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EFFECT OF PESTICIDES ON NITROGEN FIXATION ACTIVITY OF LENTIL (*LENS CULINARIS* MEDIK.)

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Abstract

Relative compatibility of two selected pesticides at two levels of application (recommended dose and four times of recommended dose) with lentil-rhizobia symbiosis was tested. Better growth was observed in the Melandaha soil than in Bhatpara soil. Detrimental effect was more pronounced in Bhatpara soil. Both the pesticides proved detrimental at four times of recommended dose to nodulation and N_2 fixation. The detrimental effect was more severe when sterile soil was inoculated. Nodulation in Bhatpara soil was decreased by more than 44 and 54% with diazinon in sterile and inoculated soils respectively, while the N_2 fixation was decreased by ca. 12 and 67%, respectively under similar conditions.

Introduction

Symbiotic nitrogen fixation depends upon a highly coordinated sequence of interaction between a legume and soil microorganisms belonging to the genera *Rhizobium* and *Bradyrhizobium*. Therefore, when considering the effects of pesticides on symbiotic nitrogen fixation emphasis should be given to the effect of both the *Rhizobium* and the host plant. Pesticides find entry in legume cultivation mainly as seed dressings or as pre-emergent soil application. In addition to their specific purpose, they may affect the inoculant rhizobia, or soil populations of rhizobia, or they may interfere with infection, nodule initiation and development.(1) Among other factors that have been investigated with respect to the survival of rhizobia, possible effect of pesticides drew attention of several authors. Kapusta and Rouwenhorst(2) studied the effects of chlorpropham, nitralin, disulfoton and carbaryl at different concentrations on the growth of six strains of *B. japonicum*. The first two are herbicides and latter two are insecticides. The *B. japonicum* strains investigated exhibited differential sensitivity to the chlorpropham, disulfoton and carbaryl but not to nitralin. Growth was found to be completely hindered by chlorpropham at 48 ppm, disulfoton at 24 ppm and carbaryl at 40

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ppm. Lin *et al.*[3] tested nine insecticides (six organophosphates and three carbamates) for their effect on growth of four species of rhizobia. *R. leguminosarum* and *R trifolii* were found to be most sensitive to the pesticides. *R. meliloti* and particularly *B. japonicum* were resistant to the insecticides. Rahman *et al.*[4] studied the inhibition by the four pesticides viz. malathion, diazinon, dursban, nogos and furadon on the growth of soil *Rhizobium* Le-735. They found that the spectrum of inhibition increased with increasing concentration of pesticides.

However, laboratory results from pure culture studies are inconclusive in predicting field behavior of the pesticides. Results vary mainly because of variation in soil types and other edaphic conditions. Using normal rate of dieldrin and lindane (59.25 kg/ha and 5.26 kg/ha, respectively), Selim *et al.*[5] observed the effects of pesticides on nodule formation of faba bean (*Vicia faba*), and concluded that although treated plants had fewer effective nodules, these rate did not harm symbiotic nitrogen fixing bacteria. Similar reduction in number of nodules was observed with aldrin on bengal gram (*Cicer arietinum*) by Kapoor and Singh.[6] But in this case the authors observed aldrin to decrease nitrogen fixation and yield even at the recommended dose. They, however, correlated the effect of aldrin with organic matter added to the soil and showed that the ill effect was completely eliminated in the presence of added organic matter.

The present study was designed to determine the extent of inhibition of selected pesticides on the nitrogen fixation activities. Pesticides were applied at two levels (recommended dose and four times of recommended dose) to lentil-rhizobia symbiosis growing on two selected soils. The objective of the experiment was to get an idea of *in vitro* effect of pesticides on pure culture of *Rhizobium* as well as the *in situ* effect of pesticides on its symbiotic relationship with its host plant.

Materials and Methods

Two soils representing Melandaha and Bhatpara series were used in the present study. Both the soils were collected from adjacent areas of Savar. A composite sample was collected to a depth of 15 cm in both the cases. Before use, each sample was screened for debris, roots and other dead plant materials and sieved in a field moist condition to pass through a 2 mm sieve. Sub samples were removed and stored at subzero temperature in sealed polyethylene bags for studies requiring field moist and freshly air-dried soil samples. The remainder of each of the samples was air dried, crushed gently to pass a 2 mm screen, and stored at room temperature in a tightly sealed plastic container.

The two soils were found as sandy loam and a silty clay loam after particle size analysis performed by hydrometer method as described by Day[7] and subsequent textural class determined by following Marshall's triangular co-ordinates as designed by USDA.[8] The water holding capacity of soils was found to be in the range 49.6 - 58.8% as determined by the method described by Harding and Ross.[9] Results of soil analysis are presented in Table 1.

Table 1. Results of analysis of two soils used in the current studies.

Soil series	Particle size distribution			Textural class	Water holding capacity (%)	CEC meq/100	pH	OC %	OM %	Total N %	C/N ratio
	Sand	Silt	Clay								
Melandaha	56.2	39.5	4.3	Sandy loam	49.6	6.98	6.7	0.91	1.59	0.09	10.11
Bhatpara	9.8	55.8	34.4	Silty clay loam	56.8	11.42	6.3	1.59	2.74	0.12	13.25

pH of the soils was measured by a combined electrode pH meter at a soil: water ratio of 1 : 2.5 as suggested by Jackson.[10] Soil organic carbon was determined by Walkley and Black's wet oxidation method as outlined by Jackson.[10] The total nitrogen content of the soils was determined by alkali distillation of the Kjeldhal digest with 40% NaOH, the NH_3 evolved being absorbed on 4% boric acid with mixed indicator and the absorbed NH_3 was estimated by back titration with H_2SO_4 as described by Jackson.[10]

Available soil phosphorus was extracted by 0.5*M* $NaHCO_3$ and phosphorus content of the extracts were determined colorimetrically using ascorbic acid as the reductant as described by Murphy and Riley.[11] C. E. C. of the soil was determined using neutral 1*N* NH_4OAc method.[12]

Two generic group *viz.* the organophosphate and chlorinated hydrocarbon, were used in the present experiment. The pesticides used in the experiment were diazinon (*O,O*-diethyl-*O*-[2 isopropyl-4-methylpyrimidyl-6] thiophosphate) and heptachlor (1,4,5,6,7,8, 8-heptachloro- 3a,4,7,7a-tetrahydro-4, 7-methanoindene) (Table 2).

Lentil (*Lens culinaris.* Medic.) was taken as the test plant and cross inoculating *Rhizobium leguminosarum* as inoculant for studying the effect of pesticides both on symbiotic nitrogen fixation and nitrogen fixing bacteria. The whole study was divided into various segments as described in the following part.

Isolation of Rhizobium from root nodule: Rhizobium strain was isolated from lentil plant growing on soil of Melandaha series. Isolation was performed as described by Clark[13] using yeast extract mannitol agar.

Isolated typical *Rhizobium* colonies were distinguished and subcultured for purification. When pure culture was obtained, it was maintained on YMA slants in tightly stoppered screw capped container.

Determination of purity of Rhizobium from other bacteria: The isolated colonies thus subcultured were tested for their purity in YMA medium containing 0.0025% Congo red.[14] Congo red of 1% solution was sterilized separately and added to the YMA medium at the rate of 2.5 ml/l. The medium was then plated and after solidification, the plates were streaked with loopful of *Rhizobium* culture and incubated at 28°C. Plates were read for reaction after three to five days. Presence of white, translucent and glistening colony was presumably considered pure and transferred to culture tubes.

Table 2. Pesticides used in the present studies.

Common name	Generic class	Class	Formulation	Recommended dose	Purity (%)	Manufacturer
Diazinon	Organo-phosphate	Insecticides	Emulsion	688 ml/Acre	60	Ciba-Geigy Ltd. Basel, Switzerland.
Heptachlor	Organo-chlorine	Insecticides	Dust	4 lb/ Acre	40	Velsical Chemical Corporation, Rosemolt, USA

Authentication of the isolate as Rhizobium : Cotton stoppered glass tubes were half filled with about 100 g acid washed coarse sand and were sterilized for 15 minutes at 121°C in an autoclave. Seeds of lentil were surface sterilized by immersing momentarily in 95% alcohol and then in 0.1% $HgCl_2$ solution, before washing in ten changes of sterilized distilled water.[15] Surface sterilized seeds were then sown asceptically in duplicate tubes containing sterilized sand. The sand was thoroughly soaked with nitrogen free nutrient solution. After germination, 1 ml of broth culture of the isolate was distributed evenly over the sand. Duplicate tubes without inoculation were added as control.

After 20 - 25 days, when satisfactory plant growth was obtained, plants were uprooted and roots were examined for the presence of nodules. Presence of nodules only in the inoculated plants was taken as the indication of the effectiveness of the isolated strain. Several representative nodules were selected and *Rhizobium* was reisolated on YMA medium containing 0.0025% Congo red. Characteristic growth on the YMA-Congo red medium profoundly proved the authenticity of the isolate.[15]

Colony and morphological characteristics of the isolate: The isolate was transferred from slant to YMA plate and different colonial characteristics, form, margin, elevation, opacity and color of the colonies were studied on the YMA plate.[15]

Following microscopic examinations were also performed: (a) Shape was observed by simple staining with crystal violate solution, (b) Gram reaction was studied by Hucker method[16], (c) motility of the isolate was tested by usual hanging drop method and (d) for spore staining, Schaeffer-Fulton method was used. Of physiological properties, catalase activity, gelatin hydrolysis and starch utilization were tested.[15] The isolates were also tested for nitrate reduction.

In vitro study of pesticide susceptibility of the isolate: Agar cup diffusion experiment was setup to determine the concentration of pesticides at which they inhibit the growth of *Rhizobium*. Loopful of culture was transferred from slant with a sterile loop to 5 ml of liquid YMA broth in 25 ml test tube. The culture was then incubated on a rotary shaker, with moderate agitation at 28°C.

YMA medium was sterilized for 15 minutes at 121°C and 15 lb pressure in an autoclave. The sterile medium was then allowed to cool down to 45 - 50°C and poured onto several sterile Petri dishes to a depth of 3.5 mm. After solidification, the plates were dried at 4°C in an incubator to drive off excess moisture. The plates were checked for contamination by incubating them overnight. Approximately 0.1 ml of the inoculum was streaked multi directionally on the plates with a sterile cotton swab to get uniform spread of organism. The plates were left to dry for 5 minutes.

Six cups (holes) per inoculated plate were made at reasonable distance by a sterile steel cork borer of 6 mm diameter. The cups were then filled successively with 50 µl of a specific pesticide solution of different concentration. Pesticides were not sterilized as Kapusta and Rouwenhorst[2] found in their study that pesticides did not require sterilization. Triplicate was done per pesticide. The plates were then left two - three hours to let the pesticide solution to diffuse into the medium. The plates were then inverted and incubated at 28°C. The zone of hydrolysis was measured after 24 hours of incubation. The diameter of hydrolysis was measured in mm by slide calipers.

Effect of pesticides on lentil-rhizobia symbiosis: Two pot experiments were carried out under natural condition using two soils and two pesticides. In the first set of experiment, plastic pots were filled with 100 g of soils. Five - six surface sterilized seeds were sown in each pot. One week after germination, half of the pots received two rates of diazinon and half heptachlor at the same rate. A control without pesticide treatment was included in each case. Second set of experiment

was arranged in the same manner with sterilized soil. Soils were sterilized in cotton stoppered large glass tubes by exposing them to prolonged heat for 72 hours at 70°C. Five to six surface sterilized seeds per glass tube were sown asceptically. Tubes were kept under laboratory condition until dressing with pesticide doses. Three days after germination, 1 ml of inoculum was distributed asceptically over the soil. To prepare the inoculum isolated strain was grown for three days at 28°C in yeast extract-mannitol broth. The strain was harvested and washed in distilled water.[17]

Pesticides were applied asceptically one week after germination. Cotton stoppers were removed when plants reached the base of the cotton stoppers. Both the sets were then left under natural condition. Additional protection was provided with iron gauge hood.

At 25, 40, and 55 days after germination, plants were carefully uprooted. The uprooted plants were then gently washed in tap water to remove the adhering soil from the roots and nodules. The numbers of nodule were then counted. Shoots and roots were dried separately in the oven at 70°C for 48 hours. Dry weight of shoots and roots were taken and ground separately in a mortar. Ground shoots and roots were then analyzed for total nitrogen by alkali distillation method. Then the amount of N fixed per plant was calculated by deducting the total N content per seed from the total N content per plant as outlined by Imamul Huq and Larher.[18]

Results and Discussion

Isolation and purification of Rhizobium from nodule of lentil: Rhizobium strain was isolated from nodule of lentil growing on Melandaha series. In the selective medium the isolates were found to produce slimy colonies within three to five days of incubation. From the growth pattern, the isolate was found to be a fast grower. Subculture was done from discrete colonies. For preliminary assessment of purity of the isolate as nodule bacteria, the isolate was grown on YMA-Congo red medium. The colonies of the isolate in Congo red medium did not assimilate red pigment throughout the incubation period. It tentatively proved the purity of the isolate.

Authentication of the isolate as an effective strain: The authenticity of the isolate as *Rhizobium* strains was conclusively proved by their ability to produce nodules in the roots of host plants under strict bacteriological control. Lentil growing on sterilized sand was inoculated with the isolated *Rhizobium* strain. Nodules were found to develop within 20 days after germination. It proved the authenticity of the isolate.

Colony characteristics of the isolates: Discrete colonies appeared within three to five days of incubation. The colonies were circular, 1 - 2 mm in diameter having a viscous texture with convex elevation and entire margin.

Morphological examination of the isolate: Iolates were motile and gram-ve short rods.

Physiological properties of the isolate: Several physiological tests were performed to charterize the isolate. It was found that the isolate was not salt tolerant. Growth was not affected at 3°C. But growth was found to be hindered at 37°C and terminated at 4°C. Catalase activity of the isolate was found to be positive. The isolates failed to utilize starch or gelatin. The isolates successfully reduced nitrate to nitrite but denitrification was not detected.

In vitro susceptibility of the isolate to pesticides: The antimicrobial activity of diazinon and heptachlor was tested against the isolated strain in the concentration range of 50 to 1200 mg a.i. kg^{-1} by agar cup diffusion method. The cup diameter was 6 mm and the inhibition of growth in terms of zone diameter (mm) was determined (Fig. 1).

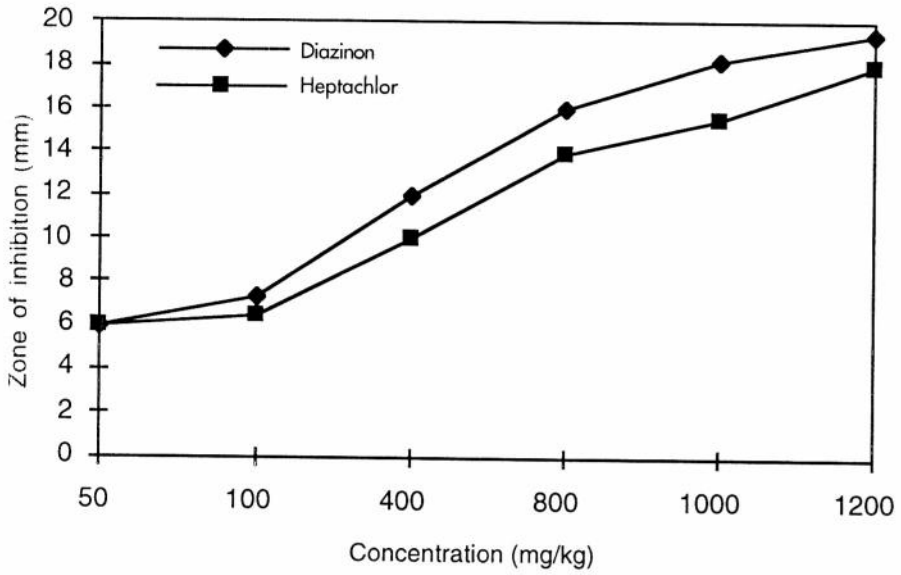

Fig. 1. Effect of diazinon and heptachlor on the growth of isolated strain of *Rhizobium* (agar cup diffusion method, cup diameter 6 mm).

Both the pesticides showed zone of inhibition at 50 mg a.i. kg^{-1} against the isolates. There is a close relationship between the extent of inhibition and the concentration of pesticides. The zone diameter increased with increasing concentration of pesticides.

However, diazinon was found to be most active in terms of inhibition zone diameter. For any given concentration, diazinon exhibited higher zone of inhibition compared to heptachlor.

Effect of pesticides on nodulation: The influences of pesticides on nodulation of lentil growing on two different soils are presented in Table 3. Each value is mean of three replicated pots, each pot contained four to five plants. First harvest was done 25 days after germination. Second and third harvests were done at 40 and 55 days after germination, respectively.

Table 3. Nodule number/plant for the three harvests of lentil grown on two different soils treated with different concentrations of diazinon and heptachlor, with and without *Rhizobium* inoculation.

Pesticides	Dose	No nudules/plant					
		Melandaha soil			Bhaipara soil		
		25 d	40 d	55 d	25 d	40 d	55 d
Uninoculated							
Diazinon	Control	7.4 bcde†	9.6 bcd	8.6 bc	7.0 bc	7.8 bcd	8.4 bc
	X*	6.7 abcd	8.0 abcd	7.3 abc	5.1 ab	6.1 ab	7.1 ab
	4 X	4.2 a	4.9 a	4.4 a	3.2 a	4.5 a	4.7 a
Heptachlor	X	6.4 abc	8.8 abcd	6.2 ab	6.0 abc	6.8 abc	6.8 ab
	4 X	5.1 ab	5.0 a	4.9 a	4.9 ab	4.7 a	6.1 ab
Inoculated							
Diazinon	Control	9.5 de	10.7 d	10.5 c	12.4 d	12.1 e	10.3 c
Diazinon	X	7.4 bcde	10.1 cd	10.7 c	7.1 bc	9.7 de	5.2 ab
	4 X	6.1 abc	5.8 ab	6.2 ab	5.0 ab	7.1 abc	4.7 a
Heptachlor	X	10.0 e	10.1 cd	10.5 c	8.9 c	9.3 cd	6.9 ab
	4 X	8.6 cde	6.1 abc	6.8 ab	4.7 ab	6.4 ab	5.2 ab

*X is the recommended dose , 0.5 mg a.i. kg^{-1} for diazinon and 0.8 mg a.i. kg^{-1} for heptachlore; 4X is four times of recommended dose. †Data followed by a common letter in the same column are not significantly different at 5% level by Duncan's New Multiple Range Test.

The results showed that nodulation of *Lens culinaris* decreased in the presence of the pesticides at both the concentrations. The deleterious effect of the pesticides on nodulation differed with soils and also with pesticides. Between the pesticides, heptachlor, at the recommended dose, had little effect on the number of nodules. However, at higher concentration, both the pesticides showed significant reduction in number of nodules.

Numbers of nodules in Melandaha soil tended to be higher than that Bhatpara soil irrespective of the types of pesticides. Thus, reduction in the number of nodules due to application of the pesticides was more evident in Bhatpara soil.

Effect of pesticides also varied due to inoculation. The inoculation of the soils significantly increased the nodule number in Bhatpara soil. The effect was not so evident in Melandaha soil. When the inoculation was made in presence of the pesticides, it was observed that in most of the cases diazinon and heptachlor significantly reduced the number of nodules at both the rates of application in Bhatpara soil. In Melandaha soil, however, only the highest doses of the pesticides showed such effects.

One interesting point to note here was that, though the pesticides treatment reduced the number of nodules, the size of the nodules was bigger. This phenomenon was observed at recommended dose of both the pesticides. However, the reverse was true for the higher doses of both the pesticides. More nodules appeared on the lateral roots of the pesticide treated plants than on taproots. In control samples the reverse was true.

When the nodules from different treatments were cut open most of them were uniformly pink indicating that there was not much variation in the leghaemoglobin content. However, the visual observation was not ascertained quantitatively.

Effect of pesticides on lentil growth: Effect of diazinon and heptachlor on lentil shoot and root dry weights are summarized in Tables 4 and 5. The results also represent the effect of pesticides in presence of inoculation.

The results had shown the usual observation that inoculated plants gained higher shoot and root weights than uninoculated plants. When inoculation was made in presence of pesticides, it was observed that both the pesticides in most of the cases had negative impact on shoot and root dry weight of lentil plants. In few stray cases heptachlor treatment elevated shoot and root dry matter production in presence or absence of inoculation.

In general diazinon was more detrimental to shoot and root dry matter production than heptachlor. Again detrimental effect of pesticides varied with the types of soil. Plants growing on Melandaha soil showed better growth than the plants growing on Bhatpara soil in control samples. When pesticides were applied, results showed that plants raised on Bhatpara soil suffered more severely.

Effect of pesticides on nitrogen fixation: Effect of pesticides on nitrogen fixation by lentil plants are presented in Table 6. At the recommended rate of application, neither of the pesticides had any significant deleterious effect on nitrogen fixation.

Table 4. Effect of pesticide treatment in presence or absence of *Rhizobium* inoculation on shoot and root growth of lentil plants growing on Melandaha soil harvested at different days after germination.

Pesticides	Dose	Shoot dry wt mg/plant			Root dry wt mg/plant		
		25 d	40 d	55 d	25 d	40 d	55 d
Uninoculated							
Diazinon	Control	12.6 ab†	30.2 bc	35.2 abc	10.9 abc	28.6 bc	30.7 abc
	X*	12.5 ab	26.5 b	33.7 ab	8.5 ab	20.66 a	30.5 abc
	4 X	8.7 a	19.1 a	30.7 a	6.9 a	17.6 a	25.6 a
Heptachlor	X	15.3 bc	32.6 bcd	32.6 a	12.5 bcd	28.7 bc	25.5 a
	4 X	9.6 a	28.4 b	30.1 a	9.5 abc	22.6 ab	27.4 ab
Inoculated							
Diazinon	Control	17.5 bc	38.1 d	43.7 d	12.8 bcd	32.3 cd	36.5 bc
Diazinon	X	16.2 bc	37.4 cd	41.6 cd	11.6 abcd	36.3 d	32.5 abc
	4 X	12.6 ab	28.2 b	35.2 abc	12.1 abcd	22.6 ab	29.3 ab
Heptachlor	X	18.7 c	37.6 cd	44.6 d	16.3 d	28.6 bc	40.5 c
	4 X	16.6 bc	32.5 bcd	40.2 bcd	14.3 cd	29.4 bc	32.5 abc

*X is the recommended dose, 0.5 mg a.i. kg^{-1} for diazinon and 0.8 mg a.i. kg^{-1} for heptachlore; 4X is four times of recommended dose. †Data followed by a common letter in the sane column are not significantly different at 5% level by Duncan's New Multiple Range Test.

Table 5. Effect of pesticide treatment in presence or absence of *Rhizobium* inoculation on shoot and root growth of lentil growing on Bhatpara soil harvested at different days after germination.

Pesticides	Dose	Shoot dry wt mg/plant			Root dry wt mg/plant		
		25 d	40 d	55 d	25 d	40 d	55 d
Uninoculated							
Diazinon	Control	9.3 a†	23.2 b	30.6 b	7.3 a	16.4 ab	28.4 abc
	X*	8.2 a	25.0 b	28.2 bc	5.5 a	16.8 ab	24.5 c
	4 X	7.4 a	13.1 ac	19.8 a	6.0 a	9.5 ad	20.6 a
Heptachlor	X	8.2 a	22.5 b	32.9 b	6.9 a	16.5 ab	20.6 b
	4 X	8.2 a	18.5 ac	23.4 ac	6.6 a	15.2 ab	18.6 b
Inoculated							
Diazinon	Control	12.6 a	34.0 d	37.0 b	8.4 a	24.0 cd	30.6 bc
	X	12.4 a	20.0 ac	34.3 b	7.8 a	21.2 ac	30.0 bc
	4 X	8.5 a	24.8 b	24.1 c	6.3 a	20.0 abc	28.5 b
Heptachlor	X	12.4 a	28.5 bd	35.2 b	9.9 a	22.6 ac	30.1 bc
	4 X	8.5 a	24.3 b	31.2 b	8.8 a	23.5 ac	28.5 b

*X is the recommended dose , 0.5 mg a.i. kg^{-1} for diazinon and 0.8 mg a.i. kg^{-1} for heptachlor; 4X is four times of recommended dose. †Data followed by a common letter in the same column are not significantly different at 5% level by Duncan's New Multiple Range Test.

However, at higher doses, diazinon significantly reduced nitrogen fixation under uninoculated condition. Heptachlor at both level of application had no significant effect on nitrogen fixation.

Table 6. Nitrogen fixed/plant by lentil grown on two different soils treated with different concentrations of diazinon and heptachlor, and with and without *Rhizobium* inoculation.

Pesticides	Dose	Amount of N_2 fixed/plant (%)†					
		Melandaha soil			Bhatpara soil		
		25 d	40 d	55 d	25 d	40 d	55 d
Uninoculated							
Diazinon	Control	2.1 de*	3.6 cde	2.9 d	1.4 ab	3.2 c	1.9 bcd
	X*	1.8 cd	3.3 cd	2.2 bcd	1.3 a	2..9 bc	2 bc
	4 X	1.1 ab	2.6 ab	1.3 a	1.1 a	1.6 a	1.7 bc
Heptachlor	X	1.9 cd	3.9 de	2.6 bcd	1.5 ab	2.9 bc	2.1 bcd
	4 X	1.5 bc	3.4 cde	2.3 bcd	1.3 a	2.5 b	1.8 bc
Inoculated							
Diazinon	Control	2.6 e	3.8 de	2.8 cd	2.9 d	3.3 c	2.7 d
	X	1.8 cd	3.1 bc	2.0 abc	1.4 a	2.8 bc	1.4 ab
	4X	0.9 a	2.5 a	1.2 a	1.1 a	1.9 a	0.9 a
Heptachlor	X	2.6 e	3.9 e	2.8 bcd	2.1 c	3.1 c	2.2 cd
	4 X	1.3 ab	3.1 bc	1.9 ab	2.0 bc	2.9 bc	1.8 bc

*X is the recommended dose, 0.5 mg a.i. kg^{-1} for diazinon and 0.8 mg a.i. kg^{-1} for heptachlor; 4X is four times of recommended dose. †Amount of N_2 fixed calculated as amount of N/plant - amount of N per grain. Amount of N_2 per grain was 0.54 mg. *Data followed by a common letter in the same column are not significantly different at 5% level by Duncan's New Multiple Range Test..

The results had shown the usual observation that inoculated plants fixed nitrogen than uninoculated plants. However, the increase was not significantly different from that of uninoculated control. When pesticides were applied under inoculated condition, they produced marked decline in nitrogen fixation. The effect of diazinon was more prominent than that of heptachlor. At both the application rate diazinon induced declining trend in N_2 fixation of lentil plants. Significant reduction was observed at both the application rate of diazinon. On the other hand, higher concentration of heptachlor was needed to produce any significant reduction in nitrogen fixation.

Yeast extract mannitol agar formulated by Fred and Waskman[19] was used to isolate root nodulating bacteria in the present investigation. The medium was found to encourage rapid growth of the isolate. This is in keeping with that reported by Jordan.[14]

Yeast extract-mannitol containing 0.025 g/l (0.0025%) Congo red was used to test purity of the isolate. The YMA medium containing 0.0025% Congo red was suggested as selective medium for *Rhizobium* by Fred and Waskman.[19] Congo red is not usually absorbed by *Rhizobium*. However, adsorption by *R. meliloti* was reported by Vincent.[20] Suitability of the dye was also reported by Jordan.[14] Once tested on YMA-Congo red medium, the authenticity of the isolate as *Rhizobium* was tested by its ability to infect roots of test plant. Ability to nodulate the roots of cross inoculating plant is a unique feature of *Rhizobium* spp. and thus plant infection method appears very convenient means of identification of the isolate as *Rhizobium*.

Morphological and colonial characteristics were also consistent with that reported by Jordan.[14] The isolates failed to grow on 2% NaCl. Most of the *Rhizobium* spp. failed to survive in such high concentration of salt. Reduction of nitrate to nitrite by the isolate was observed indicating its ability to utilize nitrate as the sole or supplementary source of nitrogen.[21]

To study the effect of selected pesticides on growth of *Rhizobium* in pure culture, pesticides were directly applied to the culture medium and zone of inhibition was observed. It was found that much higher concentration was needed to see any visible zone of inhibition. Such findings were consistent with that of Lin *et al.*[3] who found that some organophosphate, dyfonate, trithion and dursban, at lower doses did not produce any zone of inhibition in a disk inhibition study. In the present study 50 mg a.i. kg^{-1} of either of the pesticides was needed to produce any visible zone of inhibition. Recommended and four times of recommended dose, however, did not produce any visible zone of inhibition in the current study. Similar results were obtained by Kapusta and Rouwenhorst.[2] When 24 pesticides, including diazinon, were applied at ten times recommended dose of each pesticides to study the inhibition of growth of *R. japonicum* only chlorpropham, a carbamate herbicide and disulfoton, an organic phosphate insecticide were found to inhibit growth. The insecticides they used were aldrin, azinphosmethyl, carbaryl, dasanit, diazinon, dieldrin, disulfoton, lindane, malathion and methoxychlor.

In the current study diazinon was found to be more inhibitory to growth than heptachlor. Limited influence of heptachlor to growth of the isolate was either as a consequence of rhizobial tolerance or lower solubility. In fact, commercial formulations of the pesticides, as used in the current study contain a variety of chemicals in addition to the pesticides. Some of these additives are solvents, co-solvents, emulsifiers and surfactants and as well as the carrier. These additives might have contributed to the differential influence of the two pesticides in addition to the main effect of technical formulation of the two pesticides.

The high concentration of the pesticides that were used here are not likely to be present under normal conditions of application. But as mentioned earlier such concentration may be present at localized areas or build-up after several applications, at least for short duration. The situation is more likely to occur under intensive cultivation.

In recent years the use of pesticides has increased tremendously and number of pesticides have been shown to cause decreased nodulation in legumes.[6,22] Fifteen pathogen causing 17 diseases have so far been recorded in Bangladesh.[23,24] Application of pesticides for better lentil growth is thus recommended.

The pesticides used in the current study had no adverse effect at recommended dose on lentil growth and lentil-rhizobia symbiosis. But at higher rate, mild to adverse affect was observed. Pesticides were found to differ in exerting their ill effects on the parameters examined. Degree of deleterious effects of the pesticides also differed due to inoculation. Types of soil were found to be key factor influencing behavior of pesticides to lentil-rhizobia symbiosis. Pesticides used also differed in their capacity to affect nodulation. It was found that the concentration and types of pesticides would play an important role in the relation of *Rhizobium* and nodule formation.[6] In the present study it was found that in either of the inoculated or uninoculated condition, diazinon was more toxic than heptachlor to nodulation, growth and N_2 fixation.

Nodules in plants on Melandaha soil in all treatments were found, in general, to be greater in number and larger in size and healthier in appearance compared to nodules of the plants in Bhatpara soil. Bhatpara soil also showed high inhibition to nodulation at any treatment compared to Melandaha soil. When inoculated with the isolated strain, number of nodules per plant in Bhatpara soil increased sharply. It proved that either Bhatpara soil lacked sufficient number of cross inoculating *Rhizobium* or the soil contained different strain that had poor infection capacity. One interesting point to note that inoculation did not bring the number of nodule per plant in Bhatpara soil as high as the number of nodule per plant in Melandaha soil. At this point, the reason could be the nutritional status of the Bhatpara soil, especially high nitrate content compared to Melandaha soil. Phosphorus has been reported to influence the size and number of nodules. On the other hand, nitrate inhibits virtually every phase of symbiosis. Increasing concentration of nitrate reduces root hair curling and infection thread fails to develop normally. Nitrate concentration as low as 0.2 *mM* in solution may delay the appearance of the first nodule and slow the initial rate of appearance of new nodules.[25]

Regardless of the types of pesticides, it was found that at the recommended dose of application nodules were bigger and healthier in appearance compared to high dose of application and control. Kulkarni *et al.*[26] also observed similar effect with carbofuran, thimet, dasanite and heptachlor at their respective recommended dose.

There are contradictory reports on the influence of pesticides on N_2 fixation. Malik and Tesfai[27] reported that diazinon and toxaphene at the recommended dose and five times of recommended dose deleteriously affected N_2 fixation but not growth of soybean plants. Kapoor and Singh[6] reported that N_2 fixation decreased at 1, 5 and 10 ppm of aldrin, a chlorinated insecticides, in the soil in absence of farm yard manure, but in presence of farm yard manure N_2 fixation increased at 1 and 5 ppm level of aldrin. On the other hand, Kulkarni *et al.*[26] reported a slight increase in yield and N_2 content in plants raised in soil treated with dasanite and heptachlor. Similar observation was also reported by Ostwal.[28]

In the current study total N_2 fixed at different time interval were found to differ; there was an initial low N_2 fixation which then reached a pick at second observation and decreased again at final observation. The N_2 fixation profile is consistent with that proposed by Hardy *et al.*[29]

The foregoing discussion clarifies the trend of nitrogen fixation observed during the term of the experiment. It is also found that diazinon and heptachlor when used at the recommended dose have negligible ill effect *vis-à-vis* nodulation, growth and N_2 fixation. But under repeated use concentration of pesticides in the field might rise far above recommended dose that may cause fatal consequences in respect of growth, yield, nodulation and N_2 fixation.

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