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Influence of Pesticides on *Azospirillum* Populations and its Nitrogen Fixing Activity in Cotton Cultivated Black Soils of Kurnool District, Andhra Pradesh, India

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A B S T R A C T

The influence of selected insecticides, monocrotophos, cypermethrin and fungicides mancozeb and hexaconazole was evaluated on *Azospirillum* population in vertisol soils collected from cotton grown fields of Kurnool district, Andhra Pradesh, India. The influence of these pesticides on *Azospirillum* population was dose dependent; insecticide monocrotophos showed the maximum stimulation towards *Azospirillum* population at 5.0 kg ha^{-1}, whereas cypermethrin showed maximum enhancement at 2.5 kg ha^{-1} level. Likewise the fungicide mancozeb and hexaconazole showed maximum stimulation at 5.0 kg ha^{-1}. The pesticides up to 5.0 kg ha^{-1} were either stimulatory or innocuous to bacterial population. The highest stimulation in bacterial population was observed at 10-day incubation.

Introduction

In modem agriculture, it has become a common trend to apply different groups of pesticides, either simultaneously or in succession, for effective control of a variety of pests. Many of these pesticides used are persistent soil contaminants, whose impact may last for decades and adversely affect soil conservation (Babu, 2002; Quazi *et al.*, 2011; Defo *et al.*, 2011; Ahemad and khan, 2011a,b; Madhavi and Rangaswamy, 2017; Madhavi *et al.*, 2019a and 2019b). Organophosphorus insecticides are increasingly used in agriculture as a substitute for organochlorine and carbamate insecticides because of their high efficiency and lower persistence in the environment (Yang *et al.*, 2005). Pesticides are usually applied simultaneously or one after another for crop protection, and this type of pesticide application often leads to a combined contamination of pesticide residues in the soil environment (Chu *et al.*, 2008). However the majority of the applied pesticides, even if sprayed on foliage of crop plants and weeds, will eventually reach the soil, which may affect the growth and activity of soil microbial communities (Singh and Singh, 2005). Most of the studies on fungicide or insecticide effects indicate that when they are applied at recommended rates, they usually have no significant effects or have

transitory effects on soil microbial characteristics (Griffiths *et al.,* 2006 and Vig *et al.,* 2008). Soil microbes have a primary catabolic role in the environment and contributes to the global cycling of carbon, nitrogen, sulfur, phosphorus and other elements through degradation of plants and animal residues. Microorganisms in soil play an accredited role in nutrient transformations. Several studies were conducted to evaluate the effects of many pesticides on soil populations of bacteria, fungi and actinomycetes, and soil enzymes after single or repeated applications (Wyszkowska and Kucharski, 2004 and Gonzalez *et al.,* 2007).

Among the soil microflora, *Azospirillum* sp., an ecologically beneficial, diazotrophic bacteria fixes atmospheric N and maintain fertility status of soil, which has attained immense agricultural importance (Sharma, 2002). Black cotton soils are more predominant in Kurnool district a semi-arid region of Andhra Pradesh. The insect population such as cotton boll worm (*Helicoverpa armigera),* pink boll worm (*Pectinophora gossypiella),* Tobacco caterpillar (*Spodoptera litura),* Spotted boll worm (*Earias vitelia*), White fly (*Bemisia tabaci*) and Jassids (*Amrasea biguttula biguttula)* are mainly causing serious damage to the cotton (Shetty, P.K., 2000). In this context, the present work is aimed at assessing the influence of insecticides and fungicides on the population of *Azospirillum* sp. and the nitrogen-fixing activity of *Azospirillum* sp. isolated from the soils of cotton growing fields of Kurnool district.

Materials and Methods

Soils

Samples of black soils, collected at a depth of 12 cm from cotton grown fields of Kurnool district of Andhra Pradesh, India. Kurnool District lies between the Northern latitudes of 14º 15' and 16º 18' and the Eastern longitude of 76º 58' and 79º 34'. Extending over an area of 17,658 Sq.1 KM. Soil samples were air dried and sieved through a 2 mm mesh screen before use. Physicochemical characteristics of the soils were analyzed using standard methods and shown in Table 1.

Pesticides

In order to determine the influence of pesticides on *Azospirillum* population insecticides monocrotophos, cypermethrin and fungicides mancozeb and hexaconazole, were selected. For incubation studies and for estimating population of *Azospirillum* sp., commercial formulations of the pesticides diluted with distilled water were used, whereas technical grade pesticides dissolved in acetone were selected for determining nitrogen fixing ability of the selected strains of *Azospirillum*. Details of the pesticides used in the present investigation were given in Table 2.

Soil incubation

The soil ecosystem stimulating non-flooded conditions consisting of ten gram portions of soil samples were added in test tubes (25 x 150 mm) and moistened to a water potential of 0.090 MPa, in order to maintain at 60% water holding capacity. Same model was used previously to elucidate the effects of insecticides on microbial activities by Tu *et al.,* (1996); Rangaswamy and Venkateswarlu (1999); Raymond *et al.,* (2003) and Jaya Madhuri and Rangaswamy (2003).

Population of *Azospirillum* sp.

To determine the influence of insecticides monocrotophos, cypermethrin and fungicides mancozeb and hexaconazole (10, 25, 50, 75, 100 µg g^{-1} soil) on population of *Azospirillum* sp., ten gram portions of each soil were placed in 15 x 150 mm test tubes and were treated with different concentrations of pesticides, which were equivalent to 1, 2.5, 5, 7.5 and 10 kg ha^{-1} Rangaswamy and Venkateswarlu, 1999 and Jaya Madhuri and Rangaswamy, 2003. Soil samples without pesticides served as controls. The soils with and without pesticides were incubated at room temperature (28 ± 4°C) in the laboratory. Moisture content was

maintained at 60% water holding capacity (WHC) throughout the experiment. After 7 and 14 days of incubation, triplicate soil samples were withdrawn for estimation of the population of *Azospirillum* sp. following the most-probable number (MPN) method (Alexander, 1965).

MPN method for *Azospirillum* sp.

Population of *Azospirillum* sp. in soils were estimated by the most probable number technique following tenfold serial dilutions and the numbers were calculated using the probability tables (Alexander, 1965). Five ml portions of sterile nitrogen free semi solid malate medium of composition: Malic acid : 5 g; KOH : 4 g; K_2HPO_4 : 0.5 g; $MgS0_4$: 0.2 g; NaCl : 0.1 g; $CaCl_2$: 0.02 g; $FeSO_4$: 0.5 g; $NaMoO_4$: 0.002 g; $MnS0_4$: 0.01 g; bromothymol blue (5% alcoholic solution) : 2 ml; Agar : 1.75 g; Distilled water : 1000 ml; pH : 6.8. (Dobereiner *et al.,* 1976) was taken in five MPN tubes, was inoculated with 0.5 ml aliquots of the suspensions from 10^{-1} to 10^{-5} soil dilutions and incubated at 37°C. MPN tubes in which a typical white pellicle developed a few mm below the surface of the medium after 36 hours were recorded as positive for *Azospirillum* sp.

Nitrogen fixation by selected *Azospirillum* strains

Nitrogen fixation by selected strains of *Azospirillum* isolated from soils treated with pesticides was determined. Aliquots of stock solution of pesticides were transferred into sterile test tubes to provide a final concentration of 50 μgml^{-1} semisolid malate medium. 20 ml of sterilized semi solid malate medium was introduced into each test tube under aseptic conditions. The residues were equilibrated for 24h to obtain aqueous solutions of pesticides (Rangaswamy *et al.,* 1989). The test tubes with no pesticides but only semi solid malate medium served as controls. Soil suspensions (1:10 soil to sterile water) treated with pesticide (5kg ha^{-1} commercial formulations) was incubated for 7 days. Aliquots (0.1 ml) of these suspensions were used to inoculate 20 ml of malate medium with and without pesticides. After 72 h of incubation at 37°C, the medium in tubes for each treatment were digested for nitrogen (N) estimation by micro Kjeldahl method (Jackson, 1971).

Results and Discussion

Population of *Azospirillum* sp.

The influence of monocrotophos, cypermethrin and fungicides mancozeb, hexaconazole at different levels on the population of *Azospirillum* sp. in the soil was assessed. The population size of *Azospirillum* sp. was low initially in black cotton soil. The population of *Azospirillum* sp. was significantly higher in soils treated with monocrotophos and cypermethrin, than the untreated control soils during the course of experiment. The population of *Azospirillum* sp. in soils increased up to a concentration 5.0 kg ha^{-1} of pesticides thereafter the population of *Azospirillum* sp. gradually decreased as the concentration of pesticides increased to 10.0 kg ha^{-1}. Application of pesticides up to 5.0 kg ha^{-1}, profoundly enhanced the population of *Azospirillum* sp. in black cotton soil over control after 10 days of incubation (Table 3 and 4). In the present study, four pesticides applied to soil, at concentrations ranging from 1.0 to 5.0 kg ha^{-1}, had no deleterious effect on *Azospirillum* sp. and nitrogen fixing activity; rather both the bacterial population and N_2 fixing activity increased. The results are in agreement with the earlier findings of where in monocrotophos at concentrations ranging from 1.0 to 5.0 kg ha^{-1} gradually increased the population of bacteria and reached maximum at the concentration of 5.0 kg ha^{-1}. Stimulatory effect of monocrotophos, quinalphos and cypermethrin individually has also been confirmed on nitrifiers, nitrogen-fixing organisms and the population of *Azospirillum* sp. in soils (Rangaswamy *et al.,* 1989).

Table.1 Physico-chemical properties of black cotton soils used in the present study

Properties	Cultivated soil	Uncultivated soil
Texture	Clayey	Clayey
Sand (%)	20.7	21.0
Silt (%)	23.8	24.5
Clay (%)	55.5	54.5
pH[a]	8.0	8.4
Water holding capacity (ml g^{-1} soil)	0.27	0.29
Electrical conductivity (m.mhos)	265	255
Organic matter[b] (%)	1.48	1.56
Total nitrogen[c] (%)	0.086	0.091
NH_4^+ - N (µg g^{-1} soil)[d]	8.11	8.00
NO_2^- -N (µg g^{-1} soil)[e]	0.38	0.36
NO_3^- - N (µg g^{-1} soil)[f]	0.79	0.76

[a] 1:1.25 (Soil: water)
[b] Walkley-Black Method (Jackson, 1971)
[c] Micro-Kjeldhal Method (Jackson, 1971)
[d] Nesslerization method (Jackson, 1971)
[e] Diazotization Method (Barnes and Folkard, 1951)
[f] Brucine Method (Ranney and Bartlett, 1972)

Table.2 Pesticides used in the present study

Technical name	Commercial name	Chemical Class	Commercial purity	Technical purity
Monochrotophos	Monocron	Organophosphate	36%EC*	99.5%
Cypermetrin	Super kill-25	Synthetic Pyrethroid	25% EC*	94%
Mancozeb	M-45	Dithiocarbamate	75%WP**	-
Hexaconazole	Hexadhan	Triazoles	5% EC*	-

EC* : Emulsifying concentration; WP** : Wettable powder

Table.3 Population (MPN x 10^4 g^{-1} soil) of *Azospirillum* sp. in insecticide (monochrotophos and cypermethrin) treated and untreated soils after 10 days of incubation

Insecticide concentration (Kg ha^{-1})	Monochrotophos	Cypermethrin
0.0	56	52
1.0	90	85
2.5	105	125
5.0	130	105
7.5	88	82
10.0	46	42

Table.4 Population (MPN x 10^4 g^{-1} soil) of *Azospirillum* sp. in fungicide (mancozeb and hexaconazole) treated and untreated soils after 10 days of incubation

Pesticide concentration (Kg ha^{-1})	Mancozeb	Hexaconazole
0.0	68	68
1.0	85	73
2.5	120	125
5.0	155	148
7.5	95	61
10.0	65	50

Table.5 Nitrogen fixation (mg N g^{-1} malate) by *Azospirillum* sp. as influenced by selected insecticides (monochrotophos and cypermethrin)

	***Azospirillum* sp. from untreated soil**		***Azospirillum* sp. from treated soil***	
	Control	50µg ml^{-1}**	Control	50µg ml^{-1}**
		Monochrotophos		
Strain 1	5.45	9.29	5.94	10.55
Strain 2	4.05	9.86	5.67	11.05
Strain 3	4.21	8.97	6.50	11.58
Strain 4	5.02	8.54	6.10	11.34
		Cypermethrin		
Strain 1	4.94	8.99	6.25	11.55
Strain 2	4.45	9.56	5.17	10.55
Strain 3	4.58	8.67	6.30	11.28
Strain 4	5.12	7.97	5.81	11.14

*The soil sample was treated with commercial formulation of insecticide (5 kg ha^{-1}) and culture was isolated after 7 days.
** Semisolid medium was supplemented with technical sample of the insecticide (50µg ml^{-1} medium) before inoculation with the culture.

Table.6 Nitrogen fixation (mg N g^{-1} Malate) by *Azospirillum* sp. as influenced by selected fungicides (Mancozeb and Hexaconazole)

	***Azospirillum* strains from untreated soil**		***Azospirillum* strains from treated soil***	
	Control	50µg ml^{-1}**	Control	50µg ml^{-1}**
		Mancozeb		
Strain 1	5.57	8.79	5.85	10.95
Strain 2	4.15	9.56	5.27	11.15
Strain 3	4.33	8.87	6.40	11.42
Strain 4	5.02	8.64	6.18	11.32
		Hexaconazole		
Strain 1	4.76	9.10	6.28	11.15
Strain 2	4.46	8.97	5.34	10.45
Strain 3	4.78	9.82	6.53	11.36
Strain 4	5.50	8.57	5.91	11.10

*The soil sample was treated with commercial formulation of fungicide (5 kg ha^{-1}) and culture was isolated after 7 days.
** Semisolid medium was supplemented with technical sample of the insecticide (50µg ml^{-1} medium) before inoculation with the culture.

Similar effects of pyrethroid insecticides and fungicides have also been demonstrated previously on the population of *Azospirilhim* sp. (Rangaswamy and Venkateswarlu, 1999 and Jayamadhuri and Rangaswamy, 2003). However, increases in the population of *Azospirillum* sp. at high concentrations (100 ppm) of benomyl or 2-aminobenzimidazole (a hydrolysis product of benomyl) have been reported in paddy soil (Charyulu and Rao 1978 and Charyulu *et al.*, 1980).

Nayak and Rao (1980) have observed stimulation in population of *Azospirillum* sp. when treated with benomyl at lower concentration (5 ppm) in alluvial, laterite and saline soils, and carbofuran in alluvial soil only.

Nitrogen fixation by *Azospirillum* sp.

The influence of pesticides on the nitrogen fixing ability of *Azospirillum* sp. isolated after 7 days of incubation was assessed. Appreciable nitrogen-fixing activity was observed with the isolates from un-amended soils after 7 days incubation. A significant stimulation of nitrogen-fixation was noticed in the cultures treated with pesticides at the rate 5 kg ha^{-1} when compared with cultures from untreated soils (Table 5 and 6).

The amount of N_2 fixed in the current study was comparable with *Azospirillum* sp. isolated from groundnut cultivated soils amended with monochrotophos and quinolphos (Rangaswamy *et al.*, 1989). When the cultures from untreated soils were inoculated into pesticide amended soils at the rate of 50 µg ml^{-1} exhibited greater nitrogen-fixing activity. A maximum of 11.58 and 11.55 mg N g^{-1} malate was fixed by *Azopspirillum* strains treated with insecticides and fungicides respectively.

The results of the present study evidently indicate that the soil application of insecticides monochrotophos, cypermethrin and fungicides mancozeb, hexaconazole increases the *Azospirillum* population and the nitrogen fixing ability of the isolates.

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