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Antagonistic Potentials of Wheat Rhizosphere Fungi and Mode of Mycelial Interaction Behavior During Growth Inhibition of Aflatoxigenic *Aspergillus flavus* Isolates

Debalika Dalal[1] and Nandlal Mandal*[2]

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A B S T R A C T

Thirty fungi of the different taxonomic groups were isolated from the wheat rhizosphere in the present study. They were screened for their potential to inhibit the growth of aflatoxigenic *A. flavus* (BAF-4) isolates. Only eight fungi viz., WRF-4, WRF-5, WRF-7, WRF-13, WRF-20, WRF-23, WRF-27 and WRF-30 showed promising radial growth (above 50%) inhibition of toxigenic *A. flavus*. The maximum (72.35%) inhibition of BAF-4 was recorded by WRF-20 followed by WRF-27 (67.80%), WRF-5 (60.47%), WRF-23 (59.90%), WRF-4 (58.80%), WRF-13 (57.64%), WRF-7 (55.33%) and minimum (52.48%) was recorded by WRF-30. The mode of antagonistic interaction behavior was determined based on radial growth inhibition of antagonist fungi and test fungi on dual culture Petri plates. In the present study, most fungi, i.e., WRF-4, 7, 13, 20, 23, and WRF-30, showed B-type interaction, whereas WRF-5 showed C-type interaction. The antagonist fungi, i.e., WRF-27, showed an E-type of interaction and is considered the most potent antagonist. In the E type of interaction, only antagonists produce some antimicrobial compounds responsible for growth inhibition of only test fungi; hence, inhibition at a distance was recorded.

***Key words*:** Wheat rhizosphere fungi, *Aspergillus flavus*, Biological control, Radial growth inhibition, Antagonistic interaction

Aflatoxins are well-characterized mycotoxins produced by toxigenic *Aspergillus flavus* isolates on certain foods and feed on conducive environmental conditions [1]. Due to various health hazard effects of aflatoxins, viz., mutagenic, carcinogenic and teratogenicity, scientists all over the globe applied their ideas to overcome the growth of *A. flavus* and aflatoxin production [2]. Out of four well-known aflatoxin-producing *Aspergilli* viz., *Aspergillus flavus*, *A. parasiticus*, *A. niger and A. nomious* of which *A. flavus* is a more ubiquitous and potent aflatoxin producer [3-4]. In standing crops, the primary source of aflatoxin contamination is mainly due to toxigenic *A. flavus* inhabiting rhizosphere soil. Its population in the rhizosphere is directly proportional to the chance of contamination in pre-harvest stages in any crop [5]. Migration of toxigenic *A. flavus* isolates from the rhizosphere to aerial parts through different sources such as insects, mites, and airflow and where they colonize and elaborate aflatoxin. Minimizing the toxigenic *Aspergillus flavus* isolates in the rhizosphere by various physical, chemical and biological means will automatically reduce the chance of aflatoxin elaboration in standing crops [6-7]. However, a diverse assemblage of *A. flavus* strains and their aflatoxin-producing genes and regulatory sites makes it more challenging to reduce contamination with aflatoxin in standing crops.

Several earlier approaches, such as the application of fungicides and chemicals, are suitable for only decontamination but unable to detoxification of aflatoxin. These methods are also not economical and eco-friendly and increase soil and environmental pollution, resulting in an imbalance of microbial diversity in soil [6-7]. Several good cultural practices, viz., proper watering, soil solarization, and balanced fertilizer application may also reduce plant stress and injuries by pests resulting in fewer infections with *A. flavus* strains. However, these cultural practices are not an easy task and are insufficient for reducing infections of *A. flavus* and aflatoxin formation at standing crops. Similarly, producing a cultivar of resistant genotypes to *A. flavus* infections is too complicated and impossible for every crop. Recently the application of bio-control methods is one of the important techniques where microbial antagonists are applied for growth inhibition of targeted pathogen and achieve the goal of decontamination and detoxification of aflatoxin in several crops [8-9]. Keeping the above facts in mind, the present investigation aimed to isolate wheat rhizosphere fungi from Birbhum West Bengal and

* **Nandlal Mandal**
✉ nandlal.mandal@visva-bharati.ac.in

[1-2] Department of Botany (UGC- DRS & DST- FIST Sponsored), Visva- Bharati (A Central University), Santiniketan - 731 235, Birbhum, West Bengal, India

determine their antagonistic potentials and mode inhibition interaction against toxigenic *A. flavus* isolates.

MATERIALS AND METHODS

Isolation and identification of wheat rhizosphere fungi

Wheat cultivated areas of Birbhum district of West Bengal (India), viz., Bolpur, Rampurhat and Suri, were selected. The collected rhizosphere soil samples were screened for isolation of rhizosphere fungi by following the standard serial dilution technique of Waksman [10]. One ml of 10^{-4} dilution of the original soil sample was spread aseptically on the surface of previously prepared PDA Petri plates. The plates were then incubated at 28 ± 2°C for 4-7 days and visually different fungi were isolated. The pure culture was made by sub-culturing on Potato Dextrose Agar Slants and identified with the help of a manual of soil fungi [11]. The pure culture of each fungus was stored in the refrigerator at 4^0C to further study its antagonistic potential against toxigenic *A. flavus* (BAF-4) isolates.

Screening of antagonistic activity of wheat rhizosphere fungi against toxigenic Aspergillus flavus

Previously isolated *Aspergillus flavus* (BAF-4) isolates were taken from the same laboratory and after their identification by molecular methods, confirmed as *Aspergillus flavus* [12]. This isolate showed highly aflatoxigenic after screening on SMKY medium [13]. The dual culture technique was followed to determine the antagonistic potentials of different fungal isolates and their behavior against the BAF-4 [14]. For this, a loop of each fungal antagonist and the *A. flavus* were taken with the help of a sterilized inoculating needle from the edge of 3 day's old culture and then placed 2 cm apart on solidified PDA medium. Petri plates were incubated 28 ± 2^0C for 3-5 days in a BOD incubator and inhibition of % radial mycelial growth of *Aspergillus flavus* was recorded against the control set.

Interaction behavior of fungal antagonist with A. flavus

On the same dual culture PDA Petri plates, inhibition of % radial mycelial growth of each antagonist was measured by the same formula. Based on inhibition of *A. flavus and* antagonists either alone or both, through contact or distance, the mode of interaction was determined from A- E type [15]. In the A type of interaction, the mycelium of antagonists and test fungi were intermingling. In the B-type, both the antagonists and test fungi are inhibited when they contact each other. In the C-type again, both the antagonists and test fungi are inhibited but at a distance. In the D and the E-type, only test fungi are inhibited on contact and at a distance, respectively.

RESULTS AND DISCUSSION

Isolation of Rhizosphere fungi

Altogether, thirty wheat rhizosphere fungi (WRF) were isolated (WRF-1 to WRF-30) from the selected rhizosphere soil sample. Several morphological characters such as colony colour, margin, elevation, reverse plate colour and microscopic characters such as hyphae, conidiophore, conidia shape, size and attachment are considered for preliminary identification of all isolated fungi up to the generic level. Out of all fungal isolates, ten different species of *Aspergillus genera*, seven different species of *Penicillium*, two species of *Talaromyces, Fusarium,* and *Alterneria,* whereas one species of *Rhizopus, Mucor, Helminthosporium,Trichoderma, Verticill ium, Curvularia* and one unidentified fungal genera were recorded (Table 1). Abdel-Hafez, while working on rhizosphere mycoflora of wheat, *Aspergillus* and *Penicillium* were reported as significant genera of which *Aspergillus niger, A. clavatus, A. flavus, A. terreus, A. carneus, Penicillium citrinum, P. notatum, P. chrysogenum* and *Fusarium solani* was reported as dominant fungal species [16].

Table 1 Isolation and screening of antagonistic activity of wheat rhizosphere fungi against toxigenic *A. flavus* (BAF-4) isolate

Fungal isolates	Identified genera	Antagonistic activity	Fungal isolates	Identified genera	Antagonistic activity
WRF-1	*Penicillium sp. 1*	-	WRF-16	*Curvularia sp.*	-
WRF-2	*Penicillium sp. 2*	-	WRF-17	*Mucor sp.*	-
WRF-3	*Penicillium sp. 3*	-	WRF-18	*Rhizopus sp.*	-
WRF-4	*Aspergillus sp. 1*	+	WRF-19	*Aspergillus sp. 6*	-
WRF-5	*Aspergillus sp. 2*	+	WRF-20	*Trichoderma sp.*	+
WRF-6	*Aspergillus sp. 3*	-	WRF-21	*Alterneria sp.*	-
WRF-7	*Verticillium sp.*	+	WRF-22	*Aspergillus sp. 7*	-
WRF-8	*Aspergillus sp. 4*	-	WRF-23	*Aspergillus sp. 8*	+
WRF-9	*Aspergillus sp. 5*	-	WRF-24	*Alterneria sp.*	-
WRF-10	*Fusarium sp. 1*	-	WRF-25	*Helmenthosporium sp.*	-
WRF-11	*Penicillium sp. 4*	-	WRF-26	*Aspergillus sp. 9*	-
WRF-12	*Fusarium sp. 2*	-	WRF-27	*Talaromyces sp. 1*	+
WRF-13	*Penicillium sp. 5*	+	WRF-28	*Penicillium sp. 7*	-
WRF-14	*Penicillium sp. 6*	-	WRF-29	*Aspergillus sp. 10*	-
WRF-15	Unidentified	-	WRF-30	*Talaromyces sp. 2*	+

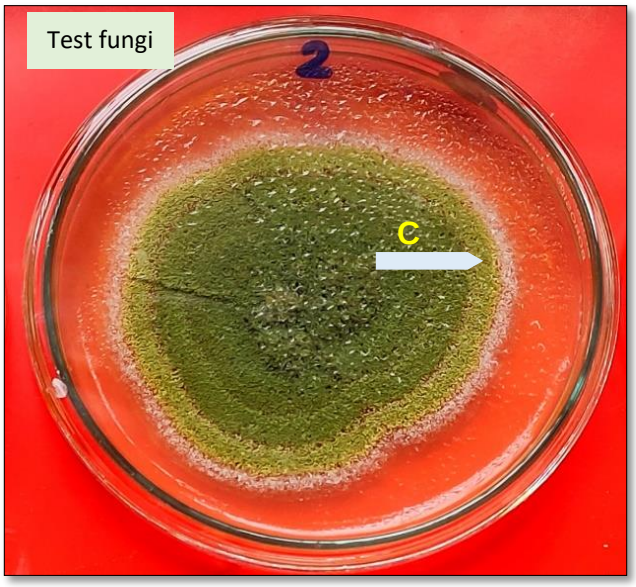
Inhibition of radial growth of BAF-4 by antagonist fungi

In dual co-culture PDA plate, while screening of antagonistic activity of all rhizosphere fungi against BAF-4, only eight fungi viz., WRF-4, WRF-5, WRF-7, WRF-13, WRF-20, WRF-23, WRF-27 and WRF-30 showed promising radial growth inhibition (above 50%) of BAF-4 isolates. The maximum (72.35%) radial growth inhibition of *Aspergillus flavus* was recorded by WRF-20 followed by WRF-27 (67.80%), WRF-5 (60.47%), WRF-23 (59.90%), WRF-4 (58.80%), WRF-13 (57.64%), WRF-7 (55.33%) and minimum (52.48%) was recorded by WRF-30 (Table 2). While working on fungi isolated from maize kernels and after screening their potentials against % inhibition of radial growth of *A. flavus* by the same dual culture methods, Choudhary obtained similar results [17]. He found that maximum (63%) inhibition was recorded by *A. niger* followed by *Cladosporium herbarum* (33.6%), *F. oxysporum* (30%) and minimum (26.5%) was recorded by *A. candidus*. He also correlated percent

inhibition of radial growth of *Aspergillus flavus* by *F. monilforme* (59.8%), *Trichoderma viride* (75.5%) and *R. nigricans* (42%) with percent reduction in aflatoxin production 73.2%, 80.9% and 45.4% respectively.

Table 2 Efficacy of radial growth inhibition of *A. flavus* (BAF-4) isolates by rhizosphere fungi and their mode of interaction behaviour

Fungal isolates	Radial growth of *A. flavus* in cm		Percent inhibition of *A. flavus* in dual culture C-T/C × 100	Radial growth of antagonist fungi in cm		Percent inhibition of antagonist C-T/C × 100	Type of interaction
	In control (C)	In dual culture (T)		In control (C)	In dual culture (T)		
WRF-4	2.125	0.875	58.82	1.53	1.35	11.76	B
WRF-5	2.201	0.875	60.47	0.85	0.8	5.88	C
WRF-7	2.128	0.95	55.33	1.112	0.837	24.73	B
WRF-13	2.125	0.90	57.64	1.425	1.08	24.21	B
WRF-20	2.261	0.625	72.35	3.25	1.97	39.38	B
WRF-23	2.120	0.85	59.90	2.0	1.1	45.0	B
WRF-27	2.252	0.725	67.80	0.65	0.65	0	E
WRF-30	2.157	1.025	52.48	0.95	0.65	31.0	B



Formula for determination of % radial growth inhibition

$$I = \frac{C - T \times 100}{C}$$

Where I = % radial growth inhibition,
C= radial growth of test fungi in control,
T= radial growth in Treatment

Type of interaction behaviour
Type A- Mutual intermingling of two organisms
Type B- Mutual inhibition on contact
Type C- Mutual inhibition at a distance
Type D- Inhibition on contact
Type E- Inhibition at a distance

Fig 1 Determination of radial growth inhibition and type of interaction behaviour

Mode of interaction behavior of antagonist fungi

For studying the mode of mycelial interaction between antagonists and test fungi, radial growth inhibition of antagonists and *Aspergillus flavus* were considered (Fig 1). The radial growth inhibition of individual antagonists by BAF-4 was recorded maximum (45%) in WRF-23, followed by WRF-20 (39.38%), WRF-30 (31%), WRF-7 (24.73%), WRF-13 (24.21%), WRF-4 (11.76%) and the minimum was noticed WRF-5 (5.88%). No radial growth inhibition was recorded in the case of an antagonist, fungi WRF-27. By following the criteria led by Johnson & curl, the interaction behaviour of all antagonists was recorded [15]. In our study, most fungi showed B-type interaction, and no fungi showed A and D type of interaction (Fig 2). In the B type of interaction, mutual inhibition of *Aspergillus flavus* and antagonists is based on contact and is shown by WRF-4, 7, 13, 20, 23, and WRF-30. Antagonist fungi (WRF-5) showed a C-type of interaction where both antagonist and *Aspergillus flavus* release antifungal compounds hence mutually inhibiting each other at a distance. Whereas in the E-type of interaction, only test fungi are inhibited at a distance because only antagonists can produce antimicrobial compounds. Only one fungus, i.e., WRF-27 showed an E-type of interaction in our present study and is considered the most potent antagonist. Chauhan obtained similar results during working on antagonistic interaction between toxigenic strains of *Aspergillus flavus* and co-existing fungi of Safed Musli [18]. He followed the same Johnson and curls fungal-fungal interaction method and observed that out of 26 co-existing rhizosphere fungi, only three fungi showed type-A interaction, eight fungi B-type, six fungi C-type, seven fungi D-Type, and only two fungi showed E-type interaction with highly toxigenic strain (CB55) of *Aspergillus flavus* isolates.

In the present study, although WRF-20 (*Trichoderma sp*) showed maximum radial growth inhibition of BAF-4 but mutually inhibited when antagonists and pathogens are in close contact. This condition may not or rarely happen in the rhizosphere region; therefore, it may not be considered a potent antagonist for minimizing the population of *Aspergillus flavus* in the rhizosphere. Antagonists WRF-27 (*Talaromyces sp*) showed second highest radial growth

inhibition at a distance and no mutual inhibition was recorded. In the overall study, *Talaromyces sp* which showed E-type of interaction is considered as most potent antagonist where production of some antimicrobial compounds is effective for inhibiting pathogens in broader areas and may undoubtedly reduce the population of *Aspergillus flavus* in the rhizosphere and ultimately reduce the chance of contamination in standing crops.

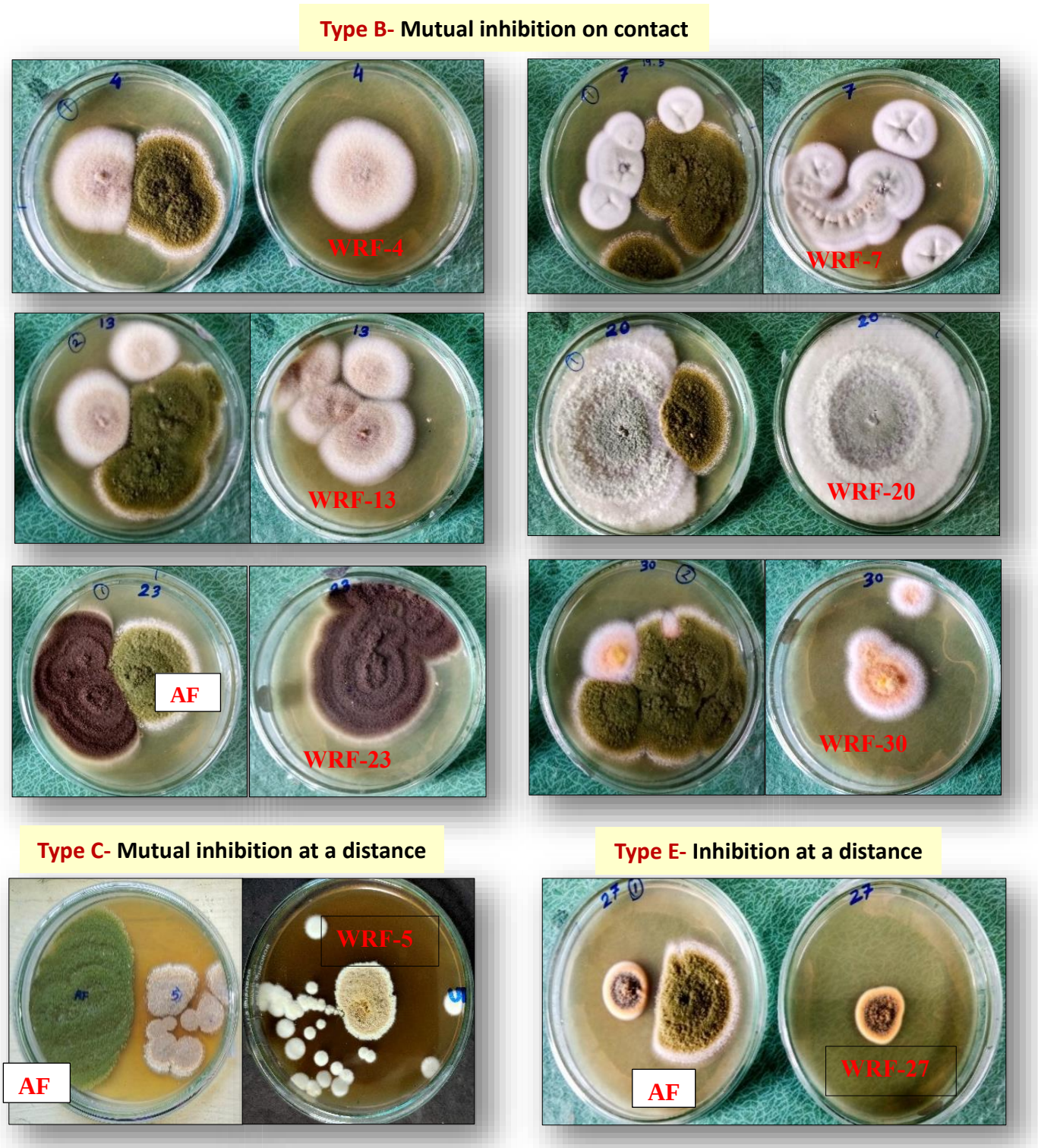


Fig 2 Radial growth inhibition of *A. flavus* in dual culture and their mode of interaction behaviour

CONCLUSION

Minimizing *A. flavus* contamination in standing crops is a cumbersome process; however, applying suitable antagonists in the rhizosphere gives better results for pre-harvest aflatoxin contamination by reducing the population of toxigenic isolates in rhizosphere soil. It is also to mention that, in any biological control program, antagonists that show E-type of interaction are considered the best antagonists because secretion of antimicrobial compounds from antagonists is responsible for inhibition of test fungi. In the present study, WRF-27 (*Talaromyces sp*) showed second maximum radial growth inhibition at a distance and is considered the most promising antagonist of toxigenic *A. flavus* isolates.

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