

1
2
3
4
5
6
7
8
9
10
11
12

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

13
14
15
16
17

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 **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. 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 **at @** 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 **four selected** pesticides such as oxydemeton methyl, emamectin benzoate, dithane Z-78 and benomyl on the population of *Azospirillum* sp. and **on** 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

18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70

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 lowering 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 hypogaeae* 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 **ample 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 the 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

71 biomass [33]. Microorganisms forms an essential part of soil food web and hence, microbial
72 biomass is considered to be a measure of potential microbiological and ecosystem
73 functioning. [34].
74

75 Bacteria that belong to the *Azospirillum* genus are known to associate symbiotically with
76 grass forming specialized structures in the roots in which there is conversion of N_2 to NH_3
77 [35]. *Azospirillum* is a free living micro-aerophilic, heterotrophic diazotrophic bacterium that is
78 involved in heterotrophic nitrogen fixation in several grass bacterial associations [36].
79

80 Agrochemicals especially pesticides and herbicides had adverse effect on *Azospirillum*
81 growth [37]. The impact of several pesticides on the growth and nitrogen fixation of
82 *Azospirillum* sp. has been scrutinized in pure culture systems by few workers [38,39,40,41].
83 Bacteria play an important role in maintaining the health status of soil ecosystem by
84 performing many biological processes. Changes on soil microbial activity may be triggered
85 by different management approaches and the study of the effects of such changes on
86 xenobiotics, of non-target populations, may represent a valuable strategy to evaluate their
87 environmental risk potential. Based on these considerations, the objective of the present
88 study was to evaluate the effect of insecticides and fungicides on *Azospirillum* sp. population
89 and its nitrogen fixation in black clay soil and red sandy loam soils of groundnut (*Arachis*
90 *hypogaeae* L.) cultivated fields of Anantapur District.
91

92 **2. MATERIALS AND METHODS**

93 **2.1 Soils**

94 Soil samples used in this investigation were collected from groundnut (*Arachis hypogaeae* L.)
95 cultivated fields of Anantapur district of Andhra Pradesh, India, to a depth of 12 cm, air dried
96 and sieved through a 2 - mm sieve before use.
97

98 **2.1.1 Chemicals**

99
100 For incubation studies and for estimating microbial populations such as *Azospirillum* sp.
101 Commercial formulations of oxydemeton methyl (25 % EC), emamectin benzoate (5 %
102 SG), dithane Z-78 and benomyl dissolved in distilled water were used. The details of the
103 pesticides can be found in Table 2.
104

105 106 **2.1.1.1 Soil incubation**

107
108 The soil ecosystem stimulating non-flooded conditions consisting of ten gram portions of soil
109 samples were added in test tubes (25 x 150 mm) and moistened to a water potential of
110 0.090 MPa, in order to maintain at 60% water holding capacity [42].
111

112 **2.1.1.1.1 Population of *Azospirillum* sp.**

113
114 To determine the influence of selected insecticides oxydemeton methyl, emamectin
115 benzoate and fungicides such as dithane Z-78 and benomyl with concentrations of 10, 25,
116 50, 75 and 100 $\mu\text{g g}^{-1}$ soil on population of *Azospirillum* sp. Ten gram portions of each soil
117 sample were placed in (25 x 150 mm) test tubes and were treated with different
118 concentrations of pesticides, (10, 25, 50, 75 and 100 $\mu\text{g g}^{-1}$ soil) which were equivalent to
119 1.0, 2.5, 5.0, 7.5 and 10 kg ha^{-1} [43,44]. Soil samples without pesticides served as controls.
120 The soils with and without pesticides were incubated at room temperature ($28 \pm 4^\circ\text{C}$) in the
121 laboratory and moisture content was maintained at 60% water holding capacity (WHC)
122 throughout the experimental period. After 7 and 14 days of incubation, triplicate soil samples

123 were used to estimate the population size of *Azospirillum* sp. using the MPN method
124 described by [45], with MPN values calculated using probability tables [45]. The growth
125 medium (sterile, nitrogen-free, semi-solid malate medium, pH=6.8 [46] contained (per L):
126 Malic acid, 5 g; KOH, 4g; K₂HPO₄, 0.5 g; MgSO₄, 0.2 g; NaCl, 0.1 g; CaCl₂, 0.02 g; FeSO₄
127 , 0.5 g; Na₂MoO₄, 0.02 g; MnSO₄, 0.01 g; 5 % Alcoholic solution of bromothymol blue, 2 ml;
128 agar, 1.75 g). Five ml aliquots of medium were added to five MPN tubes and inoculated with
129 0.5 ml of a soil suspension from 10⁻¹ to 10⁻⁵ soil dilutions, and incubated at 37° C. MPN
130 tubes in which a typical white pellicle developed a few mm below the surface of the medium
131 after incubation for 36 h were scored positive for *Azospirillum* sp.. Microscopic examination
132 of the cultures revealed the characteristic rods adhered to the flat droplets of oil.
133

134 **2.1.1.1.1 Nitrogen fixation by *Azospirillum* sp.**

135

136 Stock solutions of technical grade pesticides, prepared in acetone, were placed in sterilized
137 test tubes (25 × 200 mm) to provide a final concentration of 50µg ml⁻¹ malate medium. After
138 evaporation of carrier solvent, 20 ml portions of the steam-sterilized malate medium were
139 introduced into each test tube under aseptic conditions. The residues were equilibrated for
140 24 hrs to obtain aqueous solutions of the pesticides [47,48]. Medium, in test tubes without
141 the pesticide served as controls. Soil suspensions (1:10 soil to water ratio) from untreated
142 and pesticide-treated (5 kg ha⁻¹ level with commercial formulations) samples, incubated for 7
143 days, were prepared in sterilized distilled water. These suspensions (0.1 ml) were used to
144 inoculate 20 ml portions of malate medium with and without the pesticide. After 3 days (72 h)
145 incubation at 37°C, these test tubes for each treatment were digested with H₂SO₄ to estimate
146 in total nitrogen (N) by the Micro - Kjeldahl method as described earlier [49,50]. The amount
147 of N present in 0.1 ml soil suspensions, used for inoculation, together with that of the
148 medium was deducted from experimental values.
149

150 *Azospirillum* sp. were isolated from untreated and pesticide - treated (4 times at 10 day
151 intervals) soil samples to determine whether the increased nitrogen fixing capacity of
152 *Azospirillum* sp. isolated from soil samples treated with pesticides would continue further, the
153 isolates were subcultured in the semi - solid malate medium 3 times at an interval of 7 days,
154 and their rates of nitrogen fixation were compared with those of fresh cultures obtained
155 immediately after isolation from untreated and pesticide treated soil samples.
156

157 **3. Statistical analysis**

158

159 All data were expressed on an air dry soil basis and were averages of three replicates. Data
160 were analysed by significant difference ($P < 0.05$) between pesticide - treated and untreated
161 soils using Duncan multiple range (DMR) test [51,52]. If $A + B < AB$, the response can be
162 considered as synergistic interaction. If $A + B > AB$, the response can be considered as
163 antagonistic interaction; if $A + B = AB$, the response can be considered as additive
164 interaction (where, A = the percent stimulation in population of *Azospirillum* sp. caused by
165 pesticide X alone over the control; B = the percent stimulation in population *Azospirillum* sp.
166 caused by pesticide Y alone over the control; and AB = the percent stimulation in population
167 of *Azospirillum* sp. caused by the combination of X + Y over the control). The percent
168 stimulation values were calculated relative to population of *Azospirillum* sp.
169

170 **4. Results**

171

172 **4.1 Effect of pesticides on population of *Azospirillum* sp. in soils**

173

174 The initial size of the population of *Azospirillum* sp. was low in both soils (Table. 3 and 4).
175 The population of *Azospirillum* sp. was significantly higher in soils treated with oxydemeton

176 methyl, emamectin benzoate, dithane Z-78 and benomyl respectively, than in untreated
 177 control soils during the course of experiment. The population of *Azospirillum* sp. in soils
 178 increased when pesticides were applied at 2.5 - 5.0 kg ha⁻¹; by contrast, as the
 179 concentration of pesticides increased to 7.5 - 10.0 kg ha⁻¹, the population of *Azospirillum* sp.
 180 gradually decreased in both soils. Application of pesticides, singly and in repeated up to 5.0
 181 kg ha⁻¹, profoundly enhanced the population of *Azospirillum* sp. in vertisol soil (Table 3 and
 182 4). For the laterite soil, pesticide concentrations up to 2.5 kg ha⁻¹ increased the population of
 183 *Azospirillum* sp. after 7 and 14 days of incubation (Table 3 and 4). The increase in
 184 population of *Azospirillum* sp. in vertisol soil amended with oxydemeton methyl, emamectin
 185 benzoate, dithane Z -78 and benomyl (i.e. at 1.0, 2.5 and 5.0 kg ha⁻¹) was 100 - 300, 85 -
 186 238, 82 - 192 and 115 - 284 %, respectively, over the control treatment after incubation for 7
 187 days (Table 3). The population of *Azospirillum* sp. in vertisol soil with or without pesticides
 188 decreased gradually after 14 days (Table 3 and 4) compared to that after 7 days. The
 189 corresponding increases in population of *Azospirillum* sp. in laterite soil amended with four
 190 pesticides at 1.0 and 2.5 kg ha⁻¹ were 46 - 203, 64 - 239, 80 - 239 and 84 - 221 %, respectively,
 191 over the control treatment by the end of 7 day interval (Table 3 and 4). The
 192 population of *Azospirillum* sp. also decreased gradually under similar conditions after a 14
 193 day incubation in laterite soil (Table 4). The influence of oxydemeton methyl, emamectin
 194 benzoate, dithane Z-78 and benomyl alone, respectively, at different levels on the
 195 population of *Azospirillum* sp. in the two soils was assessed to examine interaction between
 196 pesticides. Interaction responses are generally distinguished on the basis of percent
 197 stimulation values (over control) regarding any parameter in soil treated with single pesticide
 198 or in repeated application at a specified dose in soil. In this study oxydemeton methyl,
 199 emamectin benzoate, dithane Z-78 and benomyl singly (i.e., at 1.0, 2.5, 5.0, 7.5 and 10.0 kg
 200 ha⁻¹) interacted synergistically, additively and antagonistically, respectively (Table 3,4 and 5).
 201 It is clear from these results that the occurrence of interactions between insecticides and
 202 fungicides was dose-dependent, and these interactions were prevailed in soil even after
 203 incubation for 14 days.

204
205
206
207 **Table 1. Physico-chemical properties of soils used in the present study**

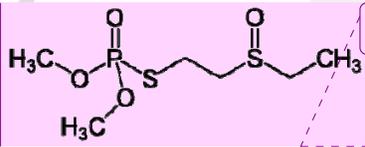
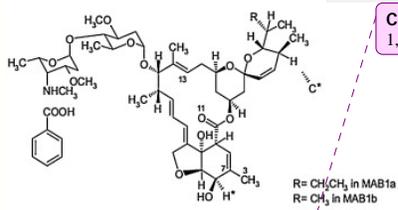
Properties	Black clay soil	Red sandy loam soil
Sand (%)	76.50	72.00
Silt (%)	18.00	25.00
Clay (%)	5.50	3.00
pH ^a	8.40	6.30
Water holding capacity (ml g ⁻¹ soil)	0.48	0.34
Electrical conductivity (m.mhos)	266.00	246.00
Organic matter ^b (%)	0.94	0.80
Total nitrogen ^c (%)	0.05	0.03
NH ₄ ⁺ - N(μ g ⁻¹ 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

209 ^a1:1.25 (soil:water)
 210 ^bWalkley-Black method (Jackson, 1971)
 211 ^cMicro-Kjeldhal method (Jackson, 1971)
 212 ^dNesslerization method (Jackson, 1971)
 213 ^eDiazotization method (Barnes and Folkard, 1951)
 214 ^fBrucine method (Ranney and Bartler, 1972)

215
 216
 217
 218
 219
 220

221 **Table 2. Particulars of the Pesticides used.**

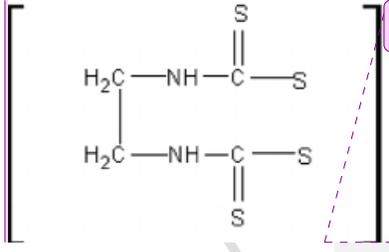
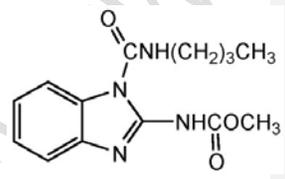
222

S.No	PESTICIDE	MOLECULAR FORMULA	STRUCTURE
1.	Oxydemeton Methyl	$C_6H_{15}O_4PS_2$	 <p>The structure shows a central phosphorus atom double-bonded to an oxygen and single-bonded to two methoxy groups (H₃C-O) and a sulfur atom. The sulfur atom is further bonded to a propyl chain, which is terminated by a methylsulfonamide group (-S(=O)-CH₃).</p>
2.	Emamectin Benzoate	$C_{51}H_{81}NO_{15}$	 <p>The structure is a complex macrocyclic lactone with multiple stereocenters, hydroxyl groups, and a benzoate ester group. It includes a methyl group and a methylsulfonamide group. The R group is defined as CH₂CH₃ in MAB1a and CH₃ in MAB1b.</p>

Comment [DJD1]: Chemical structure should be of same font size

Comment [DJD2]: Chemical structure of S.No 1,2 and 3 should be of same font size

223

3.	Dithane Z-78	$C_4H_6N_2S_4Zn$	
4.	Benomyl	$C_{14}H_{18}N_4O_3$	

Comment [DJ3]: Chemical structure should be of same font size

UNDER PEER REVIEW

225 | Table 3. Population (MPN × 10³ g⁻¹ soil) of *Azospirillum* sp. as influenced by the application of pesticides in black soil.
 226
 227

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)

228
 229 | *Initial 0-day population
 230 | **Concentration of the pesticide, *kKg ha*⁻¹
 231 | Figures, in parenthesis, indicate relative productive percentages.
 232 | Means, in each row, obtained for each sampling, followed by the same letter are not significantly different (*P* < 0.05) from each other
 233 | according to (Duncan's Multiple Range) DMR test.
 234 | Values in the table are means of triplicates.

235 | Table 4. Population (MPN × 10³ g⁻¹ soil) of *Azospirillum* sp. as influenced by the application of pesticides in red soil.

236
237

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)

238 *Initial 0-day population

239 **Concentration of the pesticide, Kg ha⁻¹

240 Figures, in parenthesis, indicate relative productive percentages.

241 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.

243 Values in the table are means of triplicates.

244 | **Table 5 : Influence of selected four pesticides on nitrogen fixation (mg N g⁻¹ malate) by**
 245 **Azospirillum sp.**
 246

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

247

248 | *The soil sample was treated with commercial formulation of the four pesticides (5 kKg ha⁻¹)
 249 and culture was isolated after 7 days.

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

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

254

255

256

257

258

259

260

261

262

263

264

265

266

267

268

269

270

271 **Table 6. Impact of subculturing of *Azospirillum* sp. isolated from pesticide-treated**
 272 **soil samples on nitrogen fixation (mg N g⁻¹ malate)**
 273

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

274
275

276 | *Soil samples were treated four times with pesticides at 5 **kKg** ha⁻¹ level.

277 | **Semi-solid malate medium was supplemented with technical sample of the pesticides
 278 | (50µg ml⁻¹ medium) before incubation with the culture.

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

281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298

299 **5. Discussion**

300

301 In the present study, four pesticides applied to soil, singly at concentrations ranging from 1.0
302 to 5.0 kg ha⁻¹, had no deleterious effect on *Azospirillum* sp. A similar individual instigate
303 effect of monocrotophos and chlorpyrifos was previously demonstrated on the population of
304 *Azospirillum* sp. [53]. Similarly, observations with other organophosphorus and pyrethroid
305 insecticides and fungicides have also been reported [44,41]. Interactions between different
306 agrochemicals applied in repeated application on microorganisms and their activities in soils
307 have received little attention in comparison to effects of a single agrochemical. There were
308 no differences in degree of diversity in bacterial populations from the application of a
309 combination of five pesticides, including chlorfenviphos and glyphosate, to field plot of 20
310 years[54]. In the present study the application of pesticides to the soils at certain
311 concentrations was not harmful to the population of *Azospirillum* sp. Some reports have
312 been published on interactions between pesticides and their solvents, pesticides and their
313 degradation products, and two different pesticides on growth of organisms in pure culture
314 studies of fungi, algae and cyanobacteria [55,56,57,58,59,60,61]. In all these studies, a
315 variety of interaction effects such as synergistic, additive and antagonistic were observed,
316 depending on concentration of the interacting chemicals. For instance, the combination of
317 permethrin and its degradation product interact to yield antagonistic, additive and synergistic
318 interactions towards the growth of fungi in pure culture [60], because the degradation rate of
319 an individual pesticide may be changed due to the combinations of pesticides, ultimately
320 leading to different types of interactions. In the present study, similar types of interactions
321 occurred by selected pesticides on population of *Azospirillum* sp. in two soils. A increase in
322 the population of *Azospirillum* sp. at high concentrations (100 ppm) of benomyl or 2-
323 aminobenzimidazole (a hydrolysis product of benomyl) were also reported in paddy soil
324 [36,38]. [39], noticed a provoking response in *Azospirillum* sp. population, when treated with
325 benomyl at lower concentration (5 ppm) in alluvial, laterite and saline soils, and carbofuran in
326 alluvial soil only.

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

352 *Azospirillum* sp. in these soils. *Azospirillum* sp. cultures obtained after 7 days of soil
353 incubation, from unamended soils exhibited appreciable nitrogen fixing activity (Table 5). A
354 significant stimulation of nitrogen fixation was evident in cultures from soils treated with the
355 four pesticides at a level of 5 kg ha⁻¹ when compared with cultures from untreated soils. The
356 extent of nitrogen fixation by the cultures observed in the present study are comparable with
357 those of *Azospirillum* cultures isolated from the same soils amended with monocrotophos
358 and quinolphos for 7 days [40], and those cultures isolated from a rice soil amended with
359 benomyl and incubated for 30 days[36]. The cultures from untreated soil, when inoculated
360 into the medium supplemented with four pesticides (Oxydemeton Methyl, Emamectin
361 Benzoate, Dithane Z-78 and benomyl) at 50 µg ml⁻¹, exhibited greater nitrogen-fixing activity.
362 However, the stimulation in nitrogen fixation was more pronounced in cultures of
363 *Azospirillum* sp. isolated from four pesticides treated (5 kg ha⁻¹) soil and inoculated to the
364 medium containing 50 µg ml⁻¹ of the pesticide (Table 5).
365 An attempt was made to determine whether the observed nitrogenase activity would
366 continue upon subsequent subcultures of the diazotroph. Although, fresh cultures from the
367 pesticide-treated soil exhibited greater nitrogen-fixation when compared with those from
368 untreated soils, subculturing of the isolates 3 times had no effect on nitrogen-fixation in the
369 cultures of *Azospirillum* sp., exposed to the selected pesticides.
370 The present study clearly shows that soil application of **four** pesticides (Oxydemeton Methyl,
371 Emamectin Benzoate, Dithane Z -78 and benomyl) increased the population of *Azospirillum*
372 sp., isolated from treated with four pesticides, last for longer periods.
373
374

375 6. CONCLUSION

376
377 The results of present investigation clearly indicate that the selected pesticides at levels
378 ranging from 2.5 to 5.0 Kg ha⁻¹ significantly increased the population of *Azospirillum* sp. and
379 nitrification in both the soils. Furthermore, increase in the concentration above 2.5 or 5.0 K g
380 ha⁻¹ exerted synergistic, additive or antagonistic interactions towards population of
381 *Azospirillum* sp. and nitrification in these soils.
382
383

384 COMPETING INTERESTS

385
386 Authors have declared that no competing interests exist.
387
388

389 REFERENCES

- 390
391
392 1. Senthil kumar, Pannrselvam A. Studies on the effect of herbicides on native strains
393 of *Azospirillum*, International journal of pharma world research. 2012;Vol.3 Issue 3.
394
- 395 2. Martinez-Toledo MY, Salmeron Y, Rodelas B, Pozo C, Gonzalez-Lopez J. Effects of
396 the fungicide Captan on some functional groups of soil microflora. Appl. Soil Ecol.
397 1998;7:245-255.
398
- 399 3. Smith MD, Hartnett DC, Rice CW. Effects of long -term applications on microbial
400 properties in tallgrass prairie soil. Soil Biol. Biochem. 2000;32:935 -946.
401
- 402 4. Chen SK, Edwards CA, Subler S. A microcosm approach for evaluating the effects
403 of the fungicides benomyl and captan on soil ecological processes and plant growth.

- 404 Appl. Soil Ecol. 2001a;18:69-82.
405
406 5. Cycon M, and Kaczynska A. Effects of selected pesticides on soil microbial activity
407 in nitrogen and carbon transformation . Pesticides.1-2 : 113-120. 2004.
408
409 6. Cycon M, Piotrowska-Seget A, Kaczynska A, Kozdroj J. Microbiological
410 characteristics of a loamy sand soil exposed to tebuconazole and λ -cyhalothrin
411 under laboratory conditions. Ecotoxicology. 2006;15(8):639-646.
412
413 7. Dubey V, Singh D. Effect of application of different pesticides to Leguminous crops
414 on soil microflora of Sidhi District (M.P.). International journal of Engineering
415 research and Development . 2012 Vol.3 Issue 12, 01-03.
416
417 8. Hajnis E, Pauke H, Negelj GD, Hanzen D. Agrohimiški v okrujaj usaej srede,
418 Moskva. 1979;357 s.
419
420 9. Ejhler V. Jadi v nasej pisae, Moskva. 1986 214 s.
421
422 10. Miligi L, Costantini AS, Veraldi A, Benvenuti A, Vineis P. Cancer and pesticides: an
423 overview and some results of the Italian multicenter case-control study on
424 hematologyphopoeitic malignancies. Ann N Y Acad Sci. 2006;1076:366-377.
425
426 11. Salmeron V, Martinez-Toledo MV, Gonzalez-Lopez J. Effects of alachlor and
427 metolachlor on the biological activity of *Azospirillum brasilense* grown in chemically
428 de@ned and dialysed-soil media. Environ. Toxicol. Chem. 1991;10,493±9.
429
430 12. Omar MNA, Berge O, Hassanein EE, Shalan SN. In vitro and in situ effects of
431 herbicide thiobencarb on rice±*Azospirillum* association. Symbiosis 1992; 13, 55 ±
432 63.
433
434 13. Bashan Y. Interactions of *Azospirillum* sp. in Soils: review. Biology and
435 Fertility of Soils. 1999;29(3)246-256.
436
437 14. Bashan Y, Vazquez P. Effect of calcium carbonate, sand, and organic matter levels
438 on mortality of five species of *Azospirillum* in natural and artificial bulk
439 soils. Biology and Fertility of Soils. 2000;30(5):450-459.
440
441 15. Dobbelaere S, Croonenborghs A, Thys A, Ptacek D, Vanderleyden J, Dutto P,
442 Labandera-Gonzalez C, Caballero-Mellado J, Aguirre JF, Kapulnik Y, Brener S,
443 Burdman S, Kadouri D, Sarig, S, Okon Y. Responses of agronomically important
444 crops to inoculation with *Azospirillum*. Australian Journal of Plant Physiology. 2001;
445 28:871-879.
446
447 16. FAO. The State of World Fisheries and Aquaculture. 2004;Rome.
448
449 17. Talawar S. Peanut in India:History, Production and Utilization. Peanut in local and
450 global food system. 2004;Series report No. 5. Department of Anthropology,
451 University of Georgia.
452

- 453 18. Dharme PK, Patil SK, Patil RB. Field evaluation of some insecticides against tobacco
454 caterpillar (*Spodoptera litura* Fabricus) on groundnut. Journal of Maharashtra
455 Agriculture University. 2001;26:164-165.
456
- 457 19. Anonymous. Agricultural production plan for Anantapuramu district for Kharif
458 season, Department of Agriculture, Anantapur, A.P, India. 2015.
459
- 460 20. Kori RN, Patil SL, Salakinakop SR, Hunshal CS, Nandagouda BT. Economics of
461 integrated weed management in irrigated groundnut (*Arachis*
462 *hypogaea* L.). J. Oil Seeds Res. 2002;17: 61 - 65.
463
- 464 21. Graebing P, Frank M, Chib JS. Effects of fertilizer and soil components on pesticide
465 photolysis. J. Agric. Food Chem. 2002;50:7332 -7339.
466
- 467 22. Balwinder Singh. Pesticide contamination of the environment in Punjab. Indian J.
468 Ecol. 2001;29(2):189-198.
469
- 470 23. Carriger JF, Rand GM, Gardinali PR, Perry WB, Tompkins MS, Fernandez AM, et al.
471 Pesticides of potential ecological concern in sediment from South Florida Canals: An
472 ecological risk prioritization for aquatic arthropods. Soil
473 Sed.Contam. 2006;15:21-45.
474
- 475 24. Pimentel D. Amounts of pesticides reaching target pests: Environmental impacts
476 and ethics. J. Agric. Environ. Ethics. 1995;8:17-29.
477
- 478 25. Pan-UK. Current pesticide spectrum, global use and major concerns. 2003 (online)
479 Available : http://www.pan-uk.org/brie.ng/sida_fil/chap1.htm
480
- 481 26. Chisholm RD, Koblitsky LK, Fahey JE, Westlake WE. DDT residues in soil. J. Econ.
482 Entomol. 1950;43:941-942.
483
- 484 27. Alexander M. Introduction to soil microbiology, 2nd edn. Wiley, Estern Ltd, New
485 Delhi ; 1961.
486
- 487 28. Singh BK, Walker A. Microbial Degradation of Organophosphorus Compounds.
488 FEMS Microbiology Review. 2006;Vol 30:No. 3.pp.428-471.
489
- 490 29. Margni M, Rossier D, Crettaz P, Jolliet O. Life cycle impact assessment of
491 pesticides on human health and ecosystems. Agric. Ecosyst. Environ. 2002;93:
492 379-392.
493
- 494 30. Vyas SC. Soil microorganisms and their activities. Nontarget Effects of Agricultural
495 Fungicides. CRC Press, FL, Boca Raton. 1988;258-275.
496
- 497 31. Edwards CA, Bater JE. An evaluation of laboratory and field studies for
498 assessment of the environmental effects of pesticides. Proceedings of the
499 Brighton Crop Protection Conference on Pests and Diseases. 1990;963-968.
500
- 501 32. Khan M, Sculion J. Effect of soil on microbial responses to metal
502 contamination. Environ. Pollut. 2000;110:115-125.
503
- 504 33. Doran JW, Parkin TB. Defining and assessing soil quality (*In: Defining Soil quality*
505 for a Sustainable Environment Soil Science Society of America, Eds:J.V. Doran,

- 506 D.C. Colema, D.F. Bezdicek, B.A. Stewart)-American Society of Agriculture,
507 Madison,1994;pp. 3–21.
508
- 509 34. Rath AK, Ramakrishnan B, Kumaraswamy S, Bharati K, Singla P, Sethunathan N,
510 et al. Effect of pesticides on microbial biomass of flooded soil,
511 Chemosphere.1998;37: 661–671.
512
- 513 35. Radwan TEE, Mahamed ZK, Reis VM. Efeito de inoculação de *Azospirillum* e
514 *Herbaspirillum* na produção de compostos indólicos em plantas de milho e arroz.
515 Pes. Agropec. Bras. 2004;39:987-994.
516
- 517 36. Charyulu PBBN, Rao VR. Nitrogen fixation by *Azospirillum* sp. isolated from
518 benomyl amended rice soil. Curr. Sci.1978;47(21):822-823.
519
- 520 37. Gadkari D. Influence of the herbicides Arelon, Goltix and Stomp on growth and
521 nitrogenase activity of *Azospirillum lipoferum*. Zentralbl. Mikrobiol.1987;142:587-594.
522
- 523 38. Charyulu PBBN, Ramakrishna C, Rao VR. Effect of 2-aminobenzimidazole on
524 nitrogen fixers from flooded soil and their nitrogenase activity. Bull Environ Contam
525 Toxicol.1980;25:482.
526
- 527 39. Nayak DN, Rao VR. Pesticides and heterotrophic N₂-fixation in paddy soils. Soil
528 Biology and Biochemistry.1980;12:1-4.
529
- 530 40. Rangaswamy V, Charyulu PBBN, Venkateswarlu K. Effect of monocrotophos and
531 quinalphos on soil population and nitrogen-fixing activity of *Azospirillum* sp. Biomed.
532 Environ. Sci.1989;2:305-314.
533
- 534 41. Rangaswamy V, Venkateswarlu K. The influence of cypermethrin and
535 fenvalerate on soil population of *Azospirillum* sp. Microbes. Agric. Indust.
536 Environ. 2000;225-230.
537
- 538 42. Alexander M. Introduction to Soil Microbiology. 2nd edn. Wiley. New York. pp.113-
539 330;1977.
540
- 541 43. Rangaswamy V, Venkateswarlu K. Microbial activities of S oxidation in
542 groundnut soils as affected by monocrotophos, quinalphos, cypermethrin and
543 fenvalerate. Poll. Res.1999;18(4):429-433.
544
- 545 44. Jaya Madhuri R, Rangaswamy V. Influence of selected fungicides on microbial
546 population in groundnut (*Arachis hypogaea* L.) soils. Poll. Res. 2003;22(2):205-212.
547
- 548 45. Alexander M. Most probable Number Method for microbial populations. In:
549 Methods of Soil Analysis'. (Ed. C.A. Black). 1965;Part 2,pp.1467-1472. Am. Soc.
550 Agr. Madison, Wisconsin, U.S.A.
551
- 552 46. Dobereiner J, Marriel IE, Neyra N. Ecological distribution of *Spirillum lipoferum*
553 Beijerinck. Can. J. Microbiol. 1976;22:1464-1473.
554
- 555 47. Megharaj M, Venkateswarlu K, Rao AS. Growth response of four species of soil
556 algae to monocrotophos and quinalphos. Environ. Pollut. 1986;A42:15-22.
557

- 558 48. Rangaswamy V, Venkateswarlu K. Ammonification and nitrification in soils and
559 nitrogen fixation by *Azospirillum* sp. as influenced by cypermethrin and
560 fenvalerate. Agric. Ecosys. Environ.1993;45:311-317.
561
- 562 49. Megharaj M, Venkateswarlu K, Rao AS. Microbial degradation and algal toxicity of
563 monocrotophos and quinalphos in flooded soil. Chemosphere. 1988;17:1033-1039.
564
- 565 50. Rangaswamy V, Venkateswarlu K. Ammonification and nitrification in soils and
566 nitrogen fixation by *Azospirillum* sp. as influenced by cypermethrin and
567 fenvalerate. Agric. Ecosys. Environ. 1993;45:311-317.
568
- 569 51. Jaya Madhuri R, Rangaswamy V. Biodegradation of selected insecticides by
570 *Bacillus* and *Pseudomonas* sps in groundnut fields. Toxicology International.2009;
571 16:127-132.
572
- 573 52. Megharaj M, Kookana K, Singleton S. Activities of fenamiphos on native algae
574 population and some enzyme activities in soil. Soil Biology and
575 Biochemistry.1999;39:1549-1553.
576
- 577 53. Rangaswamy V, Charyulu P, Venkateswarlu K. Effect of
578 monocrotophos and quinalphos on soil population and nitrogen-fixing activity of
579 *Azospirillum* sp. Biomed. Environ. Sci.1989;2:305-314.
580
- 581 54. Nicholson PS, Hirsch R. The effect of pesticides on the diversity of culturable soil
582 bacteria. Journal of Applied Microbiology.1998;84:551-558.
583
- 584 55. El-Jay A. Effects of organic solvents and solvent atrazine-interactions on two algae,
585 *Chlorella vulgaris* and *Selenastrum*. Archives of Environmental Contamination
586 and Toxicology.1996;31:84-90.
587
- 588 56. Megharaj M, Venkateswarlu K, Rao AS. Interaction effects of insecticide
589 combinations towards the growth of *Scenedesmus bijugatus* and *Synechococcus*
590 *elongatus*. Plant and Soil.1989;114:159-163.
591
- 592 57. Megharaj M, Rao AP, Rao AS, Venkateswarlu K. Interaction effects of carbaryl and
593 its hydrolysis product 1-naphthol towards three isolates of microalgae from rice soil.
594 Agriculture Ecosystem and Environment.1990;31:293-300.
595
- 596 58. Stratton GW. Effects of the herbicide atrazine and its degradation products, alone
597 and in combination on phototrophic microorganisms. Archives of Environmental
598 Contamination and Toxicology.1984;13:35-42.
599
- 600 59. Stratton GW, Corke CT. Toxicity of the insecticide permethrin and some
601 degradation products towards algae and cyanobacteria. Environmental
602 Pollution.1982a;29:71-80.
603
- 604 60. Stratton GW, Corke CT. Comparative fungitoxicity of the insecticide
605 permethrin and ten degradation products. Pesticide Science 13: 679-685.
606 *pyrenoidosa*. Environmental Contamination and Toxicology.1982b;40:736-742.
607
- 608 61. Stratton GW, Smith TM. Interaction of organic solvents with the green alga *Chlorella*
609 *pyrenoidosa*. Bull.Environ.Contam.Toxicol. 1988;40:736-742.
610

611
612
613
614
615
616
617
618
619
620

62. Auspurg B. Kombinationseffekte von Pflanzenschutz-mitteln im Boden.1985;pp.92-106.In: Pflanzenschutzmittel und Boden. Paul Parey Verlag, Hamburg
63. Lee PW, Fukuto JM, Hernandez H, Stearns SM. Fate of monocrotophos in the environment. *Journal of Agriculture Food and Chemistry*.1990;38:567- 573.
64. Chu XF, Hua P, Xuedong W, Xiao S, Bo Min F, Yunlong Y. Degradation of chlorpyrifos alone and in combination with chlorothalonil and their effects on soil microbial populations. *Journal of Environmental Sciences*. 2008;20:464- 469.

UNDER PEER REVIEW