Influence of pesticides on *Azospirillum* sp. population and its nitrogen fixation in groundnut (*Arachis hypogaea* L.) soils

ABSTRACT

Aim: To study the impact of selected pesticides on *Azospirillum* sp. population and its nitrification in groundnut (*Arachis hypogaea* L.) soils.

Study design: Black clay and red sandy loam soils with known pesticide history were collected from groundnut (*Arachis hypogaea* L.) cultivated fields and were investigated to elucidate the impact of pesticides on *Azospirillum* sp. population and its nitrification in both the soils.

Place and Duration of Study: The soil samples were collected from groundnut cultivated fields of Anantapur District, Andhra Pradesh (A.P) and the study was carried out for 3 months.

Methodology: Ten gram portions of each soil sample were placed in (25 × 150 mm) test tubes and treated with different concentrations of pesticides, (10, 25, 50, 75 and 100 µg g^{-1} soil) which were equivalent - 1.0, 2.5, 5.0, 7.5 and 10 kg ha^{-1}. Soil samples without pesticides served as controls. The soils with and without pesticides were incubated at room temperature (28 ± 4°C) in the laboratory and moisture content was maintained at 60% water holding capacity (WHC) throughout the experimental period. After 7 and 14 days of incubation, triplicate soil samples were used to estimate the population size of *Azospirillum* sp. using the MPN method. Five ml aliquots of semi – solid malate medium were added to five MPN tubes and inoculated with 0.5 ml of a soil suspension from 10^{-1} to 10^{-5} soil dilutions, and incubated at 37° C.

Results: The population of *Azospirillum* sp. in both soils increased when pesticides were applied @ 2.5 - 5.0 kg ha^{-1} and incongruity, when the pesticides concentration increased from 7.5 - 10.0 kg ha^{-1}, the *Azospirillum* sp. population gradually decreased in both soils.

Conclusion: The present study aimed at determining the influence of selected pesticides such as oxydemeton methyl, emamectin benzoate, dithane Z-78 and benomyl on the population of *Azospirillum* sp. and nitrogen fixation in black clay soil and red sandy loam soils in groundnut cultivated fields of Anantapur District, Andhra Pradesh, India. Insecticides and fungicides applied up to 5.0 kg ha^{-1}, enhanced the population of *Azospirillum* sp. and its nitrogen fixation also increased significantly after 7 and 14 days of incubation in both soils. However, the population of *Azospirillum* sp., decreased with increasing period of soil incubation in both treated and untreated soils.

Keywords: Pesticides, Groundnut (Arachis hypogaea L.*) soils, Azospirillum sp. population, nitrogen fixation activity.*

1. INTRODUCTION

Soil is an important system for the biological interactions of various microorganisms, hence the applications of pesticides in the agriculture leads to pessimistic side effects on soil micro flora leading to soil pollution and soil contamination [1]. Pesticides may perturb

microorganisms by reducing their numbers, biochemical activity, diversity and change the structure of microbial populations. [2,3,4,5,6]. According to [7], pesticides application starts from pre sowing and post sowing stages of seeds, such as treatment of pesticides includes soil treatment, seed treatment and spraying treatment. About 20% of crop farming production and 60% of fruit production are based on the utilization of pesticides [8]. According to the FAO data, discontinuation of pesticide practice, would wither agricultural crop yield by 30-50 % with the damage of about 75 billion dollars [9]. According to the type of pest which shows effectual action, pesticides are grouped into insecticides, herbicides and fungicides [10]. In pure culture and in mixed populations the impact of pesticides on the microbial activities of *Azospirillum* has been studied [11,12]. *Azospirillum* sp. are very important rhizosphere bacteria and many species has been isolated from the roots and rhizosphere of numerous host plants and successfully isolated from bulk soil [13], from the beginning of agricultural research on these species [14].

Azospirilla are free-living rhizobacteria that are able to promote plant growth and increase yields in many crops of agronomic importance. It is assumed that the bacteria affect plant growth mainly by the production of plant growth promoting substances, which leads to an improvement in root development and an increase in the rate of water and mineral uptake [15].

Among the oil yielding crops, Groundnut (*Arachis hypogeae* L.) is one of the important, major, profitable crops grown throughout the year in India and India is a World leader in groundnut farming, with 8 million hectare of cultivated area in the year 2002-03 [16]. It is the single largest source of edible oils in india and constitutes roughly about 50% of the total oil seed production [17]. Groundnut (*Arachis hypogaea* L.) is one of the major cash crops grown in dry land of India [18]. Within Andhra Pradesh state, Anantapur district, a semi-arid region occupies a predominant place in groundnut cultivation [19].

The current day agriculture involves huge cultivation of the groundnut crop because of its imperative role in edible oil seeds production [20]. The escalating increase of pest problem and demand for agricultural food production entailed the utilization of agrochemicals that ensure high quality and to crop yield [21]. The application of pesticides into the soil environment inflates concern as to their effect on ecological balance in terms of soil fertility [22,21]. The amount of applied pesticides reaching the target organism is about 0.1% while the remaining bulk contaminates the soil environment [23,24]. Globally, about 3×10^9 kg of pesticides is applied annually with a purchase price of nearly $40 billions each year [25]. According to [26], pesticide residues generally persist in the top 15 cm layer of the soil which is the area of greatest activity of soil microflora that is conducive for - interaction of pesticide residues with the flora of the soil ecosystem [27]. The interaction of pesticides with soil microorganisms and their metabolic activities may change the physiological and biochemical behavior of microorganisms in soil [28]. According to [29], the observed changes in the soil activity depend on the intensity and spectrum of activity as well as tenacity of the parent chemicals or its metabolites.

Microorganisms play a significant role in many soil biological processes, including nitrogen transformations, organic matter decomposition, nutrient release and their availability, as well as stabilize the soil structure and disturb its fertility, investigated by [30,31,32]. Soil microflora is the first biota that undergoes direct and indirect impacts of toxic substances introduced to soil. The predominant feature of soil quality is considered to be the microbial biomass [33]. Microorganisms forms an essential part of soil food web and hence, microbial biomass is considered to be a measure of potential microbiological and ecosystem functioning. [34].

Bacteria that belong to the *Azospirillum* genus are known to associate symbiotically with grass forming specialized structures in the roots in which there is conversion of N_2 to NH_3 [35]. *Azospirillum* is a free living micro-aerophilic, heterotrophic diazotrophic bacterium that is involved in heterotrophic nitrogen fixation in several grass bacterial associations [36].

Agrochemicals especially pesticides and herbicides had adverse effect on Azospirillum growth [37]. The impact of several pesticides on the growth and nitrogen fixation of *Azospirillum* sp. has been scrutinized in pure culture systems by few workers [38,39,40,41]. Bacteria play an important role in maintaining the health status of soil ecosystem by preforming many biological processes. Changes on soil microbial activity may be triggered by different management approaches and the study of the effects of such changes on xenobiotics, of non-target populations, may represent a valuable strategy to evaluate their environmental risk potential. Based on these considerations, the objective of the present study was to evaluate the effect of insecticides and fungicides on *Azospirillum* sp. population and its nitrogen fixation in black clay soil and red sandy loam soils of groundnut (*Arachis hypogeae* L.) cultivated fields of Anantapur District.

2. MATERIALS AND METHODS

2.1 Soils

Soil samples used in this investigation were collected from groundnut (*Arachis hypogeae* L.) cultivated fields of Anantapur district of Andhra Pradesh, India, to a depth of 12 cm, air dried and sieved through a 2 - mm sieve before use.

2.1.1 Chemicals

For incubation studies and for estimating microbial populations such as *Azospirillum* sp. Commercial formulations of oxydemeton methyl (25 % EC), emamectin benzoate (5 % SG), dithane Z-78 and benomyl dissolved in distilled water were used. The details of the pesticides can be found in Table 2.

2.1.1.1 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 [42].

2.1.1.1.1 Population of Azospirillum sp.

To determine the influence of selected insecticides oxydemeton methyl, emamectin benzoate and fungicides such as dithane Z-78 and benomyl with concentrations of 10, 25, 50, 75 and 100 µg g^{-1} soil on population of *Azospirillum* sp. Ten gram portions of each soil sample were placed in (25 × 150 mm) test tubes and were treated with different concentrations of pesticides, (10, 25, 50, 75 and 100 µg g^{-1} soil) which were equivalent to 1.0, 2.5, 5.0, 7.5 and 10 kg ha^{-1} [43,44]. Soil samples without pesticides served as controls. The soils with and without pesticides were incubated at room temperature (28 ± 4°C) in the laboratory and moisture content was maintained at 60% water holding capacity (WHC) throughout the experimental period. After 7 and 14 days of incubation, triplicate soil samples were used to estimate the population size of *Azospirillum* sp. using the MPN method described by [45], with MPN values calculated using probability tables [45]. The growth medium (sterile, nitrogen-free, semi-solid malate medium, pH=6.8 [46] contained (per L): Malic acid, 5 g; KOH, 4g; K2HPO4, 0.5 g; MgSO4, 0.2 g; NaCl, 0.1 g; CaCl2, 0.02 g; FeSO4

, 0.5 g; Na2MoO4, 0.02 g; MnSO4, 0.01 g; 5 % Alcoholic solution of bromothymol blue, 2 ml; agar, 1.75 g). Five ml aliquots of medium were added to five MPN tubes and inoculated with 0.5 ml of a soil suspension 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 incubation for 36 h were scored positive for *Azospirillum* sp.. Microscopic examination of the cultures revealed the characteristic rods adhered to the flat droplets of oil.

2.1.1.1.1.1 Nitrogen fixation by Azospirillum sp.

Stock solutions of technical grade pesticides, prepared in acetone, were placed in sterilized test tubes (25 × 200 mm) to provide a final concentration of 50µg ml^{-1} malate medium. After evaporation of carrier solvent, 20 ml portions of the steam-sterilized malate medium were introduced into each test tube under aseptic conditions. The residues were equilibrated for 24 hrs to obtain aqueous solutions of the pesticides [47,48]. Medium, in test tubes without the pesticide served as controls. Soil suspensions (1:10 soil to water ratio) from untreated and pesticide-treated (5 kg ha^{-1} level with commercial formulations) samples, incubated for 7 days, were prepared in sterilized distilled water. These suspensions (0.1 ml) were used to inoculate 20 ml portions of malate medium with and without the pesticide. After 3 days (72 h) incubation at 37℃, these test tubes for each treatment were digested with H_2SO_4 to estimate in total nitrogen (N) by the Micro - Kjeldahl method as described earlier [49,50]. The amount of N present in 0.1 ml soil suspensions, used for inoculation, together with that of the medium was deducted from experimental values.

Azospirillum sp. were isolated from untreated and pesticide - treated (4 times at 10 day intervals) soil samples to determine whether the increased nitrogen fixing capacity of *Azospirillum* sp. isolated from soil samples treated with pesticides would continue further, the isolates were subcultured in the semi - solid malate medium 3 times at an interval of 7 days, and their rates of nitrogen fixation were compared with those of fresh cultures obtained immediately after isolation from untreated and pesticide treated soil samples.

3. Statistical analysis

All data were expressed on an air dry soil basis and were averages of three replicates. Data were analyzed by significant difference ($P < 0.05$) between pesticide - treated and untreated soils using Duncan multiple range (DMR) test [51,52]. If A + B < AB, the response can be considered as synergistic interaction. If A + B > AB, the response can be considered as antagonistic interaction; if A + B = AB, the response can be considered as additive interaction (where, A = the percent stimulation in population of *Azospirillum* sp. caused by pesticide X alone over the control; B = the percent stimulation in population *Azospirillum* sp. caused by pesticide Y alone over the control; and AB = the percent stimulation in population of *Azospirillum* sp. caused by the combination of X + Y over the control). The percent stimulation values were calculated relative to population of *Azospirillum* sp.

4. Results

4.1 Effect of pesticides on population of *Azospirillum* sp. in soils

The initial size of the population of *Azospirillum* sp. was low in both soils (Table. 3 and 4). The population of *Azospirillum* sp. was significantly higher in soils treated with oxydemeton methyl, emamectin benzoate, dithane Z-78 and benomyl respectively, than in untreated control soils during the course of experiment. The population of *Azospirillum* sp. in soils increased when pesticides were applied at 2.5 - 5.0 kg ha^{-1}; by contrast, as the concentration of pesticides increased to 7.5 - 10.0 kg ha^{-1}, the population of *Azospirillum* sp.

gradually decreased in both soils. Application of pesticides, singly and in repeated up to 5.0 kg ha^{-1}, profoundly enhanced the population of *Azospirillum* sp. in vertisol soil (Table 3 and 4). For the laterite soil, pesticide concentrations up to 2.5 kg ha-1 increased the population of *Azospirillum* sp. after 7 and 14 days of incubation (Table 3 and 4). The increase in population of *Azospirillum* sp. in vertisol soil amended with oxydemeton methyl, emamectin benzoate, dithane Z-78 and benomyl (i.e. at 1.0, 2.5 and 5.0 kg ha^{-1}) was 100 - 300, 85 -238, 82 - 192 and 115 - 284 %, respectively, over the control treatment after incubation for 7 days (Table 3). The population of *Azospirillum* sp. in vertisol soil with or without pesticides decreased gradually after 14 days (Table 3 and 4) compared to that after 7 days. The corresponding increases in population of *Azospirillum* sp. in laterite soil amended with four pesticides at 1.0 and 2.5 kg ha^{-1} were 46 - 203, 64 - 239, 80 - 239 and 84 - 221 %, respectively, over the control treatment by the end of 7 day interval (Table 3 and 4). The population of *Azospirillum* sp. also decreased gradually under similar conditions after a 14 day incubation in laterite soil (Table 4). The influence of oxydemeton methyl, emamectin benzoate, dithane Z-78 and benomyl alone, at different levels on the population of *Azospirillum* sp. in the two soils was assessed to examine interaction between pesticides. Interaction responses are generally distinguished on the basis of percent stimulation values (over control) regarding any parameter in soil treated with single pesticide or in repeated application at a specified dose in soil. In this study oxydemeton methyl, emamectin benzoate, dithane Z-78 and benomyl singly (i.e., at 1.0, 2.5, 5.0, 7.5 and 10.0 kg ha^{-1}) interacted synergistically, additively and antagonistically, respectively (Table 3,4 and 5). It is clear from these results that the occurrence of interactions between insecticides and fungicides was dose-dependent, and these interactions were prevailed in soil even after incubation for 14 days.

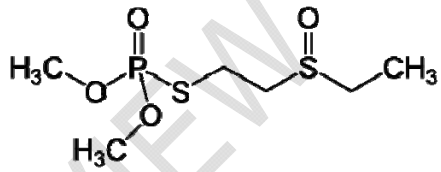
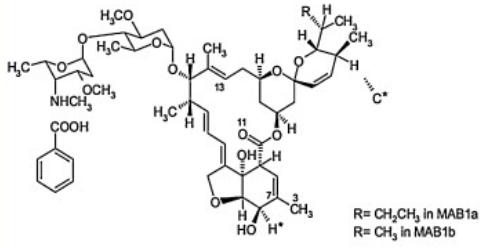
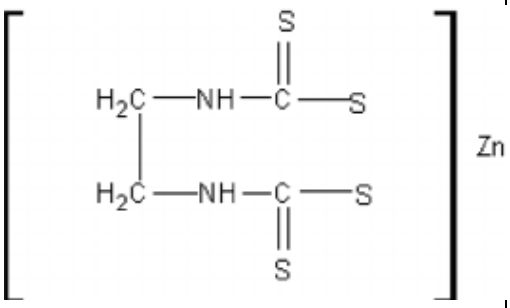
Table 1. Physico-chemical properties of soils used in the present study

Properties	Black clay soil	Red sandy loam soil
Sand (%)	76.50	72.00
Silt (%)	18.00	25.00
Clay (%)	5.50	3.00
pH[a]	8.40	6.30
Water holding capacity (ml g^{-1} soil)	0.48	0.34
Electrical conductivity (m.mhos)	266.00	246.00
Organic matter[b](%)	0.94	0.80
Total nitrogen[c] (%)	0.05	0.03
NH_4^+ - N(µ g^{-1} soil)[d]	8.95	7.80
NO_2^- - N (µ g^{-1} soil)[e]	0.51	0.35
NO_3^- -N(µ g^{-1} soil)[f]	1.04	0.19

[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 Bartler, 1972)*

Table 2. Particulars of the Pesticides used.

S.No	PESTICIDE	MOLECULAR FORMULA	STRUCTURE
1.	Oxydemeton Methyl	$C_6H_{15}O_4PS_2$	
2.	Emamectin Benzoate	$C_5H_{81}NO_{15}$	
3.	Dithane Z-78	$C_4H_6N_2S_4Zn$	

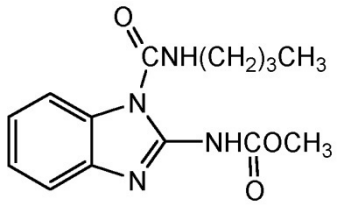
4.	Benomyl	$C_{14}H_{18}N_4O_3$	

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Table 3. Population (MPN × 10^3 g^{-1} soil) of *Azospirillum* sp. as influenced by the application of pesticides in black soil

Pesticides	Soil incubation in days, after pesticide application											
	0*	7 Days						14 days					
		0**	1.0	2.5	5.0	7.5	10.0	0**	1.0	2.5	5.0	7.5	10.0
Oxydemeton methyl	2.2	6.5 a (100)	13.0 b (200)	18.0 b (277)	26.0 c (400)	15.0 d (231)	10.0 c (154)	5.2 a (100)	9.4 b (181)	12.0 c (231)	16.0 d (308)	9.3 e (179)	8.1 f (156)
Emamectin benzoate	2.2	6.5 a (100)	12.0 b (185)	16.0 c (246)	22.0 d (338)	31.0 c (477)	8.6 f (132)	5.2 a (100)	8.5 b (163)	11.0 c (211)	14.0 d (269)	12.0 e (231)	7.3 f (140)
Dithane Z-78	2.2	6.5 a (100)	12.0 b (182)	15.0 c (231)	19.0 d (292)	13.0 e (200)	9.1 f (338)	5.2 a (100)	8.2 a (179)	11.0 b (288)	13.0 c (346)	10.2 d (188)	6.3 f (138)
Benomyl	2.2	6.5 a (100)	14.0 b (215)	18.0 c (215)	25.0 d (384)	15.0 c (231)	9.1 f (338)	5.2 a (100)	9.3 b (179)	15.0 c (288)	18.0 d (346)	9.8 e (188)	7.2 f (138)

**Initial 0-day population*

***Concentration of the pesticide, kg ha^{-1}*

Figures, in parenthesis, indicate relative productive percentages.

Means, in each row, obtained for each sampling, followed by the same letter are not significantly different ($P < 0.05$) from each other according to (Duncan's Multiple Range) DMR test.

Values in the table are means of triplicates.

Table 4. Population (MPN × 10^3 g^{-1} soil) of *Azospirillum* sp. as influenced by the application of pesticides in red soil

Pesticides	Soil incubation in days, after pesticide application												
	0*	7 Days						14 days					
		0**	1.0	2.5	5.0	7.5	10.0	0**	1.0	2.5	5.0	7.5	10.0
Oxydemeton methyl	2.2	5.6 a (100)	8.2 b (146)	17.0 c (303)	12.0 d (214)	8.5 e (152)	5.0 f (89)	4.2 a (100)	7.3 b (174)	13.1 c (312)	9.4 d (224)	6.5 e (155)	3.2 f (76)
Emamectin benzoate	2.2	5.6 a (100)	9.2 b (164)	19.0 c (339)	14.0 d (250)	12.0 e (214)	4.2 f (75)	4.2 a (100)	7.3 b (174)	14.0 c (333)	11.0 d (262)	6.8 e (162)	3.6 f (86)
Dithane Z-78	2.2	5.6 a (100)	10.0 b (180)	19.0 c (339)	16.0 d (286)	12.0 e (214)	4.3 f (76)	4.2 a (100)	7.1 b (169)	11.3 c (269)	9.4 d (224)	6.2 e (188)	3.5 f (83)
Benomyl	2.2	5.6 a (100)	10.3 b (184)	18.0 c (321)	15.0 d (268)	12.0 e (214)	4.4 f (78)	4.2 a (100)	6.2 b (147)	12.0 c (286)	7.8 d (186)	7.9 d (188)	3.7 e (88)

**Initial 0-day population*

***Concentration of the pesticide, kg ha^{-1}*

Figures, in parenthesis, indicate relative productive percentages.

Means, in each row, obtained for each sampling, followed by the same letter are not significantly different ($P < 0.05$) from each other according to (Duncan's Multiple Range) DMR test.

Values in the table are means of triplicates.

Table 5 : Influence of selected pesticides on nitrogen fixation (mg N g^{-1} malate) by *Azospirillum* sp.

Soil Type	Cultures from untreated soil		Culture from pesticide treated soil	
	Untreated	**50 μg ml^{-1}	Untreated	**50 μg ml^{-1}
		Oxydemeton methyl		
Black Soil	7.80 a	11.89 b	10.98 b	14.24 c
Red Soil	5.32 a	08.78 b	09.24 c	11.82 d
		Emamectin benzoate		
Black Soil	6.82 a	10.34 b	11.22 b	13.21 c
Red Soil	4.82 a	07.78 b	09.02 b	11.32 c
		Dithane Z-78		
Black Soil	5.78 a	09.78 a	12.01 c	12.86 c
Red Soil	4.92 a	08.71 b	09.02 b	11.32 c
		Benomyl		
Black Soil	6.24 a	10.31 b	11.24 c	11.83 c
Red Soil	4.89 a	08.24 b	09.85 b	10.54 c

**The soil sample was treated with commercial formulation of the four pesticides (5 kg ha^{-1}) and culture was isolated after 7 days.*

***Semi-solid malate medium was supplemented with technical sample of the pesticides (50 µg ml^{-1} medium) before incubation with the culture.*

Means (n = 3), in each row, are significant (P < 0.05) from each other according to Duncan's Multiple Range (DMR) test.

Table 6. Impact of subculturing of *Azospirillum* sp. isolated from pesticide-treated soil samples on nitrogen fixation (mg N g^{-1} malate)

Soil type	Fresh isolate from untreated soil**	Isolate from pesticide-treated soil*	
		Fresh	After third subculturing**
Black Soil			
1.Oxydemeton methyl	8.80 a	18.78 b	17.92 b
2.Emamectin Benzoate	9.65 a	19.24 b	19.05 b
3.Dithane Z-78	7.94 a	18.23 b	17.98 b
4.Benomyl	8.24 a	17.68 b	16.98 b
Red Soil			
1.Oxydemeton methyl	7.76 a	17.34 b	16.88 b
2.Emamectin Benzoate	8.64 a	18.34 b	17.94 b
3.Dithane Z-78	7.68 a	17.42 b	16.82 b
4.Benomyl	7.24 a	17.08 b	16.24 b

**Soil samples were treated four times with pesticides at 5 kg ha^{-1} level.*

***Semi-solid malate medium was supplemented with technical sample of the pesticides (50µg ml^{-1} medium) before incubation with the culture.*

Means (n = 3), in each row, are significant (P < 0.05) from each other according to Duncan's Multiple Range (DMR) test.

5. Discussion

In the present study, four pesticides applied to soil, singly at concentrations ranging from 1.0 to 5.0 kg ha^{-1}, had no deleterious effect on *Azospirillum* sp . A similar individual instigate effect of monocrotophos and chlorpyrifos was previously demonstrated on the population of *Azospirillum* sp. [53]. Similarly, observations with other organophosphorus and pyrethroid insecticides and fungicides have also been reported [44,41]. Interactions between different agrochemicals applied in repeated application on microorganisms and their activities in soils have received little attention in comparison to effects of a single agrochemical. There were no differences in degree of diversity in bacterial populations from the application of a combination of five pesticides, including chlorfenviphos and glyphosate, to field plot of 20 years[54]. In the present study the application of pesticides to the soils at certain concentrations was not harmful to the population of *Azospirillum* sp. Some reports have been published on interactions between pesticides and their solvents, pesticides and their degradation products, and two different pesticides on growth of organisms in pure culture studies of fungi, algae and cyanobacteria [55,56,57,58,59,60,61]. In all these studies, a variety of interaction effects such as synergistic, additive and antagonistic were observed, depending on concentration of the interacting chemicals. For instance, the combination of permethrin and its degradation product interact to yield antagonistic, additive and synergistic interactions towards the growth of fungi in pure culture [60], because the degradation rate of an individual pesticide may be changed due to the combinations of pesticides, ultimately leading to different types of interactions. In the present study, similar types of interactions occurred by selected pesticides on population of *Azospirillum* sp. in two soils. A increase in the population of *Azospirillum* sp. at high concentrations (100 ppm) of benomyl or 2-aminobenzimidazole (a hydrolysis product of benomyl) were also reported in paddy soil [36,38]. [39], noticed a provoking response in *Azospirillum* sp. population, when treated with benomyl at lower concentration (5 ppm) in alluvial, laterite and saline soils, and carbofuran in alluvial soil only.

These observations are in agreement with the results of the present study. The overall influence of pesticides on microbial activities in soil may be subject to interactions between pesticides (i.e. additive, synergistic and antagonistic) and may differ from the response of the individual pesticide components [62]. In the present study similar types of interactions occurred between selected insecticide and fungicides in two soils. Although the mechanisms of interactions are not known, interaction patterns may have a profound influence on soil microflora and their activities, thereby affecting soil fertility. Pesticides added to soil undergo degradation to metabolites in the course of time. For instance, monocrotophos is hydrolysed to N-methyl acetoacetamide [63]. Pesticides are generally applied simultaneously or serially for crop protection, hence the degradation behavior of a pesticide may be changed after it interacts with other pesticides (or their degradation products) already present in the soil; such changes in pesticide degradation may have different side effects on biological processes, such as nitrification and on microbial populations. The presence of chlorothalonil has been suggested as altering the degradation behavior of chlorpyrifos - degrading microbes [64]. The persistent interaction responses recorded in the present study cannot be attributed exclusively to parent pesticides, since metabolites may also have biological effects. Generally pesticides are recalcitrant (not easily degradable) substances, hence they persist for long periods in the soils. This may be one of the main reasons for persistent interactive effects in soil. The present study further accentuates the need for a systemic study on the interactive effects of pesticides used extensively, as well as their metabolites. The results of the present investigation clearly indicate that the selected pesticides – oxydemeton methyl, emamectin benzoate, dithane Z-78 and benomyl, respectively at levels ranging from 1.0 to 5.0 kg ha^{-1} significantly increased the population of *Azospirillum* sp. .Furthermore, these pesticides, singly and in repeated application, at levels of 1.0 to 10.0 kg ha^{-1} exerted synergistic, additive or antagonistic interactions towards population of

Azospirillum sp. in these soils. *Azospirillum* sp. cultures obtained after 7 days of soil incubation, from unamended soils exhibited appreciable nitrogen fixing activity (Table 5). A significant stimulation of nitrogen fixation was evident in cultures from soils treated with the four pesticides at a level of 5 kg ha^{-1} when compared with cultures from untreated soils. The extent of nitrogen fixation by the cultures observed in the present study are comparable with those of *Azospirillum* cultures isolated from the same soils amended with monocrotophos and quinolphos for 7 days [40], and those cultures isolated from a rice soil amended with benomyl and incubated for 30 days[36]. The cultures from untreated soil, when inoculated into the medium supplemented with four pesticides (Oxydemeton Methyl, Emamectin Benzoate, Dithane Z-78 and benomyl) at 50 µg ml^{-1}, exhibited greater nitrogen-fixing activity. However, the stimulation in nitrogen fixation was more pronounced in cultures of *Azospirillum* sp. isolated from four pesticides treated (5 kg ha^{-1}) soil and inoculated to the medium containing 50 µg ml^{-1} of the pesticide (Table 5).

An attempt was made to determine whether the observed nitrogenase activity would continue upon subsequent subcultures of the diazotroph. Although, fresh cultures from the pesticide-treated soil exhibited greater nitrogen-fixation when compared with those from untreated soils, subculturing of the isolates 3 times had no effect on nitrogen-fixation in the cultures of *Azospirillum* sp., exposed to the selected pesticides.

The present study clearly shows that soil application of pesticides (Oxydemeton Methyl, Emamectin Benzoate, Dithane Z -78 and benomyl) increased the population of *Azospirillum* sp., isolated from treated with four pesticides, last for longer periods.

6. CONCLUSION

The results of present investigation clearly indicate that the selected pesticides at levels ranging from 2.5 to 5.0 Kg ha^{-1} significantly increased the population of *Azospirillum* sp. and nitrification in both the soils. Furthermore, increase in the concentration above 2.5 or 5.0 K g ha^{-1} exerted synergistic, additive or antagonistic interactions towards population of *Azospirillum* sp. and nitrification in these soils.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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