Research Paper :

Effects of herbicides on the growth and activity of *Azospirillum lipoferum* and *Bacillus megatarium* var phosphaticum under *in vitro* conditions

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SUMMARY

The growth of *Azospirillum lipoferum* and P solubilising capabilities, *Bacillus megaterium* and growth as influenced by the presence of herbicides were studied under laboratory conditions. The results indicated that butachlor supported lesser population of *Azospirillum lipoferum* (26.10 % reduction over control) and *Bacillus megaterium* (58.18% reduction over control) when compared to other herbicides.

Key words : Herbicides, *Azospirillum lipoferum*, *Bacillus megaterium*

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In India, herbicides constituted only 15per cent of the total consumption of pesticides, compared to the worldwide consumption of 47.5 per cent. The herbicide consumption is expected to increase dramatically in future as the use of herbicides has been expanding more rapidly than that of the other pesticides. Herbicide usage, which was earlier confined to plantation crops, has now expanded to crops like wheat (42 per cent of the total consumption of herbicides) and rice (30 per cent) with the states of Punjab, Uttar Pradesh, Tamil Nadu and Andhra Pradesh leading in the consumption of more herbicides (Yadaraju and Mishra, 2002).

Research on the effect of herbicides on the soil microbial community in the rice field ecosystem is still fragmentary and inadequate to draw any major conclusions on the impact of herbicides, as majority of the research on tropical wetlands during the past decades focused mainly on the photosynthetic algae. In addition, most of the earlier studies on the impact of herbicides on soil microflora in wetland rice fields had emphasized only on short term acute impacts, while little is known about the chronic effect of repeated and long term application of herbicides under field conditions. Disappearance of components of microbial communities along with a population shift towards microorganisms more efficient in herbicide degradation have been known to occur when herbicides are applied repeatedly, leading to loss of microbial diversity and causing biological degradation of soil (Seghers *et al.*, 2003). The consequences of herbicide induced changes in microbial population would be most evident during biofertilisation, where the microbial population play an important role in maximizing the productivity of crops, as the herbicides, applied to soil persist during the development of plant roots and interact with the biofertilisers applied through seed and root inoculation (Forlani *et al.*, 1995). Moreover, the continuing introduction of new classes of herbicides and the practice of using herbicide mixtures to control weeds, demand a continued research effort to ensure that harmful effects to the ecosystem is avoided. Thus, there is a need to study the influence of herbicides on the microflora and their activities of flooded soils under realistic field conditions and cultural practices on a long term basis to derive any meaningful conclusions.

The increase in food production, till date, had come at the cost of the environment with both qualitative and quantitative degradation of land, water and bioresources (Sarkar and Ghosh, 2001). Hence, it has been advocated that, in the future, any increase in production should be obtained with practices that maintain or enhance the quality of the environment and

that the environmental security should no longer be peripheral to the food and nutritional security.

With this background, the present investigation was carried out with the objectives, to understand and predict the effect of herbicides *viz.*, 2,4-D-2ethylhexyl ester (2,4-DEE), butachlor, pretilachlor and pyrazosulfuron ethyl on rice soil microorganisms and their activities, which could lead to their judicious use and thereby reduce their negative effects, if any on the environment.

MATERIALS AND METHODS

Effect of herbicides on the growth and activity of Azospirillum lipoferum and Bacillus megaterium var. phosphaticum under in vitro conditions :

The growth and nitrogen fixation of *Azospirillum lipoferum* and P solubilising capabilities *Bacillus megaterium* and growth as influenced by the presence of herbicides were studied under laboratory conditions.

Growth responses of Azospirillum lipoferum and Bacillus megaterium var phosphaticum in the presence of herbicides :

Azospirillum lipoferum and *Bacillus megaterium* from agar slopes were transferred to a 250 ml Erlenmeyer flask containing 100 ml of nitrogen free broth and Pikovskaya's broth, respectively, and incubated for 72 h at 30 ± 1^{0}C. One ml of this uniformly growing culture was transferred into a 250 ml flask containing 100 ml of the same liquid medium. Herbicide solutions of the required concentrations were prepared by dissolving technical grade chemicals in appropriate solvents. 2,4 DEE and butachlor were dissolved in ethanol, pretilachlor in methanol, and pyrazosulfuron in acetone to obtain solutions of 0.375 µg ml^{-1}, 0.50 µg ml^{-1}, 0.15 µg ml^{-1} and 0.125 µg ml^{-1}, respectively, corresponding to the field application rates (FR) of the respective herbicides. Solutions of two fold application rates (2 FR), five fold rates (5 FR), ten fold rates (10 FR) and 100 fold rates (100 FR) were similarly prepared. The herbicide solutions were added to the broth with growing cells to give a final concentration of FR, 2 FR, 5 FR, 10 FR and 100 FR and incubated at 30 ± 1^{0}C in a rotary shaker (120 rpm). Control flasks did not receive any herbicides. After 24, 48 and 72 h of incubation, the growth of *A. lipoferum* and *B. megaterium* in the presence of herbicides was monitored by serial dilution and plating by drop plate method in N free malic acid medium and Pikovskaya's medium, respectively.

RESULTS AND DISCUSSION

The growth at different time intervals (24 h, 48 h and 72 h) and nitrogenase activity and phosphate solubilising activity of *A. lipoferum* and *B. megaterium* in the presence of different concentrations of the herbicides *viz.*, 2,4-DEE, butachlor and pretilachlor and pyrazosulfuron ethyl were studied in this experiment.

Effect of herbicides on the growth of A. lipoferum and B. megaterium :

The growth of *A. lipoferum* in N free malic acid broth amended with herbicides at different concentrations is presented in Table 1 and Fig. 1. The growth of *A. lipoferum* was observed to be highly influenced by the presence of herbicides in the growth medium. An initial decrease in growth which recovered in the later stages of incubation was observed in all the herbicides. Significant decrease in the growth of *A. lipoferum* was recorded in the presence of butachlor (5.128 log CFU ml^{-1}) compared to 2,4-DEE (5.188 log CFU ml^{-1}), pretilachlor (5.226 log CFU ml^{-1}) and pyrazosulfuron ethyl (5.258 log CFU ml^{-1}). Among the different concentrations, maximum inhibition of growth was recorded at 100FR (5.059 log CFU ml^{-1}). The least inhibition was observed in 1FR (5.244 log CFU ml^{-1}) concentration (Table 1, Fig. 1).

Growth of *B. megaterium* in Pikovskaya's broth (Table 2 and Fig. 2) amended with different concentrations of 2,4-DEE, butachlor and pretilachlor and pyrazosulfuron ethyl was measured. Among the herbicides, application of butachlor and 100FR among the concentrations were found to significantly decrease the growth of *B. megaterium* (5.384 and 5.176 log CFU ml^{-1}, respectively) in comparison with other treatments. The growth in 1FR concentrations of pyrazosulfuron ethyl (5.730 log CFU ml^{-1}) equaled that of the control (5.743 log CFU ml^{-1}) and was significantly higher than other treatments at the end of the incubation period. The lowest growth was recorded in 100FR concentrations of butachlor (5.112 log CFU ml^{-1}).

Pure culture studies are potentially important in the

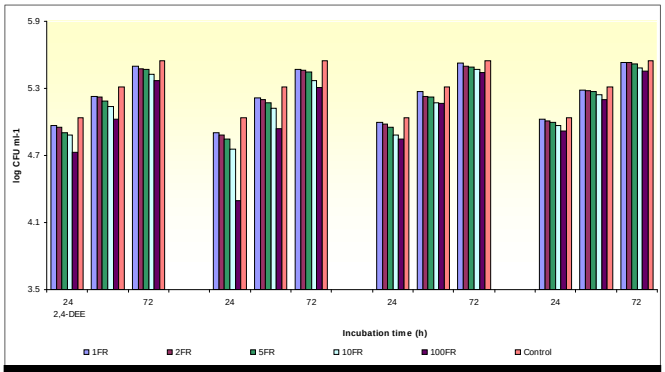

Fig. 1 : Effect of different concentrations of herbicides on the growth of *A. lipoferum* in broth culture

Table 1 : Effect of different concentrations of herbicides on growth of *Azospirillum lipoferum* in broth culture

Herbicide concentration	Population of *Azospirillum lipoferum* (x 10^4 CFU ml^{-1})												Mean
	Days after 2,4-DEE application			Days after butachlor application			Days after pretilachlor application			Days after pyrazosulfuron ethyl application			
	Incubation time (h)			Incubation time (h)			Incubation time (h)			Incubation time (h)			
	24	48	72	24	48	72	24	48	72	24	48	72	
1FR	9.33	17.00	31.33	19.22	8.00	16.33	29.66	18.00	10.00	18.66	33.66	20.77	10.66	19.33	34.33	21.44	19.86
	(4.970)	(5.230)	(5.496)	(5.232)	(4.903)	(5.213)	(5.472)	(5.196)	(5.000)	(5.271)	(5.527)	(5.266)	(5.028)	(5.286)	(5.536)	(5.283)	(5.244)
2FR	9.00	16.66	30.00	18.55	7.66	16.00	29.00	17.55	9.66	17.00	31.66	19.44	10.33	19.00	34.00	21.11	19.16
	(4.954)	(5.222)	(5.477)	(5.218)	(4.884)	(5.204)	(5.462)	(5.183)	(4.985)	(5.230)	(5.501)	(5.239)	(5.014)	(5.279)	(5.531)	(5.275)	(5.229)
5FR	8.00	15.33	29.33	17.55	7.00	15.00	28.00	16.67	9.00	16.66	31.00	18.89	10.00	18.66	33.00	20.55	18.42
	(4.903)	(5.186)	(5.467)	(5.185)	(4.845)	(5.176)	(5.447)	(5.156)	(4.954)	(5.222)	(5.491)	(5.222)	(5.000)	(5.271)	(5.519)	(5.263)	(5.207)
10FR	7.66	13.66	26.66	15.99	5.66	13.33	23.33	14.11	7.66	15.00	29.33	17.33	9.33	17.66	30.33	19.11	16.63
	(4.884)	(5.135)	(5.426)	(5.148)	(4.753)	(5.125)	(5.368)	(5.082)	(4.884)	(5.176)	(5.467)	(5.176)	(4.970)	(5.247)	(5.482)	(5.233)	(5.160)
100FR	5.33	10.66	23.33	13.11	2.00	8.66	20.33	10.33	7.00	14.66	27.66	16.44	8.33	16.00	28.66	17.66	14.39
	(4.727)	(5.028)	(5.368)	(5.041)	(4.301)	(4.938)	(5.308)	(4.849)	(4.845)	(5.166)	(5.442)	(5.151)	(4.921)	(5.204)	(5.457)	(5.194)	(5.059)
Control	11.00	20.66	35.33	22.33	11.00	20.66	35.33	22.33	11.00	20.66	35.33	22.33	11.00	20.66	35.33	22.33	22.33
	(5.041)	(5.315)	(5.548)	(5.301)	(5.041)	(5.315)	(5.548)	(5.301)	(5.041)	(5.315)	(5.548)	(5.301)	(5.041)	(5.315)	(5.548)	(5.301)	(5.301)
Mean	8.39	15.66	29.33	17.79	6.89	15.00	27.61	16.50	9.05	17.11	31.44	19.20	9.94	18.55	32.61	20.37	18.46
	(4.913)	(5.186)	(5.464)	(5.188)	(4.788)	(5.162)	(5.434)	(5.128)	(4.952)	(5.230)	(5.496)	(5.226)	(4.996)	(5.267)	(5.512)	(5.258)	(5.200)

Factors	C.D. (P=0.05)	Factors	C.D. (P=0.05)	Factors	C.D. (P=0.05)
Herbicides (H)	0.005	HXC	0.012	HXCXT	0.021
Concentration (C)	0.006	HXT	0.008		
Time (T)	0.004	TXC	0.010		

Values in parenthesis are $\log_{10}$ transformed

study of effect of pesticides on soil microorganisms because of their simplicity and the wealth of information yielded (Greaves, 1982) and is used for toxicity testing by studying the effect of an added pesticide on growth and metabolism of single microbial species in a defined medium. They are a valuable aid in rapidly screening large numbers of pesticides and in helping to decide whether further, more complicated investigations are necessary. These tests also help to determine if the herbicide tested is inhibitory to the microbial species or conversely if the herbicide can be used as nutrient source by the bacteria tested.

Typical growth response curves of both *A. lipoferum* and *B. megaterium* showing that growth was progressively inhibited as the concentrations were increased as were observed for all the herbicides used in this investigation. Butachlor at the highest concentration of 100FR was found to inhibit the growth and activities of both *A. lipoferum* and *B. megaterium* to the maximum extent.

Several *in vitro* studies in the literature report the effect of herbicides on *Azospirillum* and *Bacillus* though the herbicides are different from that used in this study. The phenoxy acid herbicide 2,4-D had no effect on the growth and nitrogenase activity of *A. brazilense* at 100, 200 and 300 µg ml^{-1} (Martinez-Toledo *et al.*, 1990). The influence of the herbicides metamitron, metribuzin, ethiozin and paraquat on growth and nitrogenase activity of *Azospirillum lipoferum* and *A. brasilense* was studied (Gadkari and Klingmuller, 1988). It was observed that metamitron (35 µg ml^{-1} and 70 µg ml^{-1}) and ethiozin (20 µg ml^{-1}) did not exert any inhibitory effect on nitrogenase activity of these strains. On the other hand, metribuzin (7 µg ml^{-1} and 14 µg ml^{-1}) and ethiozin (50 µg ml^{-1}) caused a marked decrease in nitrogenase activity. These results are contrasting to the present experiment where 0.375 µg ml^{-1}, 0.50 µg ml^{-1}, 0.15 µg ml^{-1} and 0.125 µg ml^{-1} concentrations of 2,4-DEE, butachlor, pretilachlor and

Table 2 : Effect of different concentrations of herbicides on the growth of *Bacillus megaterium* in broth culture

Population of *Bacillus megaterium* (x 10^4 CFU ml^{-1})

Herbicide concentration	Days after 2,4-DEF application			Days after butachlor application			Days after pretilachlor application			Days after pyrazosulfuron ethyl application			Mean
	Incubation time (h)			Incubation time (h)			Incubation time (h)			Incubation time (h)			
	24	48	72	24	48	72	24	48	72	24	48	72	
1FR	18.33	36.66	51.00	35.33	17.00	35.00	49.66	33.89	20.00	38.66	52.33	37.00	21.33	39.33	53.66	38.11	36.08
	(5.263)	(5.564)	(5.708)	(5.512)	(5.230)	(5.544)	(5.696)	(5.490)	(5.301)	(5.587)	(5.719)	(5.536)	(5.329)	(5.595)	(5.730)	(5.551)	(5.522)
2FR	16.66	34.00	48.66	33.11	15.33	33.66	46.33	31.77	18.66	37.33	50.66	35.55	20.00	38.00	52.00	36.67	34.27
	(5.222)	(5.531)	(5.687)	(5.480)	(5.186)	(5.527)	(5.666)	(5.460)	(5.271)	(5.572)	(5.705)	(5.516)	(5.301)	(5.580)	(5.716)	(5.532)	(5.497)
5FR	14.33	30.33	43.00	29.22	13.00	28.00	40.00	27.00	15.33	34.00	45.33	31.55	18.33	36.66	47.00	34.00	30.44
	(5.156)	(5.482)	(5.633)	(5.424)	(5.114)	(5.447)	(5.602)	(5.388)	(5.186)	(5.531)	(5.656)	(5.458)	(5.263)	(5.564)	(5.672)	(5.500)	(5.442)
10FR	11.00	22.66	34.00	22.55	10.33	19.66	31.33	20.44	13.00	27.66	39.00	26.55	14.66	30.66	40.66	28.66	24.55
	(5.041)	(5.355)	(5.531)	(5.309)	(5.014)	(5.294)	(5.496)	(5.268)	(5.114)	(5.442)	(5.591)	(5.382)	(5.166)	(5.487)	(5.609)	(5.421)	(5.345)
100FR	9.00	15.00	22.33	15.44	8.33	13.00	20.00	13.78	9.66	16.66	24.33	16.88	10.00	18.33	27.33	18.55	16.16
	(4.954)	(5.176)	(5.349)	(5.160)	(4.921)	(5.114)	(5.301)	(5.112)	(4.985)	(5.222)	(5.386)	(5.198)	(5.000)	(5.263)	(5.437)	(5.233)	(5.176)
Control	25.66	41.33	55.33	40.77	25.66	41.33	55.33	40.77	25.66	41.33	55.33	40.77	25.66	41.33	55.33	40.77	40.77
	(5.409)	(5.616)	(5.743)	(5.589)	(5.409)	(5.616)	(5.743)	(5.589)	(5.409)	(5.616)	(5.743)	(5.589)	(5.409)	(5.616)	(5.743)	(5.589)	(5.589)
Mean	15.83	30.00	42.39	29.40	14.94	28.44	40.44	27.94	17.05	32.61	44.50	31.39	18.33	34.05	46.00	32.79	30.38
	(5.174)	(5.454)	(5.609)	(5.412)	(5.146)	(5.424)	(5.584)	(5.384)	(5.211)	(5.495)	(5.633)	(5.446)	(5.245)	(5.518)	(5.651)	(5.471)	(5.429)

Factors	C.D. (P=0.05)	Factors	C.D. (P=0.05)	Factors	C.D. (P=0.05)
Herbicides (H)	0.005	HXC	0.012	HXTXC	0.021
Concentration (C)	0.006	HXT	0.008		
Time (T)	0.004	TXC	0.010		

Values in parenthesis are $\log_{10}$ transformed

pyrazosulfuron ethyl were found to reduce the growth and nitrogenase activity of *Azospirillum lipoferum*. The dissimilar results could be due to the difference in the herbicides used in the studies. The population of *Azospirillum* sp. initially decreased, then increased and reached a stationary phase in liquid medium in the presence of certain insecticides endosulfan and monocrotophos (Gadagi *et al.*, 2004). A similar trend was also observed in the present experiment where the *Azospirillum lipoferum* growth initially decreased after 24 h of incubation compared to control treatment and then increased at over time.

Inhibition of the growth of *B. megaterium* and *B. subtilis* by ioxynil and bromoxynil, diquat and paraquat was reported by Smith and Fletcher (1964). Cserhati *et al.* (1992) have reported the negative effect of benzonitrile ester herbicides on *B. megaterium*. The herbicides rimsulfuron and cinosulfuron were found to inhibit the growth of both *A. lipoferum* and *B. megaterium* when tested against concentrations ranging from 0.2 to 0.5 µmol g^{-1} (Forlani *et al.*, 1995). 2,4-D amine was found to be toxic to *B. subtilis* and the percentage survival was found to decrease with increasing concentration of the herbicide (Adeleye *et al.*, 2004). All the herbicides used in this study were observed to reduce the growth of *Bacillus megaterium* at all the concentrated tested.

Burnet and Hodgson (1991) have reported that the membrane acts as a barrier that keeps the herbicides out of the cell. So herbicides that are able to penetrate the bacterial cell or that inhibit any membrane or cell wall surface activities show inhibitory effects on bacterial growth and activity. 2,4-D has been reported to go through the cell envelope of *Rhizobium* and accumulate in the cytosol producing toxic effects (Fabra *et al.*, 1997). 2-4 D has also been shown to exhibit cytotoxicity and mutagenicity to *Rhizobium* and *Bradyrhizobium* sp (Jaiswal *et al.*, 2004). Herbicides that have more solubility in hydrophobic solvents like butachlor (Kim *et al.*, 2001) are known to increase the cell

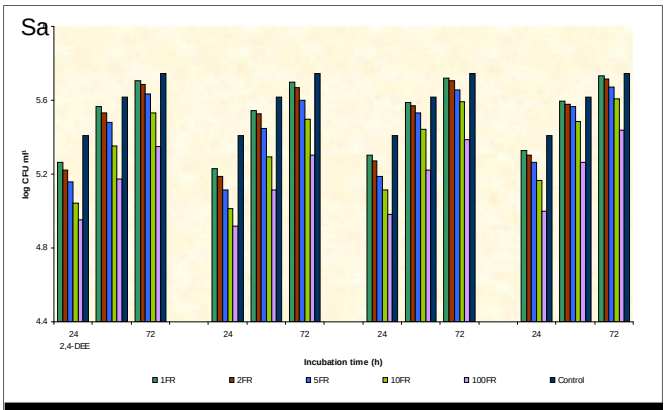

Fig. 2 : Effect of different concentrations of herbicides on the growth of *B. megaterium* in broth culture

membrane permeability and increase the toxic effects. Certain chemicals like also inhibit microbial growth and activity by inhibiting oxidative phosphorylation and ATPase activity (Ferrer *et al.*, 1986).

It was observed in this experiment that the herbicides inhibited the growth and activities of *Azospirillum lipoferum* and *Bacillus megaterium* when they were present in the growth medium where they came into direct contact with the bacterial cell. However, extrapolation of these results to the soil and rhizosphere requires caution because a number of factors including climate, soil type, agricultural practices and the composition of microbial communities also influence the effect of the applied herbicides (Gadagi, *et al.*, 2004).During *in vitro* studies, it was observed that butachlor supported lesser population of *Azospirillum lipoferum* (26.10 % reduction over control) and *Bacillus megaterium* (58.18% reduction over control) when compared to other herbicides.

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