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Journal of Postharvest Technology
2024, 12 (3): 87-94
https://doi.org/10.48165/jpht.2024.12.3.09
www.jpht.in

RESEARCH ARTICLE

Efficacy of fungicides on *Aspergillus flavus* mycelium growth and aflatoxin B_1 production in SMKY liquid medium

Diksha Sultania*, Gajendra Prasad

Mycotoxin and Pathology Laboratory, University Department of Botany, Lalit Narayan Mithila University, Darbhanga - 846 008, Bihar, India

ARTICLE INFO

Received : 28.04.2024
Accepted : 15.07.2024

ABSTRACT

Aspergillus flavus contamination and aflatoxin production in crops is a serious problem, with no promising control method available. Judicious use of fungicides can help in its limitation. This study used commercially available fungicides, REVIVE-750 (Mancozeb 63% + Carbendazim 12%), BUMPER (Propiconazole 25%) and AMISTAR TOP (Azoxystrobin 18.2% + Difenoconazole 11.4%) to know its efficacy in controlling Aspergillus flavus mycelium growth and aflatoxin B1 production in vitro conditions. Results suggest fungicide Bumper to be most effective followed by REVIVE-750 and least effective results were obtained with fungicide AMISTAR TOP for both mycelium growth and aflatoxin B1 production.

Keywords: Aflatoxin B1, *Aspergillus flavus*, Fungicides, In-Vitro

Citation: Sultania, D. and Prasad, G. 2024. Efficacy of fungicides on *Aspergillus flavus* mycelium growth and aflatoxin B1 production in SMKY liquid medium. *Journal of Postharvest Technology*, **12** (3): 87-94.

INTRODUCTION

Microorganisms are ubiquitous in nature and it interact with other living organisms for its survival, in the course of this it may provide benefits to the host or may cause harmful effects. Most of the plant disease which are economically concerning are caused by fungi, because it may produce mycotoxin in the plant products along with disease and spoilage. “Mycotoxins are toxic secondary metabolites of fungi belonging, essentially, to the *Aspergillus*, *Penicillium* and *Fusarium* genera” FAO (2015). Although the history of mycotoxin dates back thousands of years back its identification and understanding are very resent. Research on mycotoxins began in the year 1960s with the outbreak of ‘Turkey X’ disease in poultry farms of England, which led to the death of thousands of turkey poults due to consumption of mouldy groundnut cake. There after hundreds of mycotoxins have been isolated and named, but not all of it are equally toxic to humans and animals. Mycotoxins that are of more concern are aflatoxin, fumonisin, ochratoxins, trichothecenes, and zearalenones.

* *For correspondence*: D. Sultania (Email: diksha4112@gmail.com)

ISSN: 2348-4330

Among this aflatoxin is most toxic, as it causes mutagenic, teratogenic, immunosuppressive and carcinogenic effects both in humans and animals. Aflatoxin is mainly produced by fungi *Aspergillus flavus* and *Aspergillus parasiticus*. AFB_1, AFB_2, AFG_1, and AFG_2 are main ones among eighteen plus known aflatoxins (Okoth et al., 2018) and has a clear-cut aflatoxins potency series (Figure 1) is evident in majority of cases as AFB_1> AFG_1>AFB_2>AFG_2 (Wogan et al.,1970). Aflatoxin B_1 has been regarded as group 1 human carcinogen by IARC (International Agency for Research on Cancer) as it causes cancer in human.

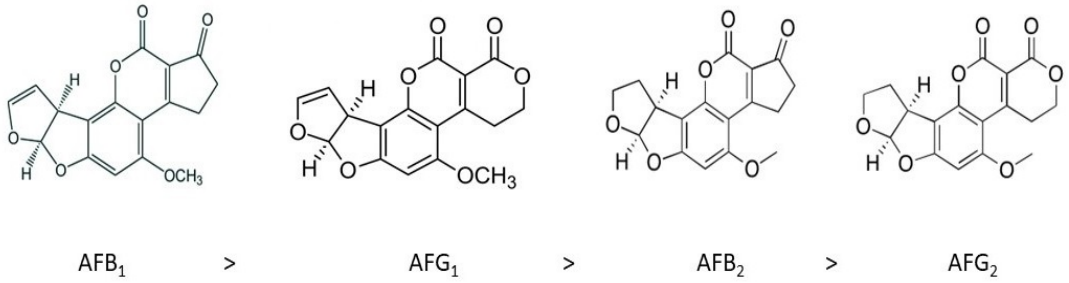

Figure 1: Aflatoxin toxicity series

Main problem with fungal contamination and mycotoxin production is that once it starts, becomes uncontrollable and thus it is better to control it at the start site. One way to achieve it is by use of chemical fungicides. Fungicides has long been used for safeguarding agricultural products form the fungal attack and is base of modern-day agriculture. But the application of fungicides must be judicious as not all fungicides may equally be beneficial in controlling the growth of *Aspergillus flavus* and aflatoxin production. Thus, this study attempts to understand the effect of three fungicides viz., REVIVE-750, BUMPER and AMISTAR TOP (Table 1) on *Aspergillus flavus* mycelium growth and aflatoxin B_1 production in vitro conditions.

Table 1: Details of fungicides used in the research work

TRADE NAME	FORMULATION	GROUP	ACTION	FORM	RECOMMENDED DOSE	COMPANY
REVIVE-750	Mancozeb 63%+ Carbendazim 12%	Dithiocarbamate +Benzimidazole	Contact +Systemic	Wettable Powder	500gm/acre	Tropical Agro India Pvt Ltd
BUMPER	Propiconazole 25%	Triazole	Systemic	Emulsifiable concentrate	200ml/acre	ADAMA India Pvt Ltd
AMISTAR TOP	Azoxystrobin 18.2% + Difenoconazole 11.4%	Strobilurin +Triazole	Systemic	Suspension Concentrate	200ml/acre	Syngenta India Ltd

MATERIALS AND METHODS

Toxigenic strain of *Aspergillus flavus* isolated from maize sample and stored in the in Mycotoxin and Pathology Laboratory, Department of Botany, Lalit Narayan Mithila University, Darbhanga, maintained on PDA (Potato Dextrose Agar) slants during the previous work by Sultania et al. (2022), strain producing aflatoxin of the range 10ppm was sub cultured on fresh PDA plates for 7 days to maintain its toxigenic potential. PDA plates were scraped to release spores and 10ml distilled water added to obtain spore suspension, filtered and subsequently diluted to obtain spore suspension of the order 10^6/ml.

0.5ml prepared spore suspension was added to 25ml SMKY (Sucrose, 200 g; MgSO4. 7H2O, 0.5 g; KNO, 3g; Yeast extract, 7 g in 1L distilled water (Diener and Davis, 1966)) liquid medium which were prepared and sterilized in conical flask of 50/100 ml capacity, modified with desired fungicide concentrations and incubated for 10days.

1µg/ml, 5µg/ml,10µg/ml fungicide concentration was used for all the tested fungicides, viz., REVIVE -750 (Mancozeb63%+ Carbendazim 12%), AMISTAR TOP (Azoxystrobin 18.2%+ Difenoconazole 11.4%), and BUMPER (Propiconazole 25%). All the experiment was performed in triplicate.

Mycelium mats were separated by filtration and allowed to dry at 60^0C until constant weight was obtained, weighed using analytical balance and recorded. Liquid portion was filtered using Whatman filter paper containing Na_2SO_4 and extracted with chloroform. Chloroform portion was separated using separating funnel and evaporated on water bath up to dryness in small vials and stored for qualitative and quantitative estimation of aflatoxin.

Quantitative and qualitative estimation of aflatoxin was performed by the method as mentioned below:

Qualitative estimation- 1 ml chloroform was added in the stored vials and spotted on TLC plate along with standard reference material and develop in TIM (Toluene: Isoamyl alcohol: Methanol :: 90:32:2) solution (Reddy et al., 1970). Allowed to dry and viewed under the UV chamber. For chemical conformation trifluoracetic acid or 25%sulphuric acid sprayed on dry TLC plate.

Quantitative estimation- spots developed on TLC plate were eluted and mixed in 5ml methanol and optical density (OD) was taken using spectrophotometer. Concentration of aflatoxin was calculated by the formula: -

$$\textbf{Concentration of aflatoxin (µg/ml)} = \frac{\mathbf{D} \times \mathbf{M} \times \mathbf{10^3}}{\mathbf{E} \times \mathbf{L}}$$

Where D= Optical Density

M= Molecular weight of aflatoxin

E= Molecular extinction coefficient

L= Path length

RESULTS AND DISCUSSION

All fungicides at all concentrations, exhibited significant inhibition of both mycelium growth and aflatoxin B_1 production at significance levels of 0.05 and 0.01 in compression to control group. Significance was determined using Dunnett's formula, which compares all treatments against the control group.

Complete inhibition of mycelium growth was observed at 10µg/ml concentrations of REVIVE-750 and BUMPER fungicides. REVIVE-750 showed 81.98% and 40.87% inhibition at 5µg/ml and 1µg/ml, respectively, while BUMPER showed 97.89% and 87.01% inhibition at 5µg/ml and 1µg/ml. Amistar Top exhibited only 23.92% inhibition of mycelium growth at 1µg/ml, 44% at 55µg/ml and 55.96% at 10µg/ml.

Complete inhibition of aflatoxin B_1 production was achieved at 10µg/ml for Revive-750 and at 5µg/ml and 10µg/ml for BUMPER fungicides. However, the highest dose of AMISTAR TOP showed only 35.33% inhibition in AFB_1 production (Table 2, Figure 2)

Table 2 Effect of fungicides on *Aspergillus flavus* mycelium growth and aflatoxin B_1 production in SMKY liquid medium

FUNGICIDE	DOSE (ug/ml)	MYCELIUM WEIGHT (mg/25ml) X±SE	% INHIBITION	AFB_1 (ppm) X ±SE	% INHIBITION
Control	–	1194.67±60.68	–	9.93±0.34	–
REVIVE -750 (Mancozeb63%+ Carbendazim 12%)	**1**	706±23.64	40.87	8.51±0.25	14.25
	5	215.33±13.61	81.98	4.41±0.17	55.59
	10	0±0	100	0±0	100
BUMPER (Propiconazole 25%)	**1**	155±12.29	87.01	3.8±0.22	61.72
	5	25.33±6.51	97.89	0±0	100
	10	0±0	100	0±0	100
AMISTAR TOP (Azoxystrobin 18.2%+ Difenoconazole 11.4%)	**1**	907.67±17.79	23.92	9.33±0.4	5.98
	5	668.33±19.6	44	8.13±0.12	18.01
	10	526±27.62	55.96	6.42±0.15	35.33
LSD (α=0.05)			**2.95**		**4.09**
LSD(α=0.01)			**4.10**		**5.69**

REVIVE 750- is a contact + systemic fungicide, composed of carbendazim 12% and mancozeb 63% in form of wettable powder, it is effective in controlling tikka leaf, collar rot and dry rot disease in groundnut, blast on paddy, early blight, late blight, black scurf of potato, blister blight, grey blight, red rust, dieback, black rot on tea, downy mildew, powdery mildew & anthracnose on grape and mango , leaf spot, fruit rot and powdery mildew on Chili, Downey mildew & leaf blight on maize, fruit scab & powdery mildew on apple (TROPICAL AGRO)

Carbendazim, belonging to the benzimidazole group, functions as a systemic fungicide renowned for its broad-spectrum activity against various fungi, including ascomycetes and basidiomycetes, though it proves ineffective against oomycetes. Introduced in

1976, carbendazim acts on a single site within the target organism, disrupting the mitotic spindle by affecting β-tubulin, a crucial protein involved in cytoskeleton formation (Oliver and Hewitt, 2014).

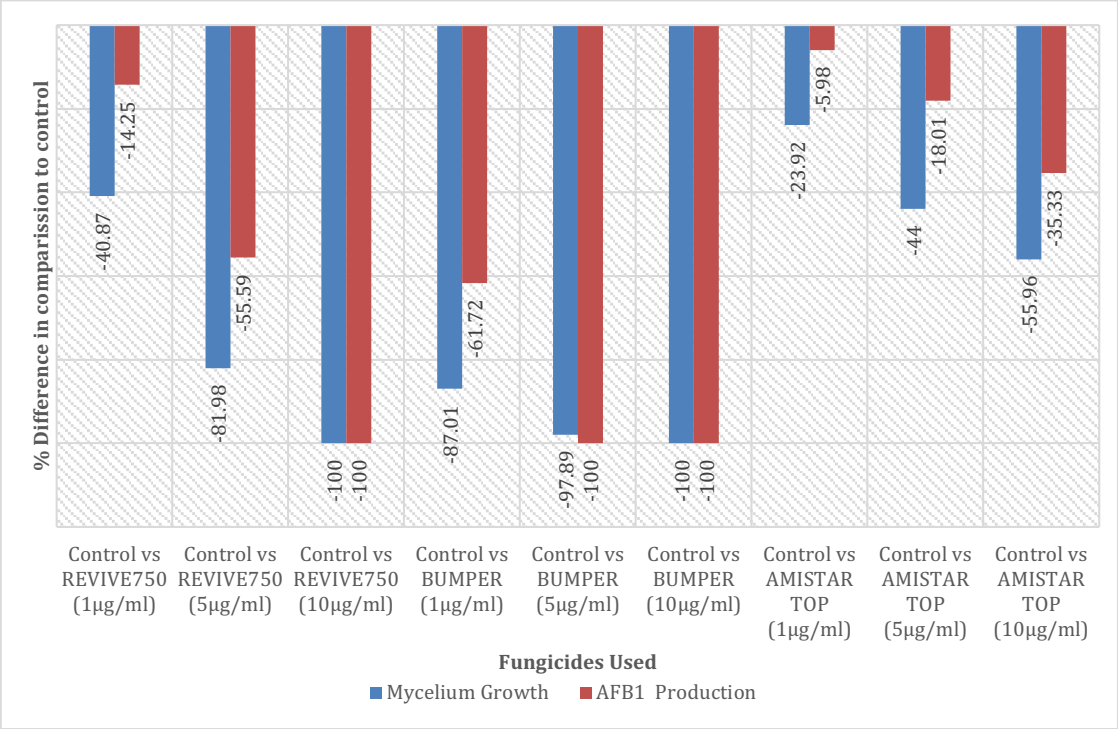

Figure 2: Effect of fungicides on Aspergillus flavus mycelium growth and aflatoxin B1 production

Mancozeb on the other hand, is an organic dithiocarbamate fungicide, operates as a contact fungicide. Comprised of maneb (Manganese ethylene bisdithiocarbamate) and zinc ion, it was introduced in 1961 and has since gained prominence due to its broad-spectrum and multi-site activity. This attribute has made it one of the most widely used chemical classes in fungicide formulations (Russell, 2005). Mancozeb's multi-site activity proves advantageous against resistance, leading to its incorporation into various fungicide combinations. Its mechanism involves reacting with and deactivating the sulfhydryl groups of amino acids and enzymes within fungal cells, disrupting lipid metabolism, respiration, and the production of adenosine triphosphate (Tomlin, 2003).

BUMPER- It is a systemic foliar fungicide containing propiconazole 25% Emulsifiable Concentrate, having both protective and curative action, translocate acropetally in the xylem. It has broad spectrum activity against a wide range of fungal diseases of wheat, barley rye oats, rice, maize, grass, turf, pecan, sugarcane, beets, stone fruits, peanuts and ornamentals. It is of low mammalian toxicity and does not affect honeybees, beneficial insects or wildlife (ADAMA, 2024).

Propiconazole belongs to the class of fungicides known as demethylation inhibitors (DMIs), also referred to as azole fungicides. DMIs work by inhibiting the biosynthesis of ergosterol, a crucial component of fungal cell membranes. Ergosterol is essential for maintaining the integrity and functionality of fungal cell membranes, similar to cholesterol in animal cells. By interfering with ergosterol biosynthesis, propiconazole disrupts the fungal cell membrane, leading to leakage of cell contents and ultimately fungal cell death. One study by Fraaije et al. (2007) investigated the mode of action of propiconazole and other triazole fungicides. They found that propiconazole inhibits the enzyme lanosterol 14α-demethylase, a key step in ergosterol biosynthesis

in fungi. This inhibition disrupts fungal cell membrane integrity, leading to cell death. Additionally, propiconazole exhibits the ability to hinder the production of crucial fungal enzymes and disrupt other metabolic pathways vital for fungal growth and reproduction. For instance, research conducted by Gao et al. (2018) revealed propiconazole's capacity to impede the activity of specific enzymes involved in mycotoxin biosynthesis in *Fusarium graminearum*, a significant plant pathogen. This multifaceted mechanism of action contributes to propiconazole's efficacy against a broad spectrum of fungal pathogens across various crops. Its effectiveness stems from its ability not only to target ergosterol biosynthesis but also to intervene in other essential metabolic pathways crucial for fungal survival and proliferation. This comprehensive approach enhances its effectiveness in managing fungal diseases in diverse crop systems.

AMISTAR TOP- It is a systemic fungicide made of Azoxystrobin 18.2% + Difenoconazole 11.4% and provide broad spectrum protection and long duration control against diseases like yellow rust, powdery mildew, late blight, sheath blight, downy mildew, leaf spots, grey mildews, red rot etc. in crops like Rice, Cotton, Sugarcane & Vegetables (Syngenta, 2024).

Azoxystrobin is a member of the strobilurin class of fungicides, operates by impeding fungal mitochondrial respiration. Specifically, it obstructs electron transfer at the Qo site of cytochrome b within the fungal respiratory chain. This interruption results in the buildup of harmful reactive oxygen species (ROS) within fungal cells, culminating in their demise. Azoxystrobin is renowned for its preventive, curative, and translaminar attributes, rendering it efficacious against a broad spectrum of fungal pathogens. The mechanism underlying azoxystrobin's function as a strobilurin fungicide has been extensively studied. For instance, research conducted by Heiser et al. (2000) delineated how azoxystrobin disrupts fungal mitochondrial respiration, elucidating the process by which it hampers electron transfer at the Qo site of cytochrome b. This disruption leads to the accumulation of reactive oxygen species, ultimately triggering fungal cell death.

Difenoconazole, categorized as a triazole fungicide and a member of the demethylation inhibitor (DMI) class, shares similarities with propiconazole in its mechanism of action. Like propiconazole, difenoconazole operates by inhibiting the synthesis of ergosterol, a critical component of fungal cell membranes, through the blockade of lanosterol 14α-demethylase enzyme activity. This disruption in ergosterol production compromises the integrity and functionality of fungal cell membranes, ultimately resulting in fungal cell death. Additionally, difenoconazole demonstrates systemic and translaminar movement within plants, affording protection to both treated foliage and emerging growth.

Aflatoxin is produced by *Aspergillus flavus* mainly in warm climatic conditions, optimally 25-30^{0}C (Schroeder and Hein, 1967; Northolt et al.,1977), thus the temperate regions which were too cool for fungal development and aflatoxin contamination may also face significant aflatoxin contamination in near future as a result of global warming, increasing the global area under aflatoxin contamination, posing serious issue to the food security. Drougt stress, high humidity, unpredictable rainfall additionally weakens the plant health, allowing easy fungal invasion. Thus, it is important to address the issue of aflatoxin contamination properly.

Some fungicides may not be suitable to control *Aspergillus flavus* growth and aflatoxin production as it may enhance the aflatoxin production due to stress as that reported by Badii and Moss (1988), all the three fungicides tridemorph, fenpropimorph and fenarimol tested in the study increased the production of aflatoxin even in cases where mycelium growth was checked suggesting that certain fungicides might enhance toxin production under stress.

The efficacy of certain fungicides like copper oxychloride 50%, triadimenol 31.2%, Sulphur 80%, trifloxystrobin 50% may be substrate, strain and temperature dependent but fungicides like tebuconazole 25% and mancozeb 80% are effective in all circumstances in controlling mycelium growth and aflatoxin production (Santos et al.,2011)

Tebuconazole 25% belongs to triazole and mancozeb 80% to dithiocarbamate similar to the fungicides REVIVE 750 containing 63% mancozeb (dithiocarbamate) and BUMPER containing 25% propiconazole (triazole) use in this study which are very effective in controlling mycelium growth and toxin production also fungicide AMISTAR TOP contains 11.4% triazole and is mildly effective in controlling growth and toxin production.

CONCLUSION

Thus, it can be stated that fungicides belonging to dithiocarbamate and triazole which are very effective in controlling *Aspergillus flavus* growth and aflatoxin production in vitro conditions irrespective of strain and environmental conditions, can be used in farm conditions to check its practical application before recommending to agricultural society.

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