directly. The concentration of diazinon within the imbibed nodules, however, was likely many times greater than what would occur from foliar application and absorption. This may explain why nitrogenase activity was significantly depressed in nodules imbibed with 1x label rate of diazinon, but it was not affected at the same rate by foliar application in previous study. The actual concentration of diazinon in plant tissues was very likely lower than 0.25x the imbibition

Table 15. Nitrogenase activity (acetylene reduction) by nodules of soybean vacuum infiltrated with diazinon.

	Rate	Nitrogenase Activity (nmoles C_2H_4/g/hr)	Prob>F
diazinon	3x	159.8**	0.00
control		2432.3	
diazinon	1x	63.3**	0.00
control		8251.3	
diazinon	0.5x	55.2**	0.01
control		1102.9	
diazinon	0.25x	119.2*	0.04
control		1102.9	

** Treatment means were significantly different (F test, 0.01) when compared with appropriate controls.

* Treatment means were significantly different (F test, 0.05) when compared with appropriate controls.

solution. This may be due to the loss during absorption, translocation, and metabolism. Previous study showed that diazinon, an organophosphate insecticide functioning as enzyme inhibitor in animal nervous system, has a short half-life (Ruzicka et al., 1967). It is rapidly metablized, in part, to the final metabolite CO_2 by series pathways in plants (Ralls et al., 1966). The study in Chapter II showed N_2 fixation of soybean was depressed 2 days after application of 3x rate of diazinon, but the depression was recovered at the 7th day. The short-term N_2 fixation inhibition may be due to the non-persistent character of diazinon in plants.

The inside of active legume nodules has a pink color because of the presence of leghemoglobin, which was first recognized by Kubo (1939). Leghemoglobin is thought to transport O_2 into the bacteroids at carefully controlled rates. Too much O_2 will inactivate nitrogenase, yet some O_2 is essential for bacteroid respiration (Salisbury & Ross, 1985). Although there is no quantitative data to support it, all of the nodules cut open after diazinon treatment were still pink, indicating that the function of leghemoglobin was probably not interrupted by diazinon. The study of Kulkarni et al. (1974) found four soil-applied insecticides had no significant effect on the leghemoglobin content in peanut nodules. Thimet and dasanite, among the four tested insecticides, are classified in the same group, organophosphate, as diazinon.

Rhizobium Study

Effects of trifluralin and methomyl on growth of two rhizobia by disc inhibition test is presented in Table 16. Both chemicals proved to be non-toxic to the tested rhizobia even at the highest concentration. Mallik & Tesfai (1985) reported that trifluralin inhibited growth of 10 strains of *R. japonicum* in their study. Many investigators (Kapustra & Rouwenhorst, 1973; Mallik & Tesfai, 1985; Tu, 1980 & 1981) agreed that the degree of pesticidal inhibition of rhizobia varies among strains. *R. japonicum* strain 61A118 used here seemed to be more resistant to trifluralin than 10 strains used by Mallik & Tesfai.

Nodulation is a symbiotic relationship, consequently, factors affecting either the plant or bacteria will affect nodule formation (Kapustra & Rouwenhorst, 1973). Since the results of "disc inhibition test" showed that these suspect chemicals did not inhibit the growth of two rhizobia, it appeared that the pesticidal influence on nodulation was due to altered plant physiological reactions rather than a direct toxicity to *Rhizobium*.

Many studies have shown that trifluralin can be absorbed by root and emerging shoot of germinating crops

Table 16. Effect of trifluralin and methomyl on growth of two rhizobia by disc inhibition test. Figures represent mean inhibitioin zones from disc in mm. Neither chemical inhibited growth of rhizobia.

Pesticide	Rate	*R. japonicum* Strain 61A118	*R.* sp. Strain 127E15
trifluralin	0x	0	0
	1x	0	0
	2x	0	0
	3x	0	0
methomyl	0x	0	--
	1x	0	--
	2x	0	--
	3x	0	--

(Hawxby et al., 1972; Parker, 1966; Penner, 1971). It is degraded very slowly in most higher plants. Much of the degradation occurs in the soil and the degradation products are subsequently absorbed by the plants. Trifluralin can interfere with cell division. It acts by binding to tubulin, which in turn prevents tubulin assembly into microtubules (Bartels & Hilton, 1973; Bayer et al., 1967; Hess & Bayer, 1974). Microtubules are functional elements of the spindle apparatus during cell division. Anatomic section of primary root of cotton made by Bayer et al. (1967) from trifluralin-treated soil showed that lateral root formation was initiated but stopped due to the inhibitory effect of trifluralin on cell division in the pericycle. Nodulation depression in legumes caused by trifluralin can be realized since nodules are from the division and growth of cortical and pericycle cells.

Depression of soybean nodulation by 3x rate of methomyl was not explained by this study, since methomyl did not inhibit the viability of R. japonicum. Previous studies proved that methomyl could be degraded to CO_2 and acetonitrile in tobacco, cotton, and cabbage (Harvey & Reiser, 1973) or in soil by microbial action (Harvey & Pease, 1973). Further investigations are needed to determine methomyl effect on soybean nodulation. Since N_2 fixation ability and nodulation were not affected by 3x and

1x rate of methomyl, respectively, it is assumed that methomyl is safe to be used in soybean.

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Appendix 1. Chemical names of the pesticides used in the studies.

Common Name	Chemical Name
Fungicides	
captan	N-(trichloromethylthio)-cyclohex-4-ene-1,2-dicarboximide
maneb	manganese ethylenebis(dithiocarbamate)
PCNB	pentachloronitrobenzene
zineb	zinc ethylenebis(dithiocarbamate)
Insecticides	
carbaryl	1-naphthyl methylcarbamate
diazinon	O,O-diethyl O-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate
malathion	diethyl mercaptosuccinate S-ester with O,O-dimethyl phosphorodithioate
methomyl	S-methyl N-(methylcarbamoyloxy) thioacetimidate
permethrin	3-phenoxybenzyl (1RS)-cis,trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate
Herbicides	
bentazon	3-(1-methylethyl)-(1H)-2,1,3-benzothiadiazin-4(3H)-one 2,2-dioxide
sethoxydim	2-[1-(ethoxyimino)butyl]-5-[2-(ethylthio) propyl]-3-hydroxy-2-cyclohexen-1-one
trifluralin	α,α,α-trifluoro-2,6-dinitro-N,N-dipropyl-p-toluidine

Sources:
1. The Pesticide Manual. 1979. BCPC Publication. Croydon. CR02TB.
2. BASF Product Information Guide. 1987. BASF Corporation, Parsippany, New Jersey.

Appendix 2.

Yeast Extract Mannitol Broth:

K_2HPO_4	0.5g
$MgSO_4 \cdot 7H_2O$	0.2g
NaCl	0.1g
Mannitol	10.0g
Yeast extract	4.0g
distilled water	900ml

Autoclave at 120°C for 15 min. The solid medium (yeast extract mannitol agar) contains 15 g agar/l.

PESTICIDE EFFECT ON NITROGEN FIXATION AND NODULATION BY SOYBEAN AND LIMA BEAN

by

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AN ABSTRACT OF A THESIS

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Effects of four fungicides, five insecticides, and three herbicides on N_2 fixation and nodulation of soybean (Glycine max L. Merrill cv. Williams 82) and lima bean (Phaseolus lunatus L. cv. Geneva) were studied. All examined pesiticides at 3x manufacturer's recommended rate were found innocuous to N_2 fixation (acetylene reduction) of both tested plants except diazinon. It decreased N_2 fixation of soybean 2 days after application, however, this decrease was not evident 7 days after treatment. Diazinon was proven to be harmless when applied at label rate. N_2 fixation of lima bean was not affected by 3x rate of diazinon.

Diazinon's possible mode of action on N_2 fixation in soybean was investigated by directly exposing nodules to the chemical. The data showed that nitrogenase activity of excised soybean nodules was severly affected by diazinon at 3x, 1x, 0.5x, and 0.25x label rates. This indicated that diazinon may have affected the function of nitrogenase directly. The concentration of diazinon within the imbibed nodules, however, was likely many times greater than what would occur from foliar application and absorption. Previous studies showed that this chemical has a short half-life and is rapidly metabolized in plants. The short-term N_2 fixation inhibition in soybean (within 2 days, but not at the 7th day) may be due to the non-persistent character of diazinon.

Soybean nodule numbers were significantly decreased by the treatments of 3x rates of methomyl and trifluralin, while lima bean nodule numbers were decreased only by trifluralin treatment. There were no difference in the average dry weights of nodules between controls and treatments in both plants. In seperate study by using the label rate of these two suspect chemicals, trifluralin still depressed nodulation of soybean, but it was safe to lima bean. Methomyl proved to be harmless to soybean nodulation at the label rate.

A "disc inhibition test" was undertaken to examine if trifluralin and methomyl affected rhizobial populations directly. Both chemicals were proved to be non-toxic to Rhizobium. It suggested the pesticidal influence on nodulation was due to altered plant physioligical reactions. Previous studies found trifluralin could interfere with cell division by binding to tubulin, thus preventing its assembly into microtubules. Nodulation depression in legumes may be realized since nodules are from the division and growth of root cortical and pericycle cells. Depression of soybean nodulation by 3x rate of methomyl was not explained by this study.