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Influence of Azoxystrobin and Difenoconazole on N_2-Fixing and Antagonistic Organisms

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ABSTRACT

The non-target effects of Amistar™ (azoxystrobin) and Score™ (difenoconazole) on soil microbial populations were expressed in terms of changes in the microbial populations and effects on symbiotic associations. The effects of Azoxystrobin and Difenoconazole on the growth of the free-living N_2-fixing bacteria *Azospirillum chroococcum* and *Azotobacter brasilense* and the antagonistic microbes *Pseudomonas flurorescens* and *Trichoderma* spp. were assessed following disc diffusion and poisoned food methods. At or below the recommended doses, the fungicides did not inhibit all these microorganisms. At higher concentrations, the sensitivity varied according to the organism and fungicide. The extent of rhizobial root nodulation and arbuscular mycorrhizal (AM) root colonization as influenced by the foliar spray with different concentrations of Azoxystrobin and Difenoconazole was studied in *Arachis hypogea* Linn. Foliar spray of both the fungicides at recommended (2.2 µg (a.i.) ml^{-1}) or lower doses generally enhanced root nodulation. Higher concentrations, however, resulted in marked decreases in the number of root nodules. A similar trend was observed in terms of AM root colonization and incidence of arbuscules in plants treated with Azoxystrobin. On the other hand, Difenoconazole spray significantly decreased both these parameters with an increasing concentration, from the lowest concentration (0.44 µg. (a.i.) ml^{-1}). The vesicular structures were however not significantly altered by different fungicide concentrations.

Keywords: Amistar, arbuscular mycorrhizal colonization, arbuscules, non-target effects, root nodulation, score
Abbreviations: AM, arbuscular mycorrhizal; **DMRT**, Duncan's multiple range test

INTRODUCTION

The beneficial and deleterious effects of fungicides, their toxicity levels in soils and their action on other macro- and microfauna should be assessed before their field application (Mallikarjunaiah 1995). Selection and examination of specific rhizobial strains in response to any newly introduced fungicide is essential for its recommendation in crop protection (Guene *et al*. 2003). Nodulation and N_2 fixation may be affected in four different ways: i) the survival of rhizobia, ii) the infection process, iii) nodule formation and development, and iv) N_2 fixation in the nodule (Diatloff 1986). Field nodulation and N_2 fixation in several legumes was induced by fungicides (Mallikarjunaiah 1995). In a field experiment, when *Rhizobium* was added to common bean (*Pha-seolus vulgaris*) treated with fungicide, a decrease in nodulation was noticed while nitrogen fixation was not significantly different to that of the control (Guene *et al*. 2003).

It is critical to study the effects of new fungicides on two important bacteria, *Azospirillum* and *Azotobacter*, which largely contribute to the process of N_2 fixation. The former enhances total N_2 fixation in cereals and Gramineae (Crossman and Hill 1987). *Azospirillum* sp. isolated from cypermethrin- and fenvalerate-treated soils at recommended doses, exhibited greater N_2 fixing activity that lasted for at least three generations (Rangaswamy and Venaketeswarlu 1992). In contrast, the fungicide captan when sprayed on soil had a negative effect on growth and nitrogenase activity of *Azospirillum brasilense* (Diciocco and Caceres *et al*. 1997). Brassicol, benlate, thiophamine and vitavax when soil-sprayed, were found to stimulate the growth of nitrogen fixation of *Azotobacter* while blitox-50, captan, duter, fytolan, dithande, thiram, ziram and thimet were toxic when soil-sprayed at recommended doses (Mallikarjuniah 1995).

Trichoderma viride, *T. harzianum* and *Pseudomonas fluorescens* are natural bio-pesticides commonly used to control plant diseases. Fungicides by their broad usage may have an influence on these antagonists present in soil. According to Transmo (1989), *T. viride* and *T. harzianum* tolerated the recommended doses of agrochemicals and seldom showed any reduced growth. Chilli (*Capsicum annum*) seeds when treated with antagonistic isolates of *T. viride*, *T. harzianum* and *P. fluorescens* and the fungicides captan and tetramethyl thiurum disulphide, recorded improved percent germination over the control (Ramanathan and Sivaprakasam 1994).

Increased P uptake is often the primary cause of growth and yield enhancement in AM (arbuscular mycorrhizal) plants (Smith and Gianiazzi-Pearson 1988). Systemic fungicides have been reported to have no effect (Nemec 1980; Ocampo and Hayman 1980), to reduce (Jalali and Domsch 1975; Fitter 1986; Knough *et al*. 1987) or stimulate the development of AM (Nemec 1980; Jabaji-Hare and Kendrick 1987). The benefits provided by AM fungi for the growth of the host plant raises concern for their survival and preservation in native soils and hence it becomes necessary to study the responses of AM fungi to fungicide application.

Amistar™ (Azoxystrobin 25% emulsifiable concentrate) and Score™ (Difenoconazole 25% emulsifiable concentrate) are two broad-spectrum, foliar, systemic fungicides yet to be released to farmers and planters in India by Syngenta India Ltd., Mumbai. The objectives of the present study were to investigate their non-target effects in terms of: (i) Inhibitory effect on *Azospirillum chroococcum*, *Azotobacter brasilense*, *Pseudomonas fluorescens* and *Trichoderma* spp. (ii) Influence on root nodulation, arbuscular mycorrhizal vesicles, arbuscules and AM colonization in *Arachis hypogea* Linn. The manufacturer's recommended dose

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for both Azoxystrobin and Difenoconazole, for foliar spray is 2.2 µg (a.i.) ml^{-1}.

MATERIALS AND METHODS

Effect of the fungicides on certain beneficial soil microorganisms

The effects of the fungicides, Azoxystrobin and Difenoconazole on *Azotobacter brasilense*, *Azospirillum chroococcum* and *Pseudomonas fluorescens* were assessed by the disc diffusion method. The effects on *Trichoderma viride* and *Trichoderma harzianum* were studied by a poisoned food technique.

Preparation of culture media

Laboratory chemicals were purchased from Himedia for the preparation of media. Culture media such as nitrogen-free bromothymol blue (NFB) medium (Subbarao 1986) for *A. chroococcum*, Ashby's mannitol agar medium (Subbarao 1986) for *A. brasilense*, King's B medium for *Pseudomonas fluorescens* (protease peptone, 20 g; glycerol, 10 ml; K_2HPO_4, 2.5 g; $MgSO_4$, 6 g; agar, 20 g; distilled water, 1 L, pH 7.2) and selective medium for *Trichoderma* (yeast, 5 g; molasses, 30 g; agar agar, 15 g; distilled water, 1 L, pH 7) were sterilized by autoclaving at 120°C for 20 min.

Disc diffusion method

About 20 mL of agar medium was dispensed into a sterilized Petri dish to yield a uniform depth of about 4 mm and allowed to cool and solidify at room temperature under sterile conditions. Discs of 6 mm diameter were prepared from Whatman No. 1 filter paper (Cat No. 1001 125). They were sterilized by autoclaving, dried at 80°C for 1 h and soaked in the respective fungicide concentrations. After drying for 3-5 min, the discs impregnated with fungicides were placed on the surface of the medium inoculated with the bacterial culture (by a spread plate technique) with the help of flame sterilized forceps and gently pressed down to ensure contact with the agar surface. The discs were spaced far enough from the edges of the Petri dishes to avoid overlapping rings of inhibition. Finally, the Petri dishes were inverted and incubated for about 18 h at 37°C, depending on the optimal growth and the inhibitory zones, if present, were measured. Clear zones of growth inhibition indicated the activity of Azoxystrobin and Difenoconazole against these beneficial bacteria.

Poisoned food technique

The fungicides were added separately into the selective medium for *Trichoderma* to obtain the predetermined concentrations. About 20 ml of agar medium was dispensed into each sterilized Petri dish to yield a uniform depth of about 4 mm and allowed to cool and solidify at room temperature. Mycelial discs (5 mm in diameter) of *T. viride* or *T. harzianum* were placed on the surface of the medium in the centre of the plates and incubated at room temperature (25 ± 1ºC) for three days. After the incubation period, the colony diameter was measured.

Effect of fungicide spray on *Rhizobium* nodulation

Seedlings of groundnut (*Arachis hypogea* Linn.) were raised in a mix of autoclaved soil and sand (1:1) under greenhouse conditions. Twenty days after sowing, the seedlings were sprayed with different concentrations of the fungicides (Azoxystrobin and Difenoconazole) and the treated seedlings were immediately transplanted to garden soil containing natural populations of *Rhizobia*. On the 30^{th} and 40^{th} day after transplantation, the number of nodules per plant was counted. Randomly selected nodules were surface sterilized, crushed and streaked on mannitol agar to confirm the rhizobial nature (Subbarao 1986).

Effect of fungicide spray on Arbuscular mycorrhizal (AM) root colonization

Sandy loam soil collected from the experimental fields of Bharathiar University, Coimbatore, was mixed with sand at a proportion of 1:1 and used as a substrate for plant growth. The substrate was sterilized in cloth bags under wet conditions in an autoclave at 1.5 kg $sq.cm^{-1}$ pressure (121°C) for 1 h on three consecutive days. This substrate was filled in to 130 polythene bags (25 cm x 30 cm) at 5 kg/bag. All pots were sown with uniform sized seeds of groundnut, 5 seeds per pot and watered regularly. After 20 days, the seedlings from each bag were sprayed with specific fungicide concentrations and three plants from each bag were immediately transferred to pots containing composite soil with an AM spore density of 79 ± 10 g^{-1} soil.

Staining of root samples and determining of AM colonization

The percent colonization of AM fungi in the feeder root samples was assessed on 3, 6, 9, 12, 15 and 30 days after transplantation. The procedure described by Hayman (1982) was adopted for clearing and staining root segments for a rapid assay of mycorrhizal colonization. The root segments were cut into 1 cm pieces and washed thoroughly. They were softened by boiling in 10% KOH at 90°C for about 30 min. Then, the root bits were washed in 3-4 changes of distilled water and bleached in 30% H_2O_2 for 5-10 min. They were again washed in distilled water, acidified with 5N HCl for 10 min and stained in 0.05% trypan blue (in lactophenol) for 3-4 h. Stained root bits were mounted on glass slides in lactophenol for microscopic examination.

A minimum of 25 root bits, each measuring about 1 cm long was randomly selected from a stained sample of each replicate and examined under a compound microscope. The number of vesicles, arbuscules and total colonization count was assessed for every sample. Percentage root length colonization was calculated using the following formula:

$$\text{Per cent root length} = \frac{\text{No. of observations showing AM infection}}{\text{Total number of observations}} \times 100$$

Statistical analysis

All measurements were repeated by conducting a separate set of measurements on a separately executed experiment. Since there was no statistical significance between the data of first and second experiments, the results of the first experiment alone is presented in this paper.

RESULTS

The effects of Azoxystrobin and Difenoconazole on certain groups of soil microorganisms were assessed in terms of changes in the general populations of free-living nitrogen fixers (*Azospirillum* and *Azotobacter*) and antagonistic microorganisms (*Pseudomonas* and *Trichoderma*). The impact of foliar spray on the beneficial symbiotic associations of plants viz., rhizobial root nodulation and AM root colonization was also assessed.

Effect on beneficial microbes

The effect of various concentrations of Azoxystrobin and Difenoconazole on the growth of *Azospirillum chroococcum*, *Azotobacter brasilense* and *Pseudomonas fluorescens* was assessed using the disc diffusion method (**Table 1**). Their effect on *Trichoderma viride* and *Trichoderma harzianum* were determined using the poisoned food technique (**Table 2**).

The growth of *A. chroococcum* was not inhibited by Azoxystrobin at all concentrations studied. Difenoconazole, however, inhibited the growth at higher concentrations as evident from an increased width of inhibition zones. Another free-living, N_2-fixing bacterium *A. brasilense*, however was more sensitive to Azoxystrobin than Difenoconazole. The antagonistic bacterium *P. fluorescens* was inhibited by both the fungicides at higher concentrations, but was relatively more sensitive to Azoxystrobin.

Table 1 Inhibitory effect of different concentrations of Amistar™ (Azoxystrobin) and Score™ (Difenoconazole) on *Azospirillum chroococcum*, *Azotobacter brasilense* and *Pseudomonas fluorescens* determined on solid media by disc diffusion assay.

Concentration (µg.a.i.ml^{-1})	Width of inhibition (mm)					
	Amistar™			Score™		
	Azospirillum chroococcum	*Azotobacter brasilense*	*Pseudomonas fluorescens*	*Azospirillum chroococcum*	*Azotobacter brasilense*	*Pseudomonas fluorescens*
Control	0 a	0 f	0 e	0 f	0 c	0 c
2.20	0 a	0 f	0 e	0 f	0 c	0 c
4.40	0 a	1 e	0 e	0 f	0 c	0 c
22.00	0 a	1 e	0 e	0 f	0 c	0 c
44.00	0 a	1 e	0 e	0 f	0 c	0 c
220	0 a	4 d	0 e	4 e	0 c	0 c
440	0 a	9 c	7 d	5 d	0 c	0 c
2200	0 a	14 b	9 c	10 c	0 c	1 b
4400	0 a	15 a	14 b	11 b	3 b	2 a
22000	0 a	15 a	15 a	14 a	4 a	2 a

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

Comparison	S.E.D.	LSD (5%)	LSD (1%)
Concentration vs species vs fungicide means	0.455	0.902	1.192

Table 2 Inhibitory effect of different concentrations of Amistar™ and Score™ on *Trichoderma* spp. determined on solid media by poisoned food technique.

Concentration (µg.a.i.ml^{-1})	% inhibition of radial growth			
	Amistar™		Score™	
	T. viride	*T. harzianum*	*T. viride*	*T. harzianum*
2.20	0 f	0 g	0 f	0 d
4.40	0 f	0 g	1.42 e	0 d
22.00	0 f	1.42 f	2.85 d	0 d
44.00	7.14 e	7.14 e	2.85 d	0 d
220	12.85 d	14.28 d	11.42 c	0 d
440	22.85 c	15.71 c	14.28 b	1.42 c
2200	22.85 c	15.781 b	14.28 b	1.42 c
4400	28.57 b	22.85 ab	15.71 a	8.57 b
22000	30.00 a	27.14 a	15.71 a	10.00 a

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

Comparison	S.E.D.	LSD (5%)	LSD (1%)
Concentration vs species vs fungicide means	0.593	1.182	1.569

Table 3 Root nodulation (number of nodules/plant) in *Arachis hypogea* Linn. as influenced by foliar spray* with different concentrations of Amistar™ and Score™.

Concentration (µg.(a.i.)ml^{-1})	Days after transplantation (DAT)			
	Amistar™		Score™	
	30 DAT	40 DAT	30 DAT	40 DAT
Control	95 a-d	102 cde	95 a	102 bc
0.44	106 a	141 a	95 a	122 a
1.10	106 a	130 ab	95 a	112 ab
1.46	102 ab	129 ab	99 a	110 ab
2.20	99 abc	120 bc	98 a	110 ab
4.40	99 abc	108 cd	96 a	109 ab
22.00	91 a-d	98 def	80 ab	89 cd
44.00	89 a-d	95 def	75 b	88 cd
220	87 a-d	94 def	68 b	90 cd
440	86 a-d	84 efg	50 c	83 cd
2200	82 bcd	80 fgh	49 c	77 d
4400	80 cd	71 gh	45 c	56 e
22000	77 d	65 h	44 c	52 e

* The seedlings were given a single foliar spray on 20th day after emergence.

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

Comparison	S.E.D.	LSD (5%)	LSD (1%)
Concentration vs DAT vs fungicide means	8.783	17.419	23.049

The growth of antagonistic fungi *T. viride* and *T. harzianum* were not inhibited at the recommended dose (2.2 µg (a.i.) ml^{-1}) of Azoxystrobin and Difenoconazole. Though *T. harzianum* exhibited a higher level of tolerance to fungicides, both *Trichoderma* species were sensitive to higher concentrations.

Effect on symbiotic associations

Table 3 shows the influence of foliar spray with different concentrations of Azoxystrobin and Difenoconazole on the extent of Rhizobial root nodulation in *Arachis hypogea*. Generally, the foliar sprays at the recommended or lower doses, increased root nodulation, though not always significantly. The higher concentrations, however, resulted in marked decreases in the number of nodules per plant.

The extent of AM root colonization in *A. hypogea*, administered with different concentrations of Azoxystrobin and Difenoconazole foliar spray is presented in **Table 4**. Azoxystrobin spray at the recommended dose (2.2 µg (a.i.) ml^{-1}) significantly increased the percentage root colonization. At concentrations higher than the recommended dose, the extent of root colonization decreased progressively. On the other hand, Difenoconazole spray significantly decreased AM root colonization with increasing fungicide concentration starting from the lowest concentration (0.44 µg

Table 4 Arbuscular mycorrhizal colonization (%) in *Arachis hypogea* as influenced by foliar sprays* with different concentrations of Amistar™ and Score™.

Concentration	Amistar™ - Days after transplantation (DAT)					Score™ - Days after transplantation (DAT)				
(µg.a.i.ml^{-1})	3 DAT	6 DAT	9 DAT	12 DAT	15 DAT	3 DAT	6 DAT	9 DAT	12 DAT	15 DAT
Control	14.33 c	20.33 d	24.66 f	28.33 e	31.66 f	14.33 a	20.33 a	24.66 a	28.33 a	31.66 a
0.44	14.33 c	20.67 d	26.00 e	31.33 c	33.00 e	13.66 a	19.00 b	23.00 b	24.66 b	29.66 b
1.10	14.66 c	23.33 c	28.00 d	34.33 b	36.33 c	12.66 b	17.66 c	20.66 c	23.66 c	28.00 c
1.46	15.66 b	24.00 b	34.33 a	35.00 b	38.00 b	12.33 b	14.00 d	20.00 d	21.33 d	26.33 d
2.20	19.33 a	25.66 a	31.33 d	35.66 a	39.66 a	10.66 c	13.00 e	18.00 e	21.00 e	25.66 e
4.40	11.66 d	18.66 e	29.33 c	34.00 b	35.33 d	9.00 d	11.66 f	17.00 f	20.66 e	25.00 f
22.00	9.33 e	18.00 f	24.66 f	29.66 d	29.00 g	7.66 e	9.00 g	13.33 g	19.66 f	22.00 g
44.00	8.33 f	17.00 g	21.00 g	23.33 f	25.66 h	5.33 f	9.00 g	13.00 g	19.00 g	22.00 g
220	8.00 f	15.66 h	19.66 g	23.00 f	25.00 i	1.66 g	6.33 h	13.00 g	18.66 g	19.00 h
440	6.33 g	11.00 i	19.00 h	21.66 g	23.66 jk	1.33 gh	4.33 i	9.33 h	16.66 h	18.66 h
2200	6.00 g	9.00 j	18.66 h	19.66 h	23.33 k	1.00 h	4.00 i	7.66 i	16.00 i	18.00 h
4400	0 h	7.66 k	17.66 i	19.00 i	23.00 j	0 i	0 j	4.33 j	11.00 j	17.00 i
22000	0 h	0 l	14.66 j	10.33 j	21.00 l	0 i	0 j	1.00 k	9.00 k	15.00 j

*- The seedlings were given a single foliar spray on 20th day after emergence.
In a column, means followed by a common letter are not significantly different at the 5% level by DMRT

Comparison	S.E.D.	LSD (5%)	LSD (1%)
Concentration vs DAT vs Fungicide means	0.259	0.510	0.672

Table 5 Number of arbuscules in the feeder roots of *Arachis hypogea* as influenced by foliar sprays* with different concentrations of Amistar™ and Score™.

Concentration	Amistar™ - Days after transplantation (DAT)					Score™ - Days after transplantation (DAT)				
(µg.a.i.ml^{-1})	3 DAT	6 DAT	9 DAT	12 DAT	15 DAT	3 DAT	6 DAT	9 DAT	12 DAT	15 DAT
Control	1.37 cd	2.17 cd	2.27 c	2.72 c	3.12 d	1.37 a	2.17 a	2.27 a	2.72 a	3.12 a
0.44	1.40 bcd	2.23 bc	2.37 b	2.80 b	3.19 cd	1.29 ab	2.08 a	2.16 ab	2.67 a	2.97 b
1.10	1.45 abc	2.25 bc	2.43 b	2.82 b	3.23 bc	1.23 bc	2.07 a	2.12 bc	2.63 ab	2.74 c
1.46	1.47 ab	2.30 ab	2.45 b	2.88 b	3.28 b	1.20 cd	1.95 b	2.05 cd	2.57 b	2.61 d
2.20	1.51 a	2.36 a	2.60 a	3.12 a	3.47 a	1.17 cd	1.89 bc	1.98 de	2.44 c	2.56 d
4.40	1.34 d	2.12 d	2.19 d	2.66 c	3.03 e	1.12 de	1.85 cd	1.94 e	2.32 d	2.43 e
22.00	1.23 e	2.02 e	2.16 d	2.53 d	2.95 f	1.06 e	1.80 d	1.80 f	2.19 e	2.32 f
44.00	1.20 e	1.94 f	2.13 d	2.46 d	2.89 fg	0.97 f	1.71 e	1.77 f	2.15 ef	2.25 f
220	1.16 e	1.84 g	2.01 e	2.34 e	2.83 g	0.94 f	1.64 e	1.72 fg	2.08 f	2.16 g
440	0.93 f	1.52 h	1.95 e	2.31 e	2.47 h	0.70 g	1.51 f	1.65 g	1.77 g	1.99 h
2200	0.91 fg	1.42 i	1.73 f	2.21 f	2.46 h	0.63 g	1.19 g	1.41 h	1.62 h	1.77 i
4400	0.84 gh	1.31 j	1.68 fg	2.14 f	2.31 i	0.49 h	0.92 h	0.96 i	1.23 i	1.63 j
22000	0.82 h	1.25 j	1.64 g	1.92 g	1.98 j	0.37 i	0.84 i	0.88 j	1.09 j	1.49 k

* The seedlings were given a single foliar spray on 20th day after emergence.
In a column, means followed by a common letter are not significantly different at the 5% level by DMRT

Comparison	S.E.D.	LSD (5%)	LSD (1%)
Concentration vs DAT vs fungicide means	0.040	0.080	0.105

Table 6 Incidence of arbuscular mycorrhizal vesicles in the feeder roots of *Arachis hypogea* as influenced by foliar sprays* with different concentrations of Amistar™ and Score™.

Concentratio	Amistar™ -Days after transplantation (DAT)					Score™ -Days after transplantation (DAT)				
n (µg.a.i.ml^{-1})	3 DAT	6 DAT	9 DAT	12 DAT	15 DAT	3 DAT	6 DAT	9 DAT	12 DAT	15 DAT
Control	1.53 a	2.51 a	2.68 a	3.11 a	3.44 a	1.53 a	2.51 a	2.68 a	3.11 a	3.44 a
0.44	1.51 a	2.42 a	2.62 a	3.06 a	3.39 a	1.42 a	2.58 a	2.41 b	3.05 a	3.29 a
1.10	1.49 a	2.31 b	2.54 a	3.01 a	3.30 a	1.36 a	2.55 a	2.33 b	2.95 a	3.23 b
1.46	1.48 a	2.30 b	2.47 b	2.89 b	3.21 b	1.36 a	2.49 a	2.28 b	2.84 b	3.15 b
2.20	1.47 a	2.12 b	2.43 b	2.84 b	3.15 b	1.30 b	2.43 a	2.24 c	2.71 b	2.88 d
4.40	1.35 a	2.02 c	2.31 b	2.75 b	3.01 c	1.25 b	2.39 a	2.13 c	2.59 c	2.84 d
22.00	1.25 b	1.83 d	2.25 c	2.63 c	2.99 c	1.20 b	2.35 b	2.06 c	2.41 d	2.60 e
44.00	1.16 b	1.53 e	1.99 d	2.44 d	2.72 d	1.16 b	2.28 b	1.72 e	2.33 d	2.55 e
220	1.02 c	1.42 f	1.94 d	2.25 e	2.68 e	1.08 c	2.22 c	1.24 g	2.25 e	2.43 f
440	0.96 c	1.23 g	1.92 d	2.20 e	2.63 e	0.99 c	1.64 e	1.10 h	2.13 e	2.33 f
2200	0.91 d	1.15 g	1.52 f	2.13 e	2.61 e	0.74 d	1.18 h	0.96 i	2.06 f	2.18 g
4400	0.84 d	1.06 h	1.45 f	1.95 f	2.49 f	0.57 e	1.12 h	0.88 i	1.96 f	1.98 h
22000	0.70 e	0.97 h	1.25 g	1.89 f	2.39 f	0.21 g	0.95 i	0.74 j	1.34 i	1.84 i

*- The seedlings were given a single foliar spray on 20th day after emergence.
In a column, means followed by a common letter are not significantly different at the **1**% level by DMRT

Comparison	S.E.D.	LSD (5%)	LSD (1%)
Concentration vs DAT vs fungicide means	1.605	0.158	0. 185

(a.i.) ml^{-1}).

The number of arbuscules and vesicles in the feeder roots of *A. hypogea* sprayed with different concentrations of Azoxystrobin and Difenoconazole are given in **Tables 5** and **6**, respectively.

The number of arbuscules (**Fig. 1**) increased significantly with increasing concentrations of Azoxystrobin foliar spray up to the recommended dose; a significant decrease with increasing concentration was noted. However, foliar spray of Difenoconazole caused a progressive decrease in the number of arbuscules with increasing concentration of the fungicides starting from the lowest concentration (0.44 µg (a.i.) ml^{-1}). On the other hand, the incidence of vesicles (**Fig. 2**) in the feeder roots of *A. hypogea* decreased with an increase in concentration of both fungicides, although not significantly.

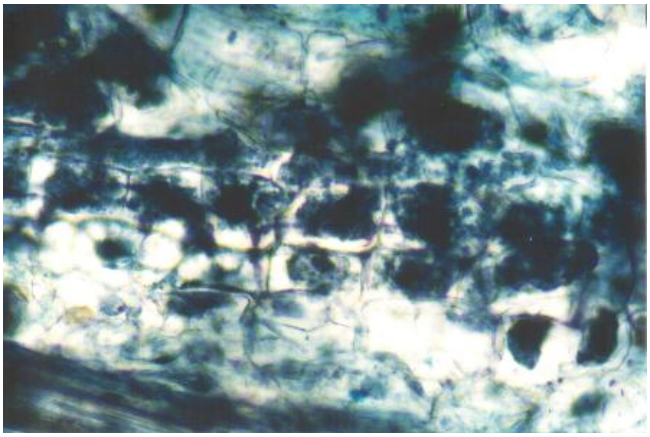

Fig. 1 Abundant arbuscules in roots of *Arachis hypogea* sprayed with Amistar™ at the recommended dose.

Fig. 2 Scarce vesicles in roots of *Arachis hypogea* sprayed with Amistar™ at the recommended dose.

DISCUSSION

Certain free-living soil microorganisms beneficial to growth and yield of crop plants viz., *Azospirillum chroococcum*, *Azotobacter brasilense*, *Pseudomonas fluorescens*, *Trichoderma viride* and *T. harzianum* were selected as models to assess the non-target effects of Azoxystrobin and Difenoconazole. At or below the recommended dose for foliar spray, all the test organisms were not inhibited by the fungicides (**Tables 1** and **2**). Similarly, a number of workers have reported that commonly used organic fungicides (copper oxychloride, dithiocarbamates, thiram, zineb and maneb) were not harmful to *Azotobacter* species in field doses in the soil (Mallikarjunaiah 1995). *Azospirillum halopraeferans* cultures also improved nitrogen fixation in the presence of organic pesticides (dithiocarbamates, copper oxy-chloride) in the soil (Jena *et al*. 1987). Suyama *et al*. (1993) reported that the predominance of *Trichoderma* species, an active decomposer of cellulose and antagonist, was not affected by the soil-application of the fungicide Chlorothalonil.

As systemic fungicides applied as foliar spray are translocated within plants, they produce a fungitoxic level of chemicals in the roots (Schwinn 1987), and it is of interest to assess whether they have any adverse effect on the development of certain beneficial symbiotic associations in the roots viz., *Rhizobium* nodulation and AM colonization. In this context, it is interesting to note that the foliar spray of Azoxystrobin or Difenoconazole to *A. hypogea* enhanced the nodulation activity in the roots at field doses (**Table 3**). The increased nodulation in the plants sprayed with Azoxystrobin and Difenoconazole may be a direct consequence of modifications in the microbial community in the root environment because of changes in the amount and the composition of root exudates.

Since AM formation and functioning are sensitive to soil fertility (Hayman 1982), physical and chemical characteristics (Menge 1982) and biotic factors (Abott and Robson 1978; Barea and Azcon-Aguilar 1985), the effect of Azoxystrobin and Difenoconazole application on naturally occurring AM fungi is of interest. In the present study, AM root colonization was significantly enhanced by the application of increasing concentrations of Azoxystrobin up to the recommended field dose. The formation of arbuscules, the functional unit of the AM symbiosis also increased. A contradictory observation was made when a foliar spray with Difenoconazole was applied resulting in significant decreases in the percentage root colonization and arbuscular incidence as the concentration increased (**Tables 4, 5**). On the one hand, it is to be expected that systemic fungicides will have detrimental effects on endomycorrhizal fungi. The fungicide benomyl was reported to inhibit spore germination and hyphal length of the arbuscular mycorrhizal fungus *Glomus mosseae* when applied at 21.25 µg/ml, 10.62 µg/ml and 10 µg/ml (Chiocchio *et al*. 2000). The mechanisms for stimulation of arbusucular mycorrhizal fungi by Azoxystrobin are unknown. Root exudation is thought to be one of many factors, which govern mycorrhizal development. The difference in the mycorrhizal root colonization in the systemic fungicide treated plants was attributed to root exudation (Jabaji-Hare and Kendrick 1987).

This study revealed that mycorrhizal colonization, rhizobial nodulation and the rhizosphere populations of certain beneficial microbes essential for the normal growth and yield of crop plants were not adversely affected by the fungicide treatment at the recommended dose. Since both fungicides are foliar spray fungicides, only a fraction of the spray is expected to reach the soil and their effect at this diluted concentration on the soil microbial populations will be highly insignificant and transient.

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