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Biotoxic Effects of Pesticides on Symbiotic Properties of Rhizobial Sps.

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The use of pesticides in agriculture is inevitable. The indiscriminate use of pesticides leads to the accumulation of these compounds in the environment disturbing the soil biological ecosystem, including the nitrogen fixing bacteria, and also causes environmental pollution (Chauhan and Sundaram 1986; Mrinalini 1986).

The Rhizobium - legume symbiosis is the most effective agricultural system for biological fixation of atmospheric nitrogen. In fast growing Rhizobium sp (R. leguminosarum, R. trifolii & R. meliloti) the genes required for nodulation (Nod) and nitrogen fixation (Nif) are located on a mega plasmid (> 100 Kb) known as the symbiosis (Sym) plasmid (Nuti et al. 1979; Prakash et al. 1980; Banfalvi et al. 1981; Rosenberg et al. 1981). The symbiotic properties of many fast growing rhizobia are reported to be unstable. This has been attributed to instability of plasmids which could be enhanced by various environmental chemical and physical agents (Zurkowski and Lorkiewicz 1978; Morrison et al. 1983).

When considering the effects of pesticides on members of the genus Rhizobium, one is concerned not only with their effect on Rhizobium sps. and nitrogen fixation, but also how they affect the genetic system of the Rhizobium.

There are a number of reports on the effect of pesticides on the growth of Rhizobia (in vitro), nodulation and nitrogen fixation but the effect of these agents on the stability of plasmid in Rhizobia was not dealt with.

In the present study, we have examined the effect of fifteen commonly used pesticides (six each of insecticides and fungicides and three herbicides) on nodulation, nitrogenase enzyme activity and sym plasmids in three rhizobial species nodulating three different cultivars. This study would also reveal the correlation between the nitrogen fixation and sym plasmid in these test strains.

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MATERIALS AND METHODS

Cultures used in the study include Rhizobium sp. IC3342 nodulating Cajanus cajan (Pigeon pea) which was obtained from International Crops Research Institute of Semi Arid Tropics, Hyderabad, India; Rhizobium leguminosarum 2001 nodulating Lens esculenta (Lentil) obtained from Indian Agricultural Research Institute, New Delhi, India; Rhizobium meliloti 4013 nodulating Medicago sativa (Alfalfa) obtained from Bhabha Atomic Research Centre, Bombay, India.

The above cultures are fast growing and possess symbiotic plasmids Rhizobium sp. IC3342 possesses three different plasmids; the middle plasmid DNA harbors symbiotic genes (Upadhyaya et al. 1985). R. leguminosarum 2001 and R. meliloti 4013 possesses only a single large symbiotic plasmid. The important characteristic feature of Rhizobium sp. IC3342 is that it induces leaf curling of the plant which is always associated with symbiotic nitrogen fixation (Kumar Rao et al. 1984).

The following pesticides, which are generally used as pest control agents, were selected in this study. These include insecticides (Monocrotophos, Acephate, Dichlorvos, Dichlorodiphenyltrichloro ethane, Danitol and Sumicidin), fungicides (Blitox, Captan, Captafol, Dithianon, Hexaconazole and Zineb) and herbicides (Butachlor, Simazine and Oxyflorfen). All compounds were technical grade. Stock solutions of each pesticide were prepared either in acetone dimethyl sulfoxide or distilled water depending on its solubility.

Pesticide treatment of bacterial cultures was done by taking an aliquot (0.1 ml) of an exponentially growing culture containing about 10^4 cells and inoculating 1 ml of yeast extract mannitol (YEM) broth having various concentrations of one of the pesticides. Control culture tubes, one consisting of medium and the other with solvent were included. The culture tubes were incubated at 28°C on a shaker for 24 hours and the minimum inhibitory concentration (MIC) was determined (Winkler et al. 1979). About 25-30 colonies (from below MIC treated culture) were isolated on YEM agar plates (with congored) and also from control cultures (Rhizobium sp. IC3342, R. leguminosarum 2001 and R. meliloti 4013). Single colonies were grown into individual cultures and tested for nodulation and nitrogenase enzyme activity by inoculating the respective cultivar and percentage loss of symbiotic properties was calculated. The same cultures were used for detection of plasmids in each strain.

Plant assays for nitrogen fixation were carried out as per the method described by Vincent (1970). The test plant seeds were surface sterilized and four seeds were sown per pot. Pesticide treated and untreated cultures were grown to the exponential stage and used to inoculate the seedlings. The growing plants were supplied with evans nitrogen free nutrient solution (Evans 1974) once a week and sterile tap water twice a week. The plants were harvested after 45 days and observed for the pre-

sence of root nodules. Nitrogenase activity in the excised roots (with or without nodules) was estimated by the acetylene reduction methods (Hardy et al. 1968).

Detection of plasmids were carried out by agarose gel electrophoresis using 0.8% agarose (Kado and Liu 1981). Plasmid DNA bands were visualized under a UV transilluminator at 274 nm and photographed. Appropriate controls using untreated cultures were maintained for comparison.

RESULTS AND DISCUSSION

The results of the effect of insecticides, fungicides and herbicides on symbiotic properties of three Rhizobium sps are summarized below (Tables 1-3).

The MIC's of pesticides for Rhizobium sp. IC3342 varied between 800-25600 ppm. The cultures prepared from below MIC pesticide treatment were inoculated into pots containing Cajanus cajan and the plants were examined for nodulation and nitrogenase enzyme activity. Of the 15 pesticides, 13 of them showed 55-100% loss of both nodulation as well as nitrogenase activity at various concentrations tested, whereas DDT and Zineb showed a maximum of 16% loss of both the characteristics at the highest concentration tested.

The above infected plants which did not show nodulation and nitrogenase enzyme also lost the leaf curling trait, a phenotypic character associated with symbiotic nitrogen fixation (Kumar Rao et al. 1984).

The MIC's of pesticides under study against R. leguminosarum, 2001 varied between 700-16000 ppm. Of the six insecticides, only sumicidin showed loss of nodulation and nitrogenase activity in infected plants of Lens esculenta at the frequency of 90%, fungicides showed a loss in both the parameters by 20-40%, whereas herbicides showed 35-75%.

Lastly, the MIC's of pesticides against R. meliloti 4013 varied between 800-22400 ppm. Of the six insecticides only sumicidin was effective (upto 35%) in the loss of nodulation and nitrogenase activity. The fungicide and herbicide treated cultures showed a reduced efficiency (20-100%) of nodulation and nitrogenase activity.

In a similar manner, the control cultures (untreated and solvent) were inoculated to their respective cultivars, 100% of plants showed symbiotic properties, i.e. nodulation and nitrogenase activity demonstrating that the loss of symbiotic properties in treated cultures were due to pesticidal affect.

The above three cultures were tested for their plasmid profile before and after pesticide treatment. It was observed that all the cultures which lost the symbiotic properties showed loss of plasmid as observed by agarose gel electrophoresis. The control

Table 1. Effect of insecticides on nodulation and nitrogenase activity in *Rhizobium* spp

Insecticides	1 *Rhizobium* sp IC3342 Pigeon pea			2 *R. leguminosarum* 2001 Lentil			3 *R. meliloti* 4013 Alfalfa		
	MIC (ppm)	Conc. used (ppm)	Loss of symbiotic properties (%)	MIC (ppm)	Conc. used (ppm)	Loss of symbiotic properties (%)	MIC (ppm)	Conc. used (ppm)	Loss of symbiotic properties (%)
Control	-	-	0	-	-	0	-	-	0
Monocrotophos	3200	1600	90	3200	1600	0	3200	1600	0
		800	90		800	0		800	0
		400	90		400	0		400	0
Acephate	25600	12800	90	12800	6400	0	6400	3200	0
		6400	90		3200	0		1600	0
		3200	90		1600	0		800	0
Dichlorvos	1600	800	100	800	400	10	800	400	0
		400	100		200	0		200	0
		200	100		100	0		100	0
DDT	12800	6400	16	12800	6400	0	12800	6400	0
		3200	0		3200	0		3200	0
		1600	0		1600	0		1600	0
Danitol	3200	1600	100	3200	1600	0	3200	1600	0
		800	100		800	0		800	0
		400	100		400	0		400	0
Sumicidin	3200	1600	100	3200	1600	90	3200	1600	33
		800	100		800	75		800	10
		400	100		400	0		400	0

Note : The control plants with nodulation showed nitrogenase activity of 1. 344.1 ± 2.1, 2. 135 ± 3.2, 3. 160 ± 2.1 nmoles/plant/hr respectively. ± denotes standard error. All the treated cultures which lost the symbiotic properties also lost the plasmid.

Table 2. Effect of fungicides on nodulation and nitrogenase activity in *Rhizobium* spp.

Fungicides	1 *Rhizobium* sp IC3342 Pigeon pea			2 *R. leguminosarum* 2001 Lentil			3 *R. meliloti* 4013 Alfalfa		
	MIC (ppm)	Conc. used (ppm)	Loss of symbiotic properties (%)	MIC (ppm)	Conc. used (ppm)	Loss of symbiotic properties (%)	MIC (ppm)	Conc. used (ppm)	Loss of symbiotic properties (%)
Control	-	-	0	-	-	0	-	-	0
Blitox	2700	2400	80	2700	2400	40	2100	1800	35
		1200	80		1200	18		900	16
		600	75		600	0		450	5
Captan	1000	900	100	900	800	20	800	700	20
		450	100		400	5		350	11
		225	80		200	0		175	10
Captafol	7200	6400	80	7200	6400	0	7200	6400	60
		3200	80		3200	0		3200	60
		1600	70		1600	0		1600	40
Dithianon	16000	14400	55	16000	14400	35	12800	11200	15
		7200	40		7200	14		5600	4
		3600	16		3600	10		2800	0
Hexaconazole	11200	9600	80	12800	11200	40	22400	19200	100
		4800	80		5600	30		9600	100
		2400	70		2800	18		4800	80
Zineb	800	700	16	700	600	20	500	400	0
		350	5		300	0		200	0
		175	0		150	0		100	0

Note : The control plants with nodulation showed nitrogenase activity of 1. 399 ± 3.4, 2. 137 ± 2.2, 3. 155 ± 3.5 nmoles/plant/hr respectively. ± denotes standard error. All the treated cultures which lost the symbiotic properties also lost the plasmid.

Table 3. Effect of herbicides on nodulation and nitrogenase activity in *Rhizobium* spp.

Herbicides	1 *Rhizobium* sp IC3342 Pigeon pea			2 *R. leguminosarum* 2001 Lentil			3 *R. meliloti* 4013 Alfalfa		
	MIC (ppm)	Conc. used (ppm)	Loss of Symbiotic properties (%)	MIC (ppm)	Conc. used (ppm)	Loss of symbiotic properties (%)	MIC (ppm)	Conc. used (ppm)	Loss of symbiotic properties (%)
Control	-	-	0	-	-	0	-	-	0
Butachlor	3600	3200	95	3200	1600	75	7200	5600	80
		1600	75		800	60		2800	56
		800	50		400	15		1400	16
Simazine	5600	2800	100	3600	1800	70	8000	4000	90
		1400	80		900	25		2000	75
		700	30		450	0		1000	20
Oxyflorfen	2400	1200	100	2000	1000	35	6400	3200	75
		600	80		500	10		1600	25
		300	30		250	0		800	5

Note : The control plants with nodulation showed nitrogenase activity of 1. 344.1 ± 2.1, 2. 134 ± 3.2, 3. 160 ± 2.1 nmoles/plant/hr respectively. ± denotes standard error. All the treated cultures which lost the symbiotic properties also lost the plasmid.

cultures exhibited symbiotic properties and harbored the plasmid.

The susceptibility of the Rhizobial strain to each pesticide was variable with regard to the parameters examined. Out of the three cultures examined, the symbiotic properties of Rhizobium sp IC3342 were more susceptible to the pesticidal treatment. Similar observations were reported by Kaszobiak (1966) working with different Rhizobial species. Further, fungicides and herbicides showed more adverse affects than the insecticides. Of all the pesticides tested DDT and Zineb were found to be least effective on the symbiotic properties of the three Rhizobial cultures. Since Rhizobial fertilizers are in vogue, the information regarding the adverse affect of pesticides on symbiotic properties of Rhizobium and its genetic system might be useful.

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