CHEMICAL AND BIOLOGICAL CONTROL OF PATHOGENIC *ASPERGILLUS* SPP.

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Abstract: Six antibiotics [viz. amphotericin-B (AP), clotrimazole (CC), fluconazole (FLC), itraconazole (IT), ketoconazole (KT) and nystatin (NS)] and six bacteria (viz. *Bacillus licheniformis*, *B. haloduran*, *B. cohnii*, *B. subtilis*, *Pseudomonas* sp., and *Rhizobium* sp.) were tested for their antifungal activities against two pathogenic fungi *Aspergillus flavus* (102566) and *A. niger*, which cause a significant yield loss in many important crops during pre- and post-harvest periods. Antibiotics susceptibility test for six antibiotics revealed the antifungal activities of five antibiotics, FLC being ineffective against the test pathogens. Out of the six bacteria, two (*Pseudomonas* sp. and *Rhizobium* sp.) were found to show antifungal activities against both the test pathogens; while, all four *Bacillus* spp. were found to be ineffective against *A. flavus* and *A. niger*, The investigation revealed that the chemical and biological agents can be effectively used against the fungal pathogens.

Keywords: *Aspergillus* spp., antifungal activity, antibiotics, biological control, *Pseudomonas* sp., *Rhizobium* sp.

INTRODUCTION

Aspergillus flavus and *A. niger* are saprophytic soil fungi that contaminate and cause severe crop losses and pose potential threat to both human and animal health as well as the environment. These fungi are both human and animal pathogens and cause many superficial infections in them such as chronic granulomatus sinusitis, cutaneous aspergillosis, keratitis, wound infections and asteomylitis, following trauma and inoculation (Hedayati *et al*., 2007). *A. flavus* infects and causes diseases in several important crops such as cotton, maize, rice, peanut before and after harvest (Klich, 2007; Michailides, 2007; Yu, 2005). *A. flavus* has been known to produce aflatoxins, the most potent naturally occuring toxic compounds known to man (Amaike and Keller, 2011; Kumar *et al*., 2005; Singh, 1997; Singh and Singh, 2007; Singh *et al*., 1992). The aflatoxins are immunocarcinogenic, teratogenic and growth retardants. *A. niger* is an opportunistic pathogen reported to produce potent mycotoxins called ochratoxins. In plants *A. niger* causes black mold of onions and also causes disease in peanuts, grapes and maize. *A. niger* is one of the most common causes of pain, temporary hair loss, and in severe cases, damage to ear canal tympanic membrane (Verweij and Brandt, 2007). Biological control of fungal pathogens has been investigated recently by Sharma et al. (2011).

Keeping in view the health and environmental concern of the fungal pathogens, the present investigation was been undertaken to screen six antibiotics [viz. amphotericin-B (AP), clotrimazole (CC), fluconazole (FLC), itraconazole (IT), ketoconazole (KT) and nystatin (NS)] and six bacteria (viz. *Bacillus licheniformis*, *B. haloduran*, *B. cohnii*, *B. subtilis*, *Pseudomonas* sp., and *Rhizobium* sp.) for their antifungal activities against *A. flavus* and *A. niger*.

MATERIAL AND METHOD

Six antibiotics [viz. Amphotericin-B (AP), Clotrimazole (CC), Fluconazole (FLC), Itraconazole (IT), Ketoconazole (KT) and Nystatin (NS)] of Hi-Media antifungal antibiotic Hexa Discs™ were used in antifungal antibiotic susceptibility test to evaluate their activity against the test fungal pathogens.

Six bacteria viz. *Bacillus licheniformis*, *B. haloduran*, *B. cohnii*, previously isolated from tannery industry; *Pseudomonas* sp., isolated from soil; *B. subtilis* obtained from Indian Agriculture Research Institute (IARI), New Delhi and *Rhizobium* sp. obtained from Centre for Environmental Management of Degraded Ecosystems (CEMDE), already maintained in the laboratory on nutrient agar slants were used in the present study. Among the pathogenic fungi, the toxigenic strain of *Aspergillus flavus* CMI (102566) was obtained from University of Strathclyde, Glasgow, U.K.; while, *A. niger* was earlier isolated from soil sample, were subcultured and maintained on Potato Dextrose Agar (PDA) medium in the laboratory.

Antibiotic susceptibility behavior of both the test pathogens i.e. toxigenic strain of *A. flavus* (102566) and *A. niger* was determined using antifungal antibiotic-impregnated discs (Himedia) consisting of 6 different antibiotics viz. amphotericin-B (AP), clotrimazole (CC), fluconazole (FLC), itraconazole (IT), ketoconazole (KT) and nystatin. Czapex Dox Agar (CDA) plates were prepared and inoculated with 0.1 ml of broth culture of the test pathogens individually. Antifungal antibiotic discs were mounted and plates were incubated overnight at 37±2°C. The plates were scored for resistance, intermediate or sensitivity after 24 h by measuring the inhibitory zone using HiAntibiotic Zone Scale™-C (Himedia).

The above mentioned bacteria were tested for their antagonistic activities against the test pathogens, using dual-culture assays (Georgakapoulos *et al*.,

2002). Dual-culture assay was done by placing a five-day old culture of the pathogen and the antagonist 3 cm apart from each other on a sterile Petriplate, containing CDA medium. Control plates with only the pathogen were also inoculated and incubated at 37±2°C, except in case of *Pseudomonas* sp. where temperature was kept at 28±2°C and all the plates were observed after seven days.

In dual-culture assays, the percentage inhibition of growth of the pathogens was calculated, using the formula: r_1-r_2/r_1×100, where r_1 denotes the diameter of radial growth of test pathogen away from the antagonist; and r_2 denotes the diameter of radial growth of test pathogen towards the antagonist.

Each observation was based on the mean of triplicate determinations.

RESULT AND DISCUSSION

In the antifungal antibiotic susceptibility test, on the basis of diameter of the inhibition zone (Fig. 1; Table 1), it was observed that among the six antibiotics used in the investigation, ketoconazole (KT) was found to be highly effective against both the fungal pathogens, followed by clotrimazole (CC). Nystatin (NS) was also found to be highly effective against, to *A. niger* while, it was intermediate in action against *A. flavus*. Itraconazole (IT) was observed to be intermediate in action against both the test pathogens. Nystatin (NS) and amphotericin-B (AP) were also intermediate in action against *A. flavus* and *A. niger* respectively. Amongst the six antibiotics tested, fluconazole (FLC) had no effect on the growth of any of the test pathogens, and amphotericin-B was also observed ineffective against category to *A. flavus*.

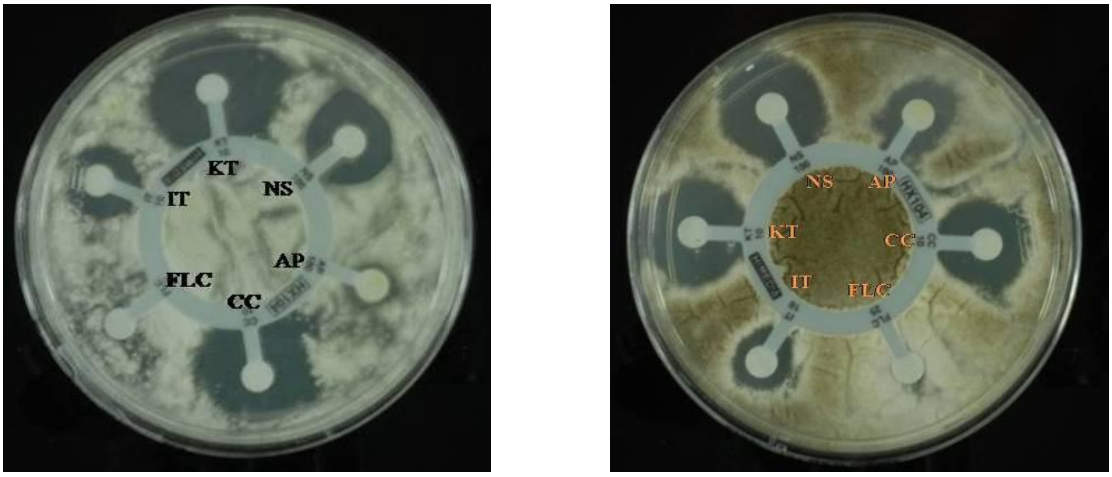
As far as mechanism of action of these antibiotics is concerned the literature depicts that amphotericin-B (AP) binds with ergosterol, the main component of fungal cell membranes, forming a transmembrane channel that leads to monovalent ion (K^+, Na^+, H^+ and Cl^-) leakage, which is the primary effect leading to fungal cell death (Gray *et al*. 2011). Like amphotericin B, nystatin (NS) also binds to ergosterol. When present in sufficient concentrations, it forms pores in the membrane that leads to K^+ leakage and death of the fungus (De Kruijff *et al*. 1974). Fluconazole (FLC) is a triazole antifungal drug used in the treatment and prevention of superficial and systemic fungal infections. Like other imidazole- and triazole-class antifungals, fluconazole inhibits the fungal cytochrome P450 enzyme 14α-demethylase. This inhibition prevents the conversion of lanosterol to ergosterol, an essential component of the fungal cytoplasmic membrane, and subsequent accumulation of 14α-methyl sterols (Sanati *et al*., 1997). Clotrimazole (CC) alters the permeability of the fungal cell wall and inhibits the activity of enzymes within the cell. Studies show that minimal concentrations of clotrimazole cause leakage of intracellular phosphorus compounds into the ambient medium, along with the breakdown of cellular nucleic acids and an accelerated K^+ efflux. This leads eventually to the cell's death (Yamaguchi and Iwata, 1979). Ketoconazole (KT) is structurally similar to imidazole and interferes with the fungal synthesis of ergosterol, a constituent of fungal cell membranes as well as certain enzymes. Similar to the mechanism of action of fluconazole, ketoconazole also works principally by inhibiting the enzyme cytochrome P-450 14-α-demethylase (P-45014DM). This enzyme participates in the sterol biosynthesis pathway that converts lanosterol to ergosterol, a main component of fungal cell wall, thus, causing fungal cell death (Edgar *et al*., 1984).

Out of the six bacteria tested, only two (*Pseudomonas* sp. and *Rhizobium* sp.) were found to show antagonistic activity against the fungal pathogens *A. flavus* (Fig.2 B & Fig.4 B) and *A. niger* (Fig.3 B & Fig.5 B) while, the *Bacillus* spp. (*B. licheniformis*, *B. haloduran*, *B. cohnii*, *B. subtilis*) were found to be ineffective against both the test pathogens. It was observed that in the present study *Rhizobium* sp. was found to show strong antifungal activity against *A. flavus* (toxigenic) and *A. niger* with 66.6% and 61.1% inhibition of their growth, respectively as revealed by dual culture assays (Table 2). Ehtashamul-haque and Ghaffar (1993) have also previously reported the biocontrol potential of rhizobia against fungi. Chakraborty and Purkayastha (1984) have reported the production of toxic metabolites by *Rhizobium* sp. which have inhibitory effect on soil borne plant pathogens. *Pseudomonas* sp. was also found to show the antagonistic activity against *A. flavus* and *A. niger* with 28.5% and 59.2% inhibition of their growth, respectively as revealed by dual-culture assays (Table 2). Antagonstic potential of *Pseudomonas* sp. might be explained on the basis of secretion of secondry metabolites which lysed chitin, the most important component of fungal cell wall (Dasgupta *et al*., 2012).

Fokkema (1978) has categorized the Percentage Growth Inhibition (PGI) on a Growth Inhibition Category (GIC) from 0 to 4, where:

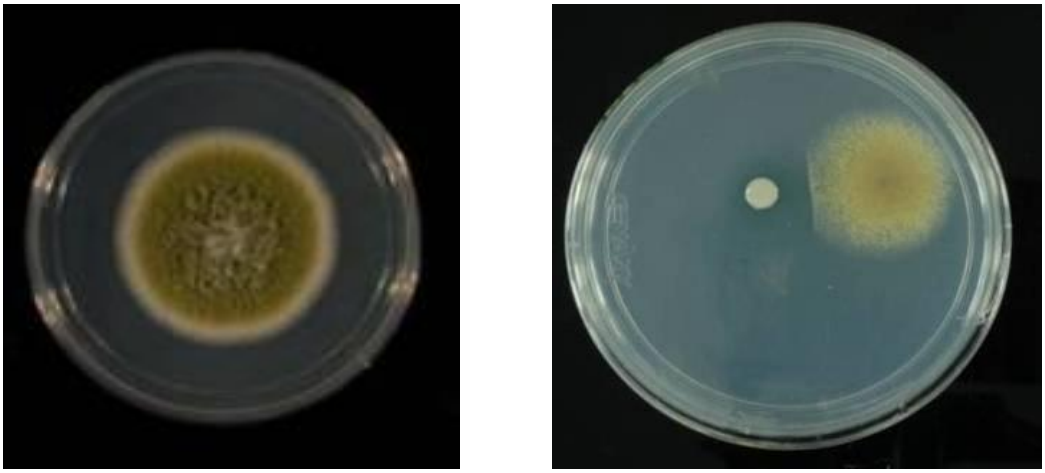
0 = no growth inhibition; 1 = 1–25% growth inhibition; 2 = 26-50% growth inhibition; 3 = 51-75% and 4 = 76-100% growth inhibition.

The PGI of pathogenic fungi i.e. *A. flavus* (toxigenic) and *A. niger* by antagonist *Rhizobium* sp. (66.6% and 61.1%) and of *A. niger* by *Pseudomonas* sp. (59.2%) was found to lie in GIC scale 3, except *P. fluorescens* against *A. flavus* (28.5%) which lie in category 2. Thus, these two bacteria can be used as biocontrol agents against the test pathogens.



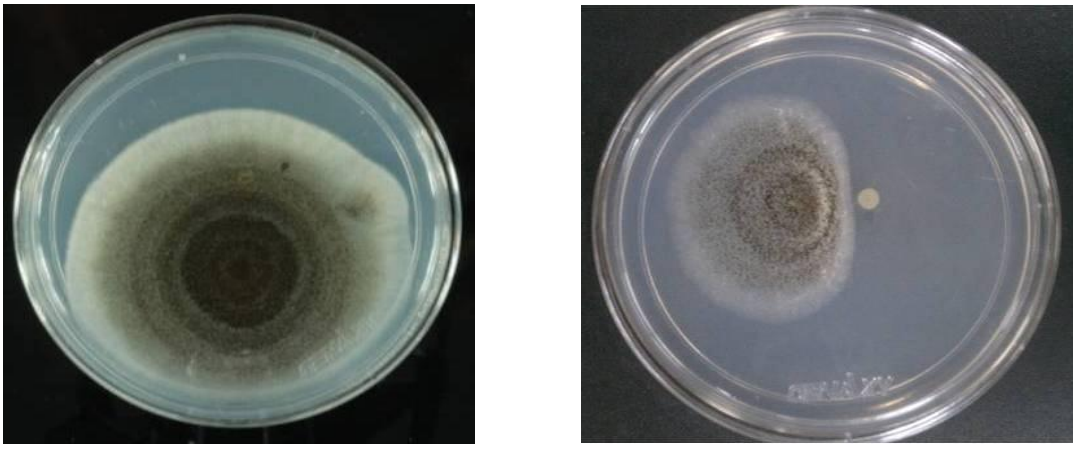
(A) **(B)**

Fig 1. Petriplates showing antibiotic susceptibility test against *A. flavus* (A) and *A. niger* (B).



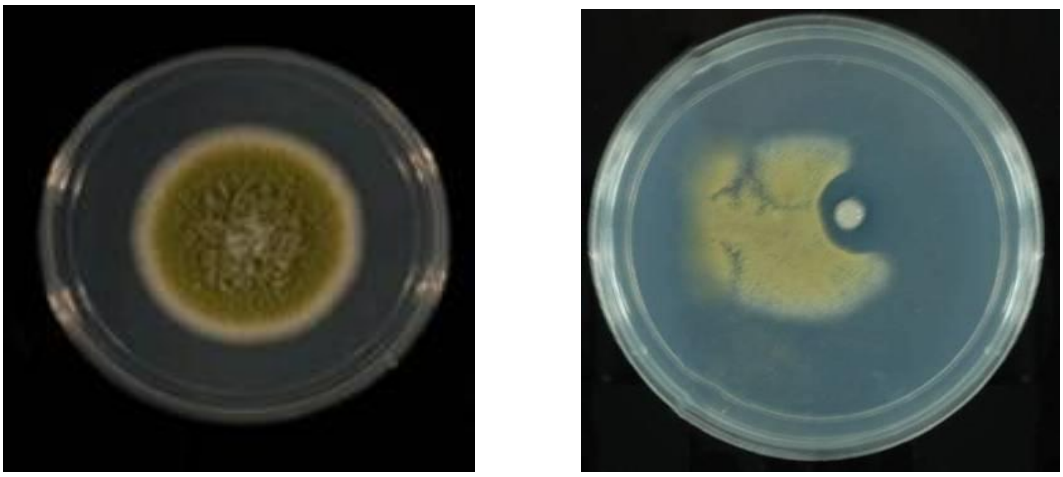
(A) **(B)**

Fig 2. Culture plates showing control culture of *A. flavus* (A) and dual-culture assay of *Pseudomonas* sp. with *A. flavus* (B).



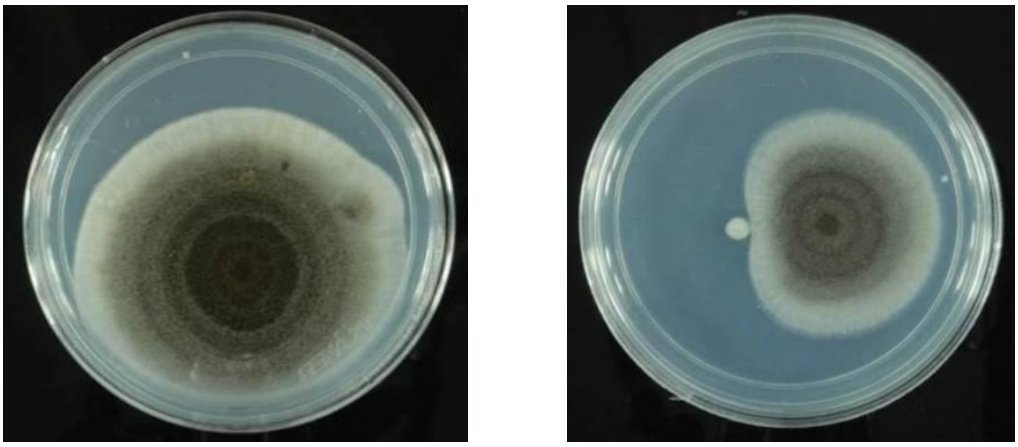
(A) **(B)**

Fig 3. Culture plates showing control culture of *A. niger* (A) and dual-culture assay of *Psuedomonas* sp. witht *A. niger* (B).



(A) **(B)**

Fig 4. Culture plates showing control culture of *A. flavus* (A) and dual-culture assay of *Rhizobium* sp. with *A. flavus* (B).



(A) **(B)**

Fig 5. Culture plates showing control culture of *A. niger* (A) and dual-culture assay of *Rhizobium* sp. with *A. niger* (B).

Table 1. Antibiotic susceptibility profile of *A. flavus* and *A. niger*.

ANTIBIOTICS USED	**SYMBOLS**	**QUANTITY**	***A. FLAVUS* (toxigenic) (Diameter of inhibition zone) (mm)**	***A. NIGER* (Diameter of inhibition zone) (mm)**
Amphotericin B	AP	100 units	12 (R)	18 (I)
Clotrimazole	CC	10 mcg	22 (S)	20 (S)
Fluconazole	FLC	25 mcg	NI (R)	NI (R)
Itraconazole	IT	10 mcg	15 (I)	14 (I)
Ketoconazole	KT	10 mcg	26 (S)	24 (S)
Nystatin	NS	100 units	18 (I)	24 (S)

NI= No inhibition

Letters in parenthesis indicate sensitivity; R= Resistance; I= Intermediate; S= Susceptible.

Table 2. Percentage inhibition of growth of test pathogens *A. flavus* and *A. niger* by the antagonistic bacteria *Pseudomonas* sp. and *Rhizobium* sp., using dual-culture assays.

TEST ORGANISM (Pathogenic fungal strains)	ANTAGONISTIC BACTERIA	DISTANCE r1 (cm)	DISTANCE r2 (cm)	PERCENTAGE-GROWTH INHIBITION (%)
Aspergillus flavus (toxigenic)	*Rhizobium* sp.	2.4	0.8	66.6
Aspergillus niger	*Rhizobium* sp.	3.6	1.4	61.1
Aspergillus flavus (toxigenic)	*Pseudomonas* sp.	2.1	1.5	28.5
Aspergillus niger	*Psuedomonas* sp.	2.7	1.1	59.2

CONCLUSION

The results of the present investigation clearly indicated that out of the six bacteria tested, two bacterial strains i.e, *Rhizobium* sp. and *Pseudomonas* sp. could be effectively used as potential biocontrol agents against aspergilli, which are known to cause severe crop losses and pose potential threat to both human and animal health as well as the environment. However, it was also observed that out of the six antifungal antibiotics used, ketoconazole (KT) and nystatin (NS) were most effective in controlling the test fungi (*A. flavus* and *A. niger*), and fluconazole (FLC) was not effective against the test fungi. And, none of the *Bacillus* spp. investigated showed any antagonistic activity against the fungal pathogens. Further, the synergistic effect of these chemical agents (antibiotics) and biocontrol agents (bacteria) needs to be investigated in order to minimize the indiscriminate use of synthetic chemicals to control pathogenic fungi, which cause severe loss to many crops all over the world.

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