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SIDE EFFECTS OF PESTICIDES ON NON-TARGET SOIL ASPERGILLI

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A B S T R A C T

Aspergilli constitute the largest fungal group present in all types of soil and also contribute in maintaining the soil structure and fertility. Fungicides that are used to control fungal plant pathogens may also be toxic to beneficial soil fungi. The present study was conducted to assess the impact of seven broad-spectrum fungicides namely Tebuconazole, Bloom, Benedict, Epic, Primacy and Hiten on growth of five different species of *Aspergillus* namely *A. nidulans* (Eidam) Wint., *A. niger* Tiegh., *A. terreus* Thom., *A. fumigatus* Fresen. and *A. flavus* Link. The results of the current study presented a comprehensive overview of the negative effects of fungicides on growth of commonly found soil Aspergilli. With the increasing concentration of all the tested fungicides from 0 to recommended dose (R), a gradual reduction in growth of all the fungal species was observed. Hiten and Epic were most effective in controlling the growth of *Aspergillus* spp. by causing complete growth arrest at only 0.05R. Bloom ceased the growth at one fourth of the recommended dose (0.25R). No growth of the *A. niger, A. terreus, A. fumigatus* and *A. flavus* was observed at 0.75R and that of the *A. nidulans* at recommended dose of Tebuconazole. In case of Benedict, *A. nidulans* and *A. terreus* were sensitive to 0.75R and *A. niger, A. fumigatus* and *A. flavus* to the recommended dose of the fungicide. Fungal Strains showed maximum resistance against Wisdom and Primacy as they continue to grow even at recommended doses of these fungicides. Thus the application of fungicides may cause alteration in soil microbial community structures.

Keywords: Aspergilli, fungicides, growth inhibition, soil microorganisms.

INTRODUCTION

Pesticides have their wide applications or even have an essential role in farming. They protect the plants from weeds, microbial pathogens as well as from insects and nematodes. In general, a pesticide could any synthesis or natural products that can be used to control the pest and has properties of discouraging the growth of pests and hence protects the plants from their damaging effect. Although use of pesticides is beneficial for plants but sometimes they have negative impact on plant, soil and also have potential toxicity for animals as well as humans (Bunce *et al.,* 1994; Akhtar *et al.,* 2009; Kalia and Gosal, 2011). Soil microorganisms are essential components of soil help in maintain soil structure and mineralization of organic matter and facilitate the uptake of nutrients by plant roots. Soil microorganisms also have the ability to degrade a lot of soil pollutants including pesticides. But there are possibilities where microbial degradation can lead to formation of more hazardous byproducts that persist in environment even longer. Fungicides that are sprayed to control fungal pests are toxic to beneficial soil fungi and this practice causes the alteration in microbial community structure of soil (Pal *et al.,* 2005). Results indicated that some organochlorine pesticides reduced the number of nitrogen fixing bacteria resulting in lesser crop productivity. Some Benomyl, Dimethoate are the pesticides reported to negatively affect the mycorrhizal fungi (Chiocchio *et al.,* 2000) as well as earthworms (Shahla and D'Souza, 2010).

Aspergilli are present in all types of environments and have direct effects on humans. In soil fungal community, species of genus *Aspergillus* constitute the largest group. Although this group is known as plant pathogens but in recent years studies proved their beneficial role in plant health. For examples *A. niger* produces Phytase that helps in mineral availability to plants (Gujar *et al.,* 2013). Such role can cause less use of fertilizers and improvement in soil health.

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In another study, *A. awamori* is known to produce growth-promoting hormone, Indole acetic acid (Mittal *et al*., 2008). The objective of the present study was to evaluate the negative impact of generally used broad spectrum fungicides on the soil microbial community.

MATERIALS AND METHODS

Procurement of fungus cultures: Five different fungal species belonging to Genus *Aspergillus* viz., *A. nidulans*, *A. niger*, *A. terreus*, *A. fumigatus* and *A. flavus* isolated from the field soils were acquired from First Fungal Culture Bank of Pakistan (FCBP), Institute of Agricultural Sciences University of the Punjab, Lahore, Pakistan. Cultivation of fungal spores was carried out on Malt Extract Agar (MEA) medium (2% w/v). Fungi were maintained on MEA slants and made fresh on petriplates when required.

Laboratory bioassays: Experiments were performed to study qualitative effect of six different fungicides on growth of *Aspergillus* spp. Initially, 0.25R, 0.5R, 0.75R and R doses of fungicides from the commercial formulation listed in Table 1 were evaluated for their efficacy to inhibit the growth of test fungi. However in case when the fungal growth was prevented at 0.25R further lower concentrations were applied until minimum inhibition concentration (MIC) achieved. Growth trials were performed on malt extract agar (MEA) growth medium (Dhingra and Sinclair, 1993) amended with different doses of fungicides to evaluate the resistance or sensitivity of the test fungi to a particular fungicide.

Growth medium was prepared in 250 conical flasks and each flask contains 100 ml of media. After sterilization by autoclaving at 121 °C and 103 kPa, media was cooled down at room temperature and appropriate amount of fungicide was added to the desired concentration. All procedures were carried out in the laminar air flow chamber to avoid the microbial contamination.

Aspergillus strains were revived from MEA slants preservation by growth on fresh media Petri plates for one week at 25 ºC. Inoculum was prepared by harvesting the spores in saline Tween 80 (0.9% (w/v) NaCl, 0.1% (v/v) Tween 80). The spore suspensions were kept refrigerated until further use in growth assays. An aliquot of 5 µL of the spore inoculum was used for inoculation. Each strain of *Aspergillus* was inoculated in the replicate of four and inoculum of five species of *Aspergillus* was given on the same Petri plate. Each treatment of fungicide was made in triplicates to avoid any confusion in studying growth responses. The Petri plates were incubated at 25 ºC ±2 for 3 days. Control treatments were prepared without addition of fungicides in the growth medium.

Growth response of test fungi to each fungicide was scored as:

(−): No growth.

(+): Cells growing normal similar to the non-treated strain

(+/−): Minimal (intermediate or poor) growth

Number of increasing + actually denote to better growth. Photographs of all Petri plates were taken for data presentation.

RESULTS

Current study was conducted to assess the effect of six fungicides viz. Bloom, Hiten, Epic, Wisdom, Benedict and Tebuconazole on growth of five different species of *Aspergillus* viz. *A. nidulans*, *A. niger*, *A. terreus*, *A. fumigatus* and *A. flavus*. A range of recommended dose of each fungicide was selected to study growth inhibition until MIC achieved.

The effect of fungicides on five different *Aspergillus* species was investigated by evaluating and comparing their phenotypic response to individual fungicide. Hiten and Epic appeared most toxic fungicides for all strains (Figure 1 and 2). All *Aspergillus* spp. except *A. fumigatus* exhibited similar growth inhibition response to both Hiten and Epic. Complete cessation of *A. fumigatus* was observed on 0.04R of Hiten while rest of the fungi failed to grow at 0.05R (Table 2 and 3). The growth of rest of the fungal species was completely inhibited by 0.05R of the recommended Hiten and Epic that showed the high efficacy of these fungicides against the *Aspergillus* species.

Table 1. List of fungicides used in the current study.

Sr. No	Brand	Active Ingredient	Chemical family	Dose/(100 L water)
1	Bloom 25 EC	Myclobutanil	Triazoles	40 mL
2	Hiten 50 SC	Fentin Hydroxide	Triphenyltin compounds	250 mL
3	Epic 12.5 SC	Epoxiconazole	Triazoles	160 mL
4	Wisdom 80 WDG	Fosetly-aluminium	Ethyl phosphonates	250 g
5	Benedict 50 EC	Iprobenfos	Organophosphate Ester	200 mL
6	Tebuconazole 6% ME	Tebuconazole	Triazoles	750 mL
7	Primacy 25 SC	Azoxystrobin	Azoxystrobin	180 mL

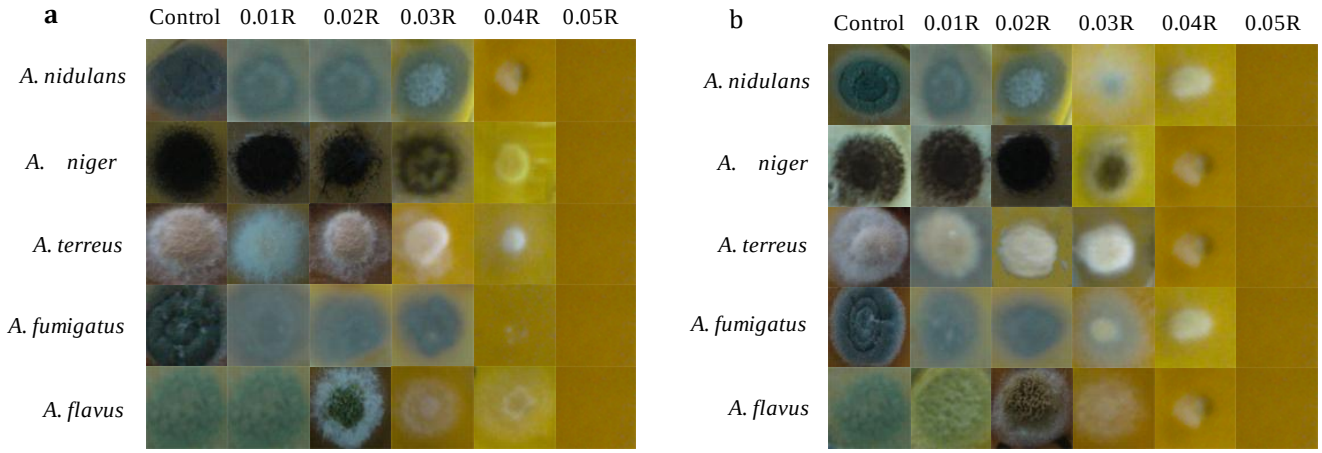

Figure 1 & 2. Fungicidal effect of a) Hiten and b) Epic on growth of *Aspergillus* spp.

Table 2. Growth responses of *Aspergillus* strains to the presence of Hiten.

Aspergillus strain	Dose of Hiten					
	Control	0.01R	0.02R	0.03R	0.04R	0.05R
A. nidulans	+++	+++	+++	++	+	–
A. niger	+++	+++	+++	++	+	–
A. terreus	+++	+++	++	++	+	–
A. fumigatus	+++	+++	++	+	–	–
A. flavus	+++	+++	++	+	+	–

Table 3. Growth responses of *Aspergillus* strains to the presence of Epic.

Aspergillus strain	Dose of Epic					
	Control	0.01R	0.02R	0.03R	0.04R	0.05R
A. nidulans	+++	+++	+++	+	+	–
A. niger	+++	+++	+++	+	+	–
A. terreus	+++	+++	++	++	+	–
A. fumigatus	+++	+++	++	++	–	–
A. flavus	+++	+++	++	+	+	–

The Fungal strains were considerably sensitive to Bloom but lesser than Hiten and Epic as they could not grow in the presence of 0.25R of recommended Bloom (Figure 3; Table 4)). Results of this study suggested that only one fourth of the recommended Bloom is enough to control the test soil fungi. Growth of the *Aspergillus* spp. was examined in the presence of Tebuconazole concentrations ranging from 0.25R to recommended dose or until complete growth inhibition was achieved. With the increasing concentration of fungicide from 0–100% of recommended dose, a gradual reduction in growth was observed. Complete growth arrest of the *A, niger, A. terreus, A. fumigatus* and *A. flavus* was recorded at 0.75R of Tebuconazole and that of the *A. nidulans* at recommended concentration of the same fungicide (Figure 4; Table 5).

Table 4. Growth responses of *Aspergillus* strains to the presence of Bloom.

Aspergillus strain	Dose of Epic					
	Control	0.01R	0.02R	0.03R	0.04R	0.05R
A. nidulans	+++	+++	+++	++	+	–
A. niger	+++	+++	++	++	+	–
A. terreus	+++	+++	++	+	+/–	–
A. fumigatus	+++	+++	++	++	+	–
A. flavus	+++	+++	++	+	+	–

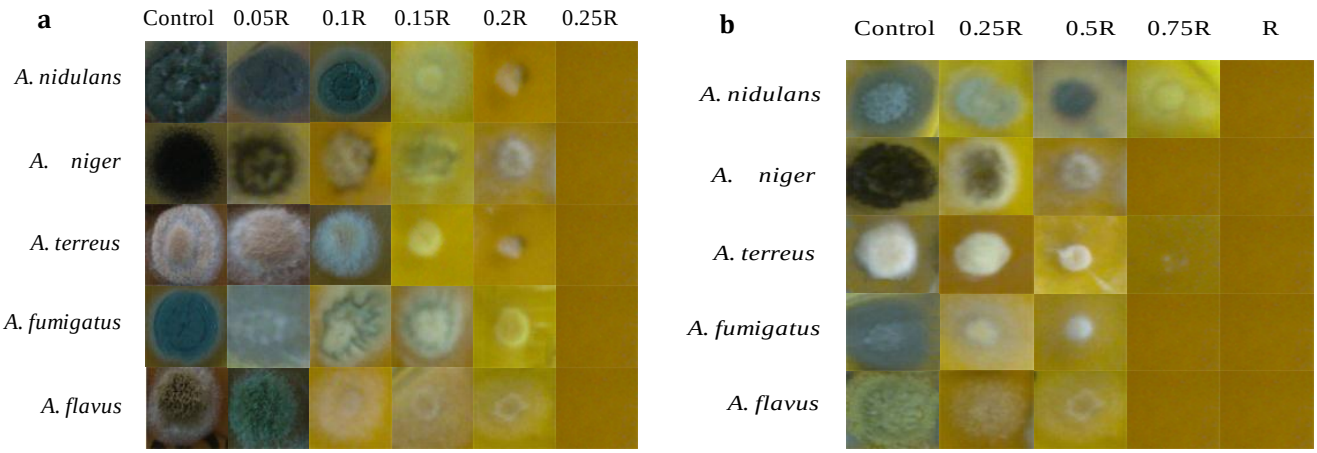

Figure 3 & 4. Fungicidal effect of a) Bloom and b) Tebuconazole on growth of *Aspergillus* spp.

Table 5. Growth responses of *Aspergillus* strains to the presence of Tebuconazole.

Aspergillus strain	Dose of Tebuconazole					
	Control	0.01R	0.02R	0.03R	0.04R	0.05R
A. nidulans	+++	++	++	++	−	+++
A. niger	+++	++	++	−	−	+++
A. terreus	+++	++	+	+/−	−	+++
A. fumigatus	+++	++	+	−	−	+++
A. flavus	+++	++	+	−	−	+++

Growth tests on revealed that *A. nidulans* and *A. terreus* were sensitive to 0.75R Benedict and Benedict above (Table 6). However *A. niger, A. fumigatus* and *A. flavus* when showed increased resistance to Benedict and growth for these strains was completely prohibited at the suggested dose of the fungicide (Figure 5). Inhibition of fungal growth by Wisdom was examined providing from one forth to full dose (250 g 100 L^{-1} water) of Wisdom (Figure 6). Lack of growth inhibition even up to suggested concentration of Wisdom was recorded for all tested strains except *A. terreus* (Table 7). The results showed unambiguously that Wisdom was not an efficient fungicide to control the fungi tested. *Aspergillus* strains were found to be resistant to recommended concentration of Primacy (Figure 7, Table 8).

Table 6. Growth responses of *Aspergillus* strains to the presence of Benedict.

Aspergillus strain	Dose of Benedict					
	Control	0.01R	0.02R	0.03R	0.04R	0.05R
A. nidulans	+++	++	+	−	−	+++
A. niger	+++	+++	+	+	−	+++
A. terreus	+++	++	+	−	−	+++
A. fumigatus	+++	++	+	+	−	+++
A. flavus	+++	+++	+	_	−	+++

Table 7. Growth responses of *Aspergillus* species to the presence of Wisdom.

Aspergillus strain	Dose of Wisdom					
	Control	0.01R	0.02R	0.03R	0.04R	0.05R
A. nidulans	+++	++	++	+	+	+++
A. niger	+++	+++	++	+	+	+++
A. terreus	+++	+++	++	+	+/−	+++
A. fumigatus	+++	++	++	+	+	+++
A. flavus	+++	++	+	+	+	+++

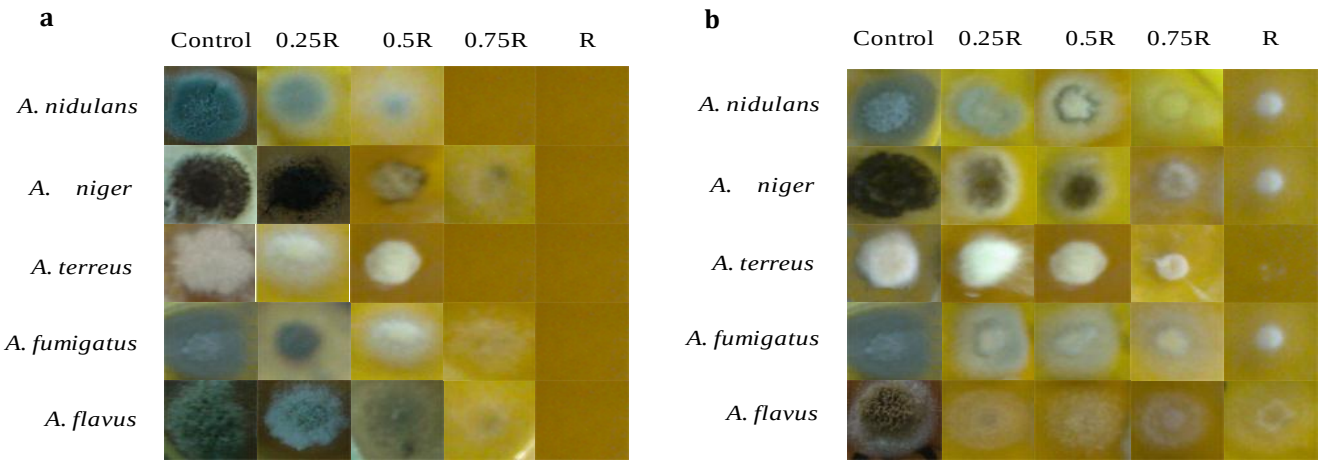

Figure 3 & 4. Fungicidal effect of a) Benedict and b) Wisdom on growth of *Aspergillus* spp.

Table 8. Growth responses of *Aspergillus* strains to the presence of Primacy.

Aspergillus strain	Dose of Primacy				
	Control	0.25R	0.5R	0.75R	R
A. nidulans	+++	++	++	++	+
A. niger	+++	+++	+++	++	++
A. terreus	+++	+++	++	++	+
A. fumigatus	+++	+++	+++	++	+
A. flavus	+++	+++	++	+	+

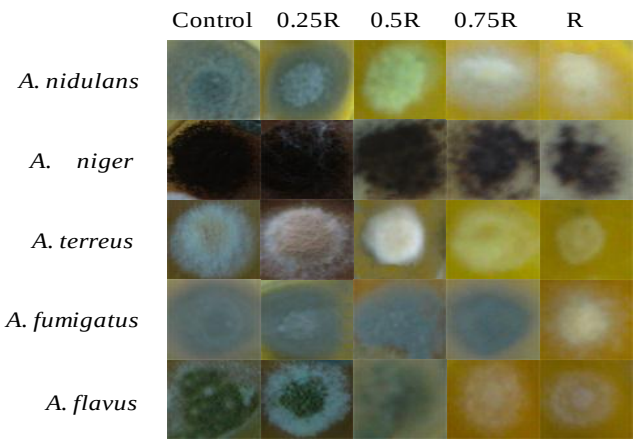

Figure 7. Fungicidal effect of Primacy on growth of *Aspergillus* spp.

DISCUSSION

The present study was designed to delve deeper into the crucial environmental and agriculturally important practice of using pesticides. A modest level of productivity and exploration has been achieved on several aspects of extensive use of pesticides and their effect on soil microorganisms when seven fungicides viz. Wisdom, Tebuconazole, Bloom, Benedict, Epic, Primacy and Hiten were tested against five different species of *Aspergillus* namely *A. nidulans*, *A. niger*, *A. terreus*, *A. fumigatus* and *A. flavus*. The results of current study present a comprehensive overview of the negative effects of fungicides on growth of commonly found soil fungi. Wisdom Benedict and Primacy inhibited or reduced to a great extend the growth of strains when used according to their recommended dose. However Tebuconazole controlled fungal growth at 0.75R and Bloom at 0.25R. Most deleterious results were observed in case of Hiten and Epic fungicides where minimum fungal growth inhibition concentration was just 0.05R of the suggested dose.

Fungicides are mostly studied for their positive role in agriculture by discussing their role to control fungal pathogens (Saladin *et al.,* 2003). However awareness regarding the detrimental impacts of chemical fungicides increased as they are reported to enhance plant defenses by producing phytoalexin synthesis, stimulate synthesis of phenolic compounds, cell wall lignification (Awmack and Leather, 2002), CO_2 assimilation, photosynthetic efficiency that strongly inhibits biomass production and growth rates (Untiedt and Blanke, 2004).

During a study using fungicides benomyl, captan and chlorothalonil, it was observed that soil microbial diversity, activity and nitrogen dynamics is badly affected by applying these fungicides (Chen *et al.,* 2001). The inhibitory effects of some pesticides on non-target organisms have already been confirmed (Rodriguez and

Curl, 1980). Incidences of *F. oxysporum, A. flavus* and some other pathogens were decreased after application of some chemicals to the field soil and growth media (Das *et al*., 2003; Tubajika and Damann, 2002).

Presently, diverse activity of different fungicides could be attributed to various active ingredients in them. So far, Fosetyl aluminium is the organic phosphate compound and active ingredient of Wisdom. The antifungal activity of Fosetyl aluminium has been previously reported against number of biotrophs and nectrotrophs. The antifungal activity appears to be based on its phosphate compound (Fenn and Coffey, 1984) that probably act systematically by inhibiting spore germination and by blocking mycelial growth and spore production. Tebuconazole, Bloom, and Epic belong to Triazole group. Trizole has been reported to interfere with sterol biosynthesis that results in insufficient availability of ergosterol in fungi. Insufficiency of ergosterol in fungal membranes likely to disturb membrane functions including activity of membrane-bound enzymes and proper synthesis of new hyphal cell walls. Consequently, severe effects of Triazol on the development of hyphal and haustorial structures possibly lead to reduced growth of fungi (Han *et al*., 2006). Even though different Triazole fungicides have a similar mechanism of action, they may show marked differences in their activity against different fungal pathogens. The considerable antifungal activity of Benedict is due to Iprobenfos (active ingredient), that was reported to act against fungi by inhibiting the synthesis of phopholipids (Roberts and Hutson, 1999). Fentin hydroxide in Hiten is known to inhibit oxidative phophorylation and ATP synthesis in fungi (FRAC, 2006).

The inherent toxicity of fungicides against the fungi probably catalyzes the production of highly reactive hydroxyl radicals, which can subsequently damage lipids, proteins, DNA and other biomolecules along with inhibition in sterol synthesis. Alterations in the conformational structure of nucleic acids and proteins likely to interfere in oxidative phosphorylation and osmotic balance along with inhibition in normal functioning of enzymes. Therefore, exposure of the cells to elevated levels of hydrogen peroxide resulted in increase in protein and proline contents that may facilitate in metabolization of reactive oxygen species in peroxysomes (Ercal *et al*., 2001).

CONCLUSION

It is concluded from the present study that recommended doses of fungicides have detrimental effects on soil fungal population and their activities and ultimately result in loss of soil fertility.

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