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Degradation of the Fungicides, Azoxystrobin and Difenoconazole in Soil and their Influence on Soil Microbial Activity

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ABSTRACT

The non-target effects of Amistar™ (azoxystrobin) and Score™ (difenoconazole) were studied in terms of changes in the soil microbial populations, soil respiration and activity of soil enzymes. Both the fungicides significantly reduced the fungal population in the soils even at the lowest concentration used (0.44 µg. (a.i.) g^{-1} soil) and totally eliminated it at the recommended dose for field application (2.2 µg. (a.i.) g^{-1} soil). On the other hand, the populations of bacteria and actinomycetes in the treated soil increased with increasing concentrations of the fungicides. Soil respiration decreased significantly with increasing concentrations of Azoxystrobin and Difenoconazole. However, there was an increase in the rate of soil respiration when up to 0.44 µg. (a.i.) g^{-1} soil of Difenoconazole was applied. The activities of soil enzymes cellulase, xylanase and protease increased with increasing concentrations of the fungicides Azoxystrobin and Difenoconazole. On the other hand, the activities of urease and acid phosphatase decreased with the application of these fungicides. Both the rate of soil respiration and the activities of the tested soil enzymes reached the same level as controls after prolonged incubation. Degradation of Azoxystrobin and Difenoconazole in two different soils was estimated using HPLC. Azoxystrobin was degraded by 95% in a period of 96 hours in the slightly alkaline Coimbatore soil, whereas in the acidic Valparai soil, the degradation was only 70% during the same period. Difenoconazole, however, was quickly degraded in both Coimbatore (95%) and Valparai (97%) soils after 96 hours.

Keywords: Amistar, degradation of fungicides, microbial population, score, soil enzymes, soil respiration
Abbreviations: DMRT, Duncan's multiple range test

INTRODUCTION

An ideal fungicide is one that selectively kills or inhibits pathogenic fungi in soil when used at concentrations non-toxic to all other soil microorganisms. The remaining micro-organisms could then serve as a biological buffer against the reestablishment of plant pathogens and also contribute activities essential for soil fertility.

Extensive use of fungicides can lead to marked changes in the microbial equilibrium of agricultural soils, both in terms of numbers of microorganisms and their activity. The changes may bring either beneficial or detrimental effects on plant growth, either by ameliorating or limiting the various microbial populations and their activities (Ingham and Coleman 1984; Akinyanju and Fadayomi 1989; El-Abyad and Gharrel 1991).

Efforts to quantify soil quality on the basis of soil biology are not new (Dick 1994; Turco *et al* 1994; Staben *et al.* 1997). Numerous reports have been published on the maintenance or improvement of soil quality on the basis of soil physical and chemical characteristics (Papendick and Parr 1992; Karlen and Stott 1994). In the past decade also microbiological, chemical and physical soil properties have all been used to obtain a more complete and precise definition of soil fertility (Schinner *et al.* 1991). Soil biological parameters are potential and sensitive indicators of soil ecological stresses or restoration (Dick 1994; Gregorich *et al.* 1994). Continuous inputs of pesticides might affect the soil microflora and so impair soil fertility (Schuster and Schroder 1990). Agricultural management practices may influence the size and composition of microflora in soils (Arden-Clarke and Hodges 1988). The combined metabolism of mixed populations of microorganisms in complex substrates such as soil can be quantitatively determined by measurements of microbial respiration, which is characterized by the production of CO_2 or the uptake of O_2 as a result of metabolism (Parkin *et al*. 1996).

Enzyme activities are useful for assessing the influence of fertilizers and agrochemicals on the metabolic properties of the soil (Lethbridge and Burns 1976; Bayer *et al*. 1982; Schinner *et al*. 1990; He *et al*. 2001). The relationship between enzyme content and the levels of microbial biomass appeared to vary with sampling time (Banerjee *et al.* 1999). Treatment of soils with agrochemicals sometimes produces no changes in soil microflora and hence no change in enzymatic activity as reported by earlier workers (Gianfreda *et al.* 1993; Mohanty and Padhan 1993; Tu 1993). Even the most hazardous pollutant, diesel oil, was found to have only a slight effect on the activity of acid and alkaline phosphatases (Wyszkowska 2002). However, some of the tested agrochemicals were said to have a positive co-relation with soil enzymes. Urease was induced by nematicides tetone II, telone C17, vorlex, vorlex CP, fenamiphos and fungicides propamocarb (Tu *et al*. 1995), benomyl and captan (Chen *et al*. 2001); Phosphatases were induced by mefenoxam, metalaxyl (Monkiedje and Spiteller 2002) and fonofos (Stepniewska *et al*. 2003); Proteases were induced by imazethapyr (Perucci 1994) and metsulfuron-methyl (Ismail *et al*. 1998).

On the other hand, a decrease in enzymatic activity was also reported by many workers. Inhibition of urease and phosphatase was reported with 32 pesticides (Tu 1981), metolachlor (Ismail *et al*. 1996), benomyl, copperoxychloride, mancozeb (Shukla and Mishra 1996), treflan 480 EC (Wyszkowska 2002), and protease, cellulase and xylanase by linuron (Hasan and AbdelSater 2000) and metasulphuron-methyl (Ismail *et al*. 2000).

Use of fungicides is on the rise and their extensive and

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indiscriminate use is a cause for concern as: (i) they may have a longer half-life, (ii) their residues are also toxic and (iii) a major part of fungicides reaches non-target organisms. In order to safeguard the environment, degradation of fungicides and their residues is essential. Banerjee and Dey (1992) observed an initial decrease in the fungal population followed by an increase as a result of the degradation of fungicide. Degradation of agrochemicals may be possible by photolytic, chemical or biodegradation methods (Gowrisankar *et al*. 2002). Many physical, chemical and biological parameters like potency of hydrogen (Hance 1979; Blumhorst and Weber 1994), soil properties (Seta and Karathanasis 1997; Jenks *et al*. 1998), humic matter content (Johnson *et al*. 1998), moisture content (Topp *et al*. 1995; Bollag *et al*. 1996), and the presence of alternative nutrients (Mg, Mn, and P) (Javanjal and Deopurkar 1994) play an important role on the fate of the pesticides in soil (Gowrisankar *et al*. 2002). The lower the half-life, the safer the fungicide. The fungicide pefurazoate is said to have a half-life of less than a week (Takenaka *et al*. 1991) and the fungicide diethofencarb a half-life of 0.3 to 6.2 days (Sakata *et al*. 1992).

Amistar™ (Azoxystrobin 25% EC) and Score™ (difenoconazole 25% EC) are two broad spectrum, foliar, systemic fungicides, yet to be released to farmers and planters in India by Syngenta India Limited, Mumbai. The objective of the present study was to investigate the effects of these fungicides on soil microbial population, soil respiration and soil enzyme activities. Besides, degradation of the fungicides at their recommended dose (2.2 µg (a.i.) g^{-1} soil) over a period of 96 hours was studied using HPLC. Experiments were carried out mostly at concentrations ranging from 0.44-44 µg (a.i.) g^{-1} soil.

MATERIALS AND METHODS

This study was conducted in the Department of Botany, Bharathiar University, Coimbatore during 2001-2004.

Fungicide treatment of soil

Five hundred grams of garden soil samples (from Coimbatore) were mixed with the respective fungicide concentrations ranging from 0.44 to 44 (µg (a.i.). g^{-1} soil) of Azoxystrobin and Difenoconazole and filled separately in cotton cloth bags of 15 x 15 cm. The soil samples in bags were watered regularly in order to maintain the soil moisture content to field capacity with tap water. Three replicates were maintained for each treatment. Required amounts of soil samples were removed from the treatment bags periodically and used for various analyses.

Effect of fungicides on total microbial population in soil

A dilution plate method was employed for the enumeration of microbial population in the soil samples. The slimy, colourless and coloured bacterial colonies appeared in the nutrient agar medium after 24 h incubation. The fungal colonies appeared in the potato dextrose agar – rose bengal medium after 3 days incubation and small white chalky colonies of actinomycetes appeared in Kuster's agar medium after 6 days incubation. Three replicates were maintained for each of the organisms studied.

Procedure for dilution plate method

Soil dilutions of 10^{-3} for fungi, 10^{-7} for bacteria and 10^{-6} for actinomycetes were used for plating. One ml each from the soil dilutions were pipetted into sterile Petri-dishes and about 20 ml of the respective molten, warm medium was poured into each Petri dish, rotated gently by hand so that the diluted sample was uniformly dispersed in the agar medium, labeled and incubated at room temperature in an inverted position. All these procedures were carried out under aseptic conditions. Observations were made on the 4th day for fungi, the 7th day for actinomycetes and after 24 h for bacteria. The microbial populations per ml samples were calculated as follows:

Colony forming units (CFU) ml^{-1} = Average number of colonies per plate X dilution factor

Determination of soil dry matter

Ten grams of moist soil was weighed into a porcelain dish (determine the tare) and the sample was dried to constant weight at 105°C for at least 3 hours. The sample was allowed to cool for a few minutes and was re-weighed.

$$\text{Per cent dry matter (dm)} = \frac{\text{Soil dry matter}}{\text{Initial soil weight}} \times 100$$

Estimation of soil respiration

Soil samples were incubated in closed vessels at 25°C. The CO_2 evolved was absorbed by potassium hydroxide and was quantified by titration following the method of Jaggi (1976).

Enzyme assays

The colorimetric assay of carboxymethyl cellulase activity was carried out according to the procedure of Schinner *et al*. (1990). CM-cellulase activity was expressed as µg of glucose equivalents (GE) g^{-1} dry matter and incubation time. The method developed by Schinner *et al*. (1990) was employed for estimating xylanase activity. Xylanase activity was expressed as µg of glucose equivalents (GE) g^{-1} dry matter and incubation time. Using casein as substrate, protease activity was assayed following the method of Ladd (1978). Protease activity was expressed as µg tyrosine equivalents (tyr) per gram dry matter and incubation time. Assay of urease activity was according to the method of Kandeler and Gerber (1988) and the urease activity was expressed as µg N per gram dry matter and incubation time. The method of Eivazi and Tatabai (1976) was used for assaying acid phosphatase activity. Acid phosphatase activity was expressed as µg *p*-nitrophenol (pNP) per gram dry matter and incubation time.

Degradation of Azoxystrobin and Difenoconazole in soil

Two soil samples one from Coimbatore (garden soil) and the other from a tea plantation, Valparai were collected from A_1 horizon, ensuring that they were not exposed to previous application of either Azoxystrobin or Difenoconazole. The physico-chemical and biological properties of the soils are given in **Table 1**. These soils were sieved through 1 mm mesh and the recommended doses of Azoxystrobin and Difenoconazole per gram of soil were amended separately and incubated at field moisture level.

Fungicides were estimated in a HPLC (Hewlett Packard series 1100, USA) using micro Bondapak C18 column. Stock solutions of Azoxystrobin and Difenoconazole containing 100 mg (a.i.) l^{-1} were prepared from which working standards of 0.1, 1, 5 and 10 mg (a.i.) l^{-1} were prepared using volumetric flasks for each fungicide.

Mobile phase A was prepared with methanol, acetic acid and water (1: 0.1: 8.9) and phase B with the same solvents but at a ratio of 8.9: 0.1:1. The flow rate was 1 ml min^{-1}. The UV detector was set at 255 nm. 20 µl of each working standard was injected into the column.

Soil (~1 g) from the fungicide-treated soil samples was placed in a centrifuge tube and was mixed with 9 ml water, shaken on an

Table 1 Physico-chemical and biological properties of the soils.

Parameter	Coimbatore soil	Valparai soil
pH	7.2	4.35
Moisture content	13.295%	19.53%
Total nitrogen	0.37%	0.27%
Available phosphorous	83.68 mg/kg	112.47 mg/kg
Exchangeable potassium	114.99 mg/kg	141.50 mg/kg
Bacterial CFU	15.8 x 10^{-6} CFU/g	12.5 x 10^{-6} CFU/g
Fungal CFU	12.2 x 10^{-3} CFU/g	14 x 10^{-3} CFU/g
Actinomycetes CFU	10.2 x 10^{-6} CFU/g	7.3 x 10^{-6} CFU/g

orbital shaker for 1 h, spun at 5000 rpm (1960 × *g*) for 5 min and the supernatant was filtered through a filter of 0.45 µm pore size. 20 µl of the filtrate was injected into the column. Periodic samplings of the soil at 24 h intervals were made for 4 days to determine the left over Azoxystrobin and Difenoconazole in the soil samples. Each sample was injected thrice.

Statistical analysis

All sets of 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

Effect on fungal, bacterial and actinomycete populations of soil

The changes in the population of fungi, bacteria and actinomycetes in soils treated with different concentrations of Azoxystrobin and Difenoconazole are presented in **Tables 2**, **3** and **4**, respectively. Both the fungicides significantly reduced the fungal populations with increasing concentrations of their active ingredients in the treated soil (**Table 2**). The toxic effects of the fungicides were more pronounced immediately after the period of application of the fungicides. With an increasing incubation period, the fungal population tended to revive. Among the two fungicides, Difenoconazole was more fungitoxic than Azoxystrobin.

The bacterial (**Table 3**) and actinomycete (**Table 4**) populations of the soil were not adversely affected by Azoxystrobin and Difenoconazole. On the other hand, the populations tended to increase with an increase in the concentration of the fungicides tested. Though the bacterial and actinomycete populations in the fungicide treated soils were significantly higher than the untreated control, their populations tended to decrease slightly after 12 days of incubation. Among the two fungicides, Difenoconazole favoured bacterial populations while the actinomycetes were favoured more by Azoxystrobin.

Table 2 Effect of various concentrations of Azoxystrobin and Difenoconazole on the fungal populations (x10^3 CFU g^{-1} dry soil) of the treated soil.

Conc.	Days after treatment					
(µg.a.i.g^{-1} soil)	**0**	**4**	**8**	**12**	**16**	**20**
Control	52 a	53 a	53 a	54 a	51 a	50 a
Azoxystrobin						
0.44	16 b	18 b	20 b	21 b	22 b	24 b
1.10	12 bc	13 bc	15 c	16 bc	16 bc	17 bc
1.46	10 c	11c	12 c	14 c	15 c	15 c
2.20	-	-	-	5 d	6 d	7 d
4.40	-	-	-	1 d	2 de	2 de
22.00	-	-	-	-	-	-
Difenoconazole						
0.44	9 c	10 c	11 c	12 c	12 c	13 c
1.10	5 d	8 c	8 cd	10 c	11 c	11 c
1.46	-	2 de	3 d	4 d	4 d	5 cd
2.20	-	-	-	-	-	1 e
4.40	-	-	-	-	-	-
22.00	-	-	-	-	-	-

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 days vs fungicide means	2.77	5.46	7.21

Table 3 Effect of various concentrations of Azoxystrobin and Difenoconazole on the bacterial populations (x10^6 CFU g^{-1} dry soil) of the treated soil.

Conc.	Days after treatment					
(µg.a.i.g^{-1} soil)	**0**	**4**	**8**	**12**	**16**	**20**
Control	25 cd	26 d	22 e	26 c	29 ab	29 a
Azoxystrobin						
0.44	25 cd	28 cd	29 d	29 bc	29 ab	28 a
1.10	26 cd	28 cd	30 cd	29 bc	28 a	28 a
1.46	27 c	30 c	33 c	30 bc	28 a	29 a
2.20	29 c	32 c	32 c	31 b	28 a	27 a
4.40	29 c	29 c	30 cd	31 b	27 a	28 a
22.00	30 c	30 c	30 cd	28 b	26 a	29 a
Difenoconazole						
0.44	30 c	31 c	32 c	28 b	29 ab	29 a
1.10	30 c	33 c	36 bc	28 b	29 ab	29 a
1.46	32 bc	35 c	39 b	32 b	29 ab	29 a
2.20	38 b	40 b	42 b	39 a	29 ab	28 a
4.40	39 ab	44 b	50 a	44 a	32 a	28 a
22.00	44 a	49 a	52 a	45 a	33 a	29 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 days vs fungicide means	3.363	6.639	8.761

Table 4 Effect of various concentrations of Azoxystrobin and Difenoconazole on the actinomycete populations (x10^5 CFU g^{-1} dry soil) of the treated soil.

Conc.	Days after treatment					
(µg.a.i.g^{-1} soil)	**0**	**4**	**8**	**12**	**16**	**20**
Control	39 ab	35 bc	39 de	40 c	40 b	40 a
Azoxystrobin						
0.44	35 b	36 bc	40 c	40 c	40 b	40 a
1.10	37 b	38 bc	42 cd	42 c	40 b	40 a
1.46	39 ab	40 b	44 cd	43 c	42 ab	40 a
2.20	41 a	42 b	49 bc	46 bc	41 ab	39 a
4.40	42 a	49 a	55 ab	52 ab	49 a	39 a
22.00	44 a	46 a	62 a	55 a	42 ab	38 a
Difenoconazole						
0.44	39 ab	40 b	40 cd	40 b	40 b	40 a
1.10	39 ab	40 b	41 cd	40 b	38 bc	39 a
1.46	41 a	43 ab	43 cd	42 ab	40 b	40 a
2.20	42 a	42 ab	48 bc	42 ab	41 b	39 a
4.40	43 a	45 a	49 bc	47 ab	43 ab	39 a
22.00	45 a	48 a	51 b	49 a	46 ab	40 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 days vs fungicide means	4.098	8.089	10.674

Effect on soil respiration

Table 5 shows soil respiration as influenced by the different concentrations of Azoxystrobin and Difenoconazole. In the Azoxystrobin-treated soil, respiration decreased significantly with an increase in concentration of the active ingredients on the 7th and 14th day after treatment. However, on the 21st day after fungicide treatment, respiration was similar to that of the untreated control soil. A similar trend was also observed in Difenoconazole-treated soil.

Table 5 Effect of Azoxystrobin and Difenoconazole on soil respiration (mg CO_2.g^{-1} dm.24 h^{-1}).

Concentration	Days after treatment					
(µg.(a.i.)g^{-1}	**Azoxystrobin**			**Difenoconazole**		
soil)	**7**	**14**	**21**	**7**	**14**	**21**
Control	2.102 a	2.102 a	2.102 a	2.102 d	2.102 c	2.102 a
0.44	1.887 b	2.098 a	2.086 a	4.349 b	2.281 b	2.102 a
1.10	1.305 c	1.826 b	2.086 ab	7.152 a	2.385 a	2.102 a
1.40	1.202 d	1.826 c	2.086 ab	3.216 c	2.136 c	2.102 a
2.20	1.190 d	1.489 c	2.067 ab	1.638 e	1.891 d	2.102 a
4.40	1.142 d	1.535 b	2.048 ab	1.332 f	1.687 e	2.102 a
22.00	1.045 e	1.389 d	2.010 b	0.482 g	1.585 f	2.102 a
44.00	0.827 f	1.229 e	2.010 b	0.244 h	1.576 f	2.102 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 days of incubation vs fungicide means	0.0378	0.0750	0.0632

Table 6 Cellulase activity (μg GE.g^{-1} dm.24h^{-1}) in Azoxystrobin- and Difenoconazole-treated soils.

Concentration (μg.a.i.g^{-1} soil)	Days after treatment							
	Azoxystrobin				Difenoconazole			
	7	14	21	28	7	14	21	28
Control	324.859 f	324.859 e	324.859 e	324.859 a	324.859 f	324.859 d	324.859 a	324.859 a
0.44	383.061 e	326.544 de	326.084 d	324.859 a	347.254 e	326.482 cd	324.859 a	324.859 a
1.10	396.539 d	329.760 d	327.310 d	324.859 a	396.539 c	327.922 c	324.859 a	324.859 a
1.46	505.132 d	332.364 c	331.598 c	324.859 a	397.918 b	332.057 b	324.859 a	324.859 a
2.20	530.474 a	386.737 b	345.077 b	325.499 a	408.349 a	385.165 a	324.859 a	324.859 a
4.40	589.525 b	432.839 a	383.061 a	325.679 a	376.950 d	327.616 c	325.165 a	324.859 a
22.00	413.387 c	327.157 f	325.625 e	324.866 a	79.951 g	317.967 e	321.183 b	324.859 a
44.00	146.883 g	306.326 g	324.706 e	324.706 a	43.804 h	271.099 f	307.245 c	324.859 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 Days of incubation vs Fungicide means	0.864	1.709	2.259

Effect on soil enzyme activity

Changes in the activities of the soil enzymes cellulase, xylanase, protease, urease and acid phosphatase as influenced by different concentrations of the fungicides Azoxystrobin and Difenoconazole are given in **Tables 6**, **7**, **8**, **9** and **10**, respectively.

The activity of cellulase increased progressively with an increase in Azoxystrobin concentration up to 44 μg (a.i.) g^{-1} soil. A similar trend was observed on the 14th day of incubation, but cellulase activity was lower than equivalent values at 7 days incubation. The difference in the cellulase activity in the treated soils on 21st and 28th day, however, was not statistically significant when compared with that of control soil. Treatment with difenoconazole also enhanced cellulase activity, but the increases were noted only up to 2.20 μg (a.i.) g^{-1} soil and were less pronounced. Upon prolonged incubation, the enzyme activity reached the same level as controls.

Xylanase and protease activities were also enhanced by increase in the concentration of Azoxystrobin and Difenoconazole on the 7th day after treatment. Azoxystrobin treatment resulted in highest activity of xylanase while Difenoconazole enhanced protease activity. Activities of both these enzymes were at approximately the same level as the untreated control on the 14th day of incubation.

Urease activity generally decreased with an increase in concentration of both Azoxystrobin and Difenoconazole on the 7th day of treatment. However, at its lowest concentration (0.44 μg (a.i.) g^{-1} soil), Azoxystrobin enhanced the activity of urease. On 14th day of incubation, the urease activity was more or less equal to that of control soil. The acid phosphatase activity also attenuated with increasing concentrations of Azoxystrobin and Difenoconazole on the 7th and 14th day of incubation. Difenoconazole, however, enhanced the enzyme activity at its lower concentrations (0.44-1.46 μg (a.i.) g^{-1} soil) as observed on 7th and 14th days of incubation. On the 21st day of incubation, the enzyme activity in treatment soils was more or less equal to that of control soil.

Degradation of fungicides in soil

The retention time for Azoxystrobin and Difenoconazole were 19.4 min and 16.1 min, respectively. The left over fungicides in the soil at the end of different incubation periods was quantified from the peak area from which the per cent degradation was arrived at.

Table 11 shows the percentage degradation of the systemic fungicides Azoxystrobin and Difenoconazole in two different soils over a period of time. Azoxystrobin was degraded by 95% in a period of 96 hours in the slightly alkaline Coimbatore soil whereas in acidic Valparai soil, the degradation was only 70% during this period. Difenoconazole, however, was almost completely degraded in both Coimbatore (95%) and Valparai (97%) soils after 96 hours.

Table 7 Xylanase activity (μg GE.g^{-1} dm.24 h^{-1}) in Azoxystrobin- and Difenoconazole-treated soils.

Concentration (μg.a.i.g^{-1} soil)	Days after treatment			
	Azoxystrobin		Difenoconazole	
	7	14	7	14
Control	8.270 h	8.270 b	8.270 h	8.270 a
0.44	52.994 g	8.576 ab	12.865 g	8.270 a
1.10	60.499 f	8.729 ab	15.049 f	8.270 a
1.46	62.950 e	8.730 ab	20.064 e	8.270 a
2.20	65.247 d	8.730 ab	28.181 d	8.117 a
4.40	140.144 b	8.883 ab	81.520 b	8.117 a
22.00	257.314 a	8.883 ab	171.389 a	8.270 a
44.00	135.089 c	9.189 a	76.581 c	8.270 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%)
Conc. vs Days of inc. vs Fungicide means	0.337	0.673	0.894

Table 8 Protease activity (μg tyr.g^{-1} dm) in Azoxystrobin- and Difenoconazole-treated soils.

Concentration (μg.a.i.g^{-1} soil)	Days after treatment			
	Azoxystrobin		Difenoconazole	
	7	14	7	14
Control	26.915 e	28.248 a	28.248 f	28.248 a
0.44	109.220 d	28.248 a	170.838 e	28.248 a
1.10	217.585 e	28.248 a	315.143 c	28.248 a
1.46	218.258 c	28.248 a	435.425 b	28.248 a
2.20	223.122 c	28.248 a	451.376 b	28.248 a
4.40	274.268 c	28.248 a	672.565 a	28.248 a
22.00	511.829 a	28.248 a	362.452 c	28.248 a
44.00	445.020 b	28.248 a	241.102 c	28.248 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 Days of incubation vs Fungicide means	1.802	3.600	4.784

Table 9 Urease activity (μg N.g^{-1} dm.24 h^{-1}) in Azoxystrobin- and Difenoconazole-treated soils.

Concentration (μg.a.i.g^{-1} soil)	Days after treatment			
	Azoxystrobin		Difenoconazole	
	7	14	7	14
Control	210.078 b	210.078 d	210.078 a	210.078 a
0.44	229.744 a	212.008 a	186.736 b	209.894 a
1.10	178.741 c	211.089 bc	178.833 c	209.435 b
1.46	177.822 d	210.997 b	175.892 d	206.219 c
2.20	165.355 e	210.997 bc	144.555 e	204.289 d
4.40	143.820 f	210.813 b	115.240 f	195.467 e
22.00	34.277 g	210.629 b	23.985 g	195.466 e
44.00	30.418 h	210.078 d	6.616 h	195.375 e

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 Days of incubation vs Fungicide means	0.127	0.254	0.338

Table 10 Acid phosphatase activity (μg NP.g^{-1}.dm.h^{-1}) in Azoxystrobin- and Difenoconazole-treated soils.

Concentration (μg.a.i.g^{-1} soil)	Days after treatment					
	Azoxystrobin			Difenoconazole		
	7	14	21	7	14	21
Control	2.102 a	2.102 a	2.102a	2.102 d	2.102 cd	2.102a
0.44	1.887 b	2.098 a	2.086 a	4.349 b	2.281 b	2.102 a
1.10	1.305 c	1.826 b	2.086 ab	7.152 a	2.385 a	2.102 a
1.40	1.202 d	1.826 c	2.086 ab	3.216 c	2.136 c	2.102 a
2.20	1.190 d	1.489 c	2.067 ab	1.638 e	1.891 d	2.102 a
4.40	1.142 d	1.535 b	2.048 ab	1.332 f	1.687 e	2.102 a
22.00	1.045 e	1.389 d	2.010 b	0.482 g	1.585 f	2.102 a
44.00	0.827 f	1.229 e	2.010 b	0.244 h	1.576 f	2.102 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 Days of incubation vs Fungicide means	0.0378	0.0750	0.0993

Table 11 Percentage degradation of Azoxystrobin and Difenoconazole in soil over a period of incubation.

Period of incubation (hrs)	Azoxystrobin		Difenoconazole	
	Coimbatore (Plain area)	Valparai (Hill area)	Coimbatore (Plain area)	Valparai (Hill area)
0	0 e	0 d	0 d	0 e
24	38.451 d	38.802 c	48.510 c	68.825 d
48	66.181 c	46.409 c	69.905 b	75.311 c
72	92.173 b	60.550 b	92.046 a	89.943 b
96	95.066 a	70.047 a	95.218 a	97.419 a

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

DISCUSSION

Determination of microbially mediated reactions is limited because present assays for determining the overall rate of entire metabolic processes (such as respiration) or specific enzyme activities (such as urease, protease and phosphomonoesterase activity) do not allow any identification of the microbial species directly involved in the measured processes (Nannipieri *et al*. 2003). However, the only accurate way to determine the combined metabolism of mixed populations of microorganisms is by measuring soil respiration (Parkin *et al*. 1996) and/or enzyme activities (He *et al*. 2001). Singh *et al*. (2002) found that the soil microbial parameters (enzyme activities and total microbial biomass) were stable in the pesticide-free control soils throughout the 90-d incubation period, but they were all adversely affected by the presence of chlorothalonil in the soil. The effects from fenamiphos or chlorpyrifos on the soil microbial characteristics were either very small or insignificant (Singh *et al*. 2002).

In the present investigation, data on the population density of microorganisms in soils treated with Azoxystrobin and Difenoconazole indicate a significant reduction in fungi even at the lowest concentration used (0.44 μg (a.i.) g^{-1} soil). At the recommended dose (2.2 μg (a.i.) g^{-1} soil) and above, the fungal population was totally eliminated. The outcome is expected as both the chemicals are broad-spectrum fungicides. On the other hand, the populations of bacteria and actinomycetes in the treated soil tended to increase with an increase in concentration of the fungicides. This can be attributed to the elimination of fungal competitors for nutrition in the treated soils or increased substrate in the form of dead hyphae (Ingham and Coleman 1984). The increase in the population of bacterial and actinomycete components of the soil may also be due to their ability to degrade the organic fungicides Azoxystrobin and Difenoconazole for nutrition, which is supported by the faster disappearance of the applied fungicides from the soil (**Table 11**). The present study also recorded the tendency of fungal populations to recover over a period of time. In a similar study with the soils amended with fungicides captan, thiram and verdasan, Anderson *et al*. (1981) recorded the restoration of fungal to bacterial balance within 8 days of incubation.

A dose-dependant and significant inhibition of soil respiration by treatment with increasing concentrations of Azoxystrobin and Difenoconazole was noticed (**Table 5**). The decrease in respiration may be related to a marked reduction in the population of fungi, an important component of soil microflora. However, the sharp increase in the rate of respiration at lower concentrations of Difenoconazole treatment is perplexing. The increased CO_2 liberation may be due to the oxidative degradation of Difenoconazole by microorganisms. The immediate depression in the respiratory activity of the fungicide treated soils tended to recover with an increase in incubation period. This means that, the deleterious effects of the added fungicides gradually disappeared, possibly because of the degradation of the toxic substances (**Table 11**).

The enzyme activity in the soil might be a direct reflection of microbial activity. Furthermore the activities of cellulase, xylanase, protease, urease and acid phosphatase release easily utilizable carbon, nitrogen and phosphorous sources for microbial growth. Hence, their assay in soil may serve as a useful pointer in the assessment of soil health. Experiments on the influence of fungicides on soil enzymatic activity are very limited (Gowrisankar *et al.* 2002). In the present study, the activities of cellulase, xylanase and protease increased significantly with an increase in Azoxystrobin and Difenoconazole concentrations (**Tables 6-8**). As the fungal populations were inhibited at higher doses of fungicides, the other probable microbial source of these enzymes might be bacteria and actinomycetes, whose populations improved with an increase in Azoxystrobin and Difenoconazole concentrations (**Tables 3-4**). Reduction in the activities of enzymes such as urease, xylanase and protease following fungicide (metachlor and metasulfuron-methyl) treatment in the soil is also known (Ismail *et al*. 1996, 1998, 2000).

A significant decrease in the activity of urease and acid phosphatase with an increase in Azoxystrobin and Difenoconazole concentrations applied to soils (**Tables 9, 10**) are in line with the observations made by other workers (Shukla and Mishra 1996; Nowak *et al*. 2000; Wyszkowska 2002; He *et al*. 2003). The decreased activities of these enzymes were concomitant with a decrease in fungal population and an increase in bacterial and actinomycete populations as a consequence of fungicide treatment. As such, the predominant contribution of the fungal population to the urease and phosphatase enzyme pool in soil is obvious. Direct inhibition of their activities by fungicides is another possibility.

Pesticides or their left over residues in the soil can be detected using three different methods. The first method involves the use of gas chromatography (GC) (Slade and Fullerton 1992). The second method of analysis employs Liquid Scintillation (LSC) Spectrometry (Saxena *et al*. 1987; Sakata *et al*. 1992). The third and the most recent one use High Performance Liquid Chromatography (HPLC) (Hodgeson *et al*. 1992). Mitchell and Cain (1999) found that enhanced degradation of the dicarboximide fungicides, iprodione and vinclozolin, was stimulated by only one application of the fungicides in a soil with no previous history of any pesticide input and concluded that agent(s) of enhanced degradation was probably bacterial. In the present investigation, the faster degradation of Azoxystrobin and Difenoconazole within a week as revealed by the HPLC studies, qualify them to be environmentally friendly.

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REFERENCES

Akinyanju JA, Fadayomi O (1989) Effect of diuron on sugarcane rhizosphere microbial populations. *Nigerian Journal of Botany* **2**, 49-58

Anderson JPE, Armstrong RA, Smith SN (1981) Methods to evaluate pesti-

cide damage to the biomass of the soil microflora. *Soil Biology and Biochemistry* **13**, 149-153

Arden-Clarke C, Hodges RD (1988) The environmental effects of conventional and organic/biological farming systems: II. Soil ecology, soil fertility and nutrient cycles. *Biological Agriculture and Horticulture* **5**, 223-285

Banerjee MR, Burton DL, Grant CA (1999) Influence of urea fertilization and urease inhibitor on the size and activity of the soil microbial biomass under conventional and zero tillage at two sites. *Canadian Journal of Soil Science* **79**, 255-263

Banerjee MR, Dey BK (1992) Effects of different pesticides on microbial populations, nitrogen mineralisation and thiosulphate oxidat in the rhizosphere of jute (*Corchorus capsularis* L.). *Biology of Fertilized Soils* **14**, 213-218

Bayer H, Mitterer M, Schinner F (1982) Der fin flub von insektiziden auf mikrobiogene prozesse in ah-materialien eines land wirtschaftlich genutzten bodens. *Pedobiologia* **23**, 311-319

Blumhorst MR, Weber JB (1994) Chemical Vs Microbiological degradation of Cyanazine and Atrazine in soils. *Pesticide Science* **42**, 79-84

Bollag JM, McGahen L, Minard L, Liu SY (1996) Bio-conversion of alachlor in an anaerobic stream sediment. *Chemosphere* **15**, 153-162

Chen SK, Edwards CA, Subler S (2001) A microcosm approach for evaluating the effects of the fungicides benomyl and captan on soil ecological processes and plant growth. *Applied Soil Ecology* **18(1)**, 69-82

Dick RP (1994) Soil enzymes as assays indicators of soil quality. In: Doran JW, Coleman DF, Bezdicek DF, Stewert BA (Eds) *Defining Soil Quality for a Sustainable Environment*, SSSA Inc., Madison, W.I. Special Publication. No. 35, pp 175-200

Eivazi F, Tatabai MA (1976) Phosphatases in soils. *Soil Biology and Biochemistry* **9**, 167- 172

El-Abyad MS, Gharrel M (1991) Changes of tomato rhizosphere microflora following application of the herbicide diphenamid to soil infested with *Fusarium oxysporum* f. sp. *lycopersci*. *Phytoparasitica* **113**, 89-94

Gianfreda L, Sannino F, Filazzola MT, Violante A (1993) Influence of pesticides on the activity and kinetics of invertase, urease and acid phosphatase enzymes. *Pesticide Science* **39**, 237-244

Gowrisankar R, Palaniappan R, Ramasamy K, Ramesh S (2002) Microbial degradation of herbicides. *Asian Journal of Microbiology, Biotechnology and Environmental Sciences* **4**, 187-196

Gregorich EG, Carter MR, Angers DA, Monreal CM, Ellert BH (1994) Towards minimum data set to assess soil organic matter quality in agricultural soils. *Canadian Journal of Soil Science* **74**, 367-385

Hance RJ (1979) Effect of pH on the degradation of atrazine, linuron and propyzamide in soil. *Pesticide Science* **10**, 83-86

Hasan HAH, AbdelSater MA (2000) Responses of mycoflora and sorghum to pre-planting soil incorporation with linuron herbicide. *Rostlinna Vyroba* **46**, 417-422

He WX, Lai HX, Wu YJ, Zhu MG (2001) Study on soil enzyme activities affected by fertilizing cultivation. *Journal of Zhejiang University Agriculture and Life Sciences* **27**, 265-268

He WX, Jiang Xin, Yu GuiFen, Bian YongRong, Jiang X (2003) Effect of dimehypo on soil urease activity. *Acta Pedologica Sinica* **40**, 750-755

Hodgeson JW, Bashe WJ, Eichelberger JW (1992) Method 549.1: Determination of diquat and paraquat in drinking water by liquid-solid extraction and high performance liquid chromatography with ultraviolet detection. *EPA/600/R-92/129.* USEPA Environ. Monitoring Systems Lab, Cincinnati, 10 pp

Ingham ER, Coleman DC (1984) Effects of streptomycin, cycloheximide, fungizone, captan, carbofuran, cygon, and PCNB on soil microorganisms. *Microbial Ecology* **10**, 345-358

Ismail BS, Ingon D, Omar O (1996) Effects of metachlor on activities of enzymes in a Malaysian soil. *Journal of Environmental Science and Health. Part B: Pesticides, Food Contaminants, and Agricultural Wastes* **31**, 1267-1278

Ismail BS, Yapp KF, Omar O (1998) Effect of metasulfuron-methyl on amylase, urease and protease activities in two soils. *Australian Journal of Soil Research* **36**, 449-456

Ismail BS, Ampong N, Omar O (2000) Effect of metsulphuron-methyl on amylase, invertase and xylanase activities in two soil types. *Microbiology* **103**, 73-83

Jaggi W (1976) Die bestimmung der CO_2-bildung als MaB der bodenbiologischen aktivitat. *Schw Landw Forsch.* **15**, 371-380

Javanjal SJ, Deopurkar RL (1994) Biodegradation of *p*-nitropohenol by indigenously isolated bacteria from pesticide amended soil. *Indian Journal of Microbiology* **34**, 125-129

Jenks BM, Roeth FW, Martin AR, McCallister DL (1998) Influence of surface and subsurface soil properties on atrazine sorption and degradation. *Weed Science* **46**, 132-138

Johnson RM, Pepperman AB, Selim HM (1998) Field mobility and persistence of alginate controlled release formulation of atrazine. *Soil Science* **163**, 46-52

Kandeler E, Gerber H (1988) Short-term assay soil urease activity using calorimetric determination of ammonium. *Biology of Fertilized Soils* **6**, 68-72

Karlen DL, Stott DE (1994) A framework for evaluating physical and chemical indicators of soil quality. In: Doran JW (Ed) *Defining Soil Quality for a Sustainable Environment*, SSSA Spec. Pub. No. 35, SSSA linc, WI, pp 15-20

Ladd JN (1978) Origin and range of enzymes in soil. In: Burns RG (Ed) *Soil Enzymes*, Academic Press Inc., London, UK

Larson WE, Pierce FJ (1994) The dynamics of soil quality as a measure of sustainable management. In: Doran JW, Coleman DC, Bezdicek DF, Stewart BA (Eds) *Defining Soil Quality for a Sustainable Environment*, SSSA Spec. Pub. No. 35, SSSA linc, WI, pp 37-51

LethbridgeG, Burns RG (1976) Inhibition of soil urease by organophosphorus insecticides. *Soil Biology and Biochemistry* **8**, 99-102

Mitchell JA, Cain RB (1999) Rapid onset of the accelerated degradation of dicarboximide fungicides in a UK soil with a long history of agrochemical exclusion. *Pesticide Science* **48**, 1-11

Mohanty RK, Padhan S (1993) Effect of nematicides on enzyme activities in soil planted with two types of crops. *Environment and Ecology* **11**, 7-12

Monkiedje A, Spiteller M (2002) Effects of phenylamide fungicides, mefenoxam and metalaxyl, on the microbiological properties of a sandy loam and a sandy clay soil. *Biology of Fertilized Soils* **35**, 393-398

Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara G, Renella G (2003) Microbial diversity and soil functions. *European Journal of Soil Science* **54**, 655-670

Nowak J, Klodka D, Biernacka TM, Haas TU (2000) Influence of combined and single applications of herbicides and adjuvents on the activity of dehydrogenase and phosphatase in soil. *Pflanzenkrankheiten und Pflanzenschutz* **17**, 769-774

Papendick RI, Parr JF (1992) Soil quality – the key to a sustainable agriculture. *American Journal of Alternative Agriculture* **7**, 2-3

Parkin TB, Doran JW, Franco-Vizcaino E (1996) Field laboratory tests of soil respiration. In: Dosan JW, Jones AJ (Eds) *Methods for Assessing Soil Quality*, SSSA Special Publ. 49, Madison, WI, pp 231-245

Perucci PS (1994) Effects of the herbicide imazethapyr on soil microbial biomass and various soil enzyme activities. *Biology of Fertilized Soils* **17**, 237-240

Sakata S, Katagi T, Yoshimura J, Mikami N, Yamada H (1992) Degradation and leaching behaviour of the carbamate fungicide diethofencarb in soils. *Journal of Pesticide Science* **17**, 221-230

Saxena A, Zhang R, Bollag JM (1987) Microorganisms capable of metabolizing the herbicide Metachlor. *Applied and Environmental Microbiology* **53**, 390-396

Schinner F, Mersi W Von (1990) Xylanase-, CM-cellulase and invertase activity in soil, an improved method. *Soil Biology and Biochemistry* **22**, 511-515

Schinner F, Ohlinger R, Kandeler E (1991) *Bodenbiiologische Arbeits Methoden*, Springer, Berlin, 120 pp

Schuster E, Schroder D (1990) Side-effects of sequentially applied pesticides on non-target soil microorganisms: Field experiments. *Soil Biology and Biochemistry* **22**, 367-373

Seta AK, Karathanasis VD (1997) Atrazine adsorption by soil colloids and co-transport through subsurface environments. *Soil Science Society of America Journal* **61**, 612-617

Shukla AK, Mishra RR (1996) Response of microbial population and enzyme activities to fungicides in potato field soil. *Proceedings of the Indian National Science Academy, Part B Biological Sciences* **62**, 435-438

Singh BK, Walker A, Wright DJ (2002) Degradation of chlorpyrifos, fenamiphos, and chlorothalonil alone and in combination and their effects on soil microbial activity. *Environmental Toxicology and Chemistry* **21**, 2600-2605

Slade EA, Fullerton RA (1992) Degradation of the dicarboximide fungicides iprodione, vinclozolin and procymidone in patumahoe clay loam soil, New Zealand. *Pesticide Science* **35**, 95-100

Staben ML, Bezdicek DF, Smith JL, Fauci MF (1997) Assessment of soil quality in conversion reverse program and wheat-fallow soils. *Soil Science Society of America Journal* **15**, 45-47

Stepniewska Z, Charytoniuk P, Fornal E, del Re AAM (2003) Fonofos influence on soil phosphomonoesterase and dehydrogenase activity under flooded and non flooded conditions. *Pesticide in air, plant, soil and water system. Proceedings of the XII symposium- Pesticide chemistry.* Piacenza, Italy, 4th to 6th June, 2003, pp 115-121

Takenaka M, Sakai S, Nishida H, Kimura S, Hase H (1991) Degradation and adsorption of a seed fungicide pefurazoate in paddy soil. *Journal of Pesticide Science* **16**, 631-639

Topp ET, Vallaeys T, Soulas G (1995) Pesticides. Microbial degradation and effects on microorganisms. In: Elsas JDV, Trevors JT, Wellington JMH (Eds) *Modern Soil Microbiology*, Marcell Dekker, Inc., New York, pp 547-575

Tu CM (1981) Effects of some pesticides on enzyme activities in an organic soil. *Bulletin of Environmental Contamination and Toxicology* **27**, 109-114

Tu CM (1993) Effect of fungicides captafol and chlorothalonil on microbial and enzymatic activities in mineral soil. *Journal of Environmental Science and Health* **B28**, 67-80

Tu CM, Marks CF, Johnson PW, Gayed SK, Elliot JM (1995) Degradation of the fungicides, captafol and chlorothalonil in the soil. *Journal of Environmental Science and Health. Part B: Pesticides, Food Contaminants and Agricultural Wastes* **30**, 141-162

Turco RF, Kennedy AC, Jawson MD (1994) Microbial indicators of soil quality. In: Doran JW, Coleman DC, Bezdicek DF, Stewart BA (Eds) *Defining Soil Quality for a Sustainable Environment*, SSSA Inc., Madison, WI, Special Publication No. 35, pp 25-75

Vyas SC (1993) *Hand Book of Systemic Fungicides*, Tata McGraw Hill Publishing Co., Ltd.

Wyszkowska (2002) Effect of soil contamination with treflan 480 EC on biochemical properties in soil *Polish Journal of Environmental Studies* **11**, 71-77