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ASSESSMENT OF GLUCONIC ACID PRODUCING FUNGI AS BIOCONTROL AGENTS AGAINST AFLATOXIGENIC *ASPERGILLUS FLAVUS* AND *ASPERGILLUS PARASITICUS*

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Abstract – Mycotoxin producing fungi such as *Aspergillus flavus* and *Aspergillus parasiticus* are a serious menace in crop fields as they cause contamination of staple foods and make them unfit for consumption. Biocontrol measures have been effective for combating many fungal infections. In this study, gluconic acid producing fungi such as *Gliocladium virens*, *Scopulariopsis canadensis* and *Penicillium funiculosum* were chosen as candidates and were assessed for their biocontrol activity against toxin producing *Aspergillus flavus* and *Aspergillus parasiticus* under *in vitro* conditions. This involved the evaluation of radial growth reduction of *A. flavus* and *A. parasiticus* in various solid media when cocultured with the chosen candidate fungi. Monitoring aflatoxin concentrations, mycelial dry weight and spore morphology changes of the toxigenic fungi, along with pH measurements during coculture facilitated to discern the potential of each antagonist fungi. A maximum antagonism of 80.1% was exhibited by *G. virens* against *A. parasiticus* compared to other chosen fungi. The efficacy of the candidate fungi was also evaluated by simultaneous co-culture on corn kernels as substrate which resulted in about 95.5% inhibition in toxin production by*A. parasiticus* with *G. virens*. This indicates that *G. virens* proves to be an efficient competing strain against aflatoxigenic fungi.

INTRODUCTION

Mycotoxigenic filamentous fungi are involved in causing a number of diseases in plants which result in contamination and loss of cereal crops (Nguyen *et al.*, 2017). The mycotoxins produced by such fungi are toxic secondary metabolites which contaminate crops and threaten human and animal health on consumption (Iqbal *et al*., 2015). Among the different types of mycotoxins, aflatoxins have gained much importance. They are produced predominantly by *A. flavus* and *A. parasiticus* and also by *A. parvisclerotegus*, *A. minisclerotigenes* and *A. nomius* (Pleadin *et al*., 2014). The four major aflatoxins produced are B1, B2, G1 and G2 which are classified based on the blue or green fluorescence they emit and not all fungi produce all the four toxins. *A. parasiticus* produces all the four types of aflatoxin (Waliyar *et al*.,2015). The International Agency for Research on Cancer has classified the B and G toxins as Group I mutagens which lead to liver cancer in case of chronic toxin exposure (IARC, 2015). Among the different strategies existing for aflatoxin management, biological control, which involves the use of an antagonist microbe, proves to be a promising approach during pre-harvest stages (Udomkun *et al*., 2017). Over the years, efforts have been taken to search for new antifungal compounds in microorganisms which contribute to enhanced biocontrol. Gluconic acid is one such compound which has antifungal activity. *Pseudomonas* strain AN5 which produces it has been developed as a biocontrol agent against 'take all' disease in wheat caused by *Gaeumannomyces graminis* (Kaur *et al*., 2006). Thus gluconic acid producing fungi serve to be a potential untapped resource which can be assessed for their biocontrol efficiency against *Aspergillus flavus* and *Aspergillus parasiticus*.

MATERIALS AND METHODS

Collection and maintenance of fungi

Gluconic acid producing fungi such as *Gliocladium virens* MTCC 3835, *Scopulariopsis canadensis* MTCC

567 and *Penicillium funiculosum* MTCC 2000 were chosen as candidate strains in this study to assess their biocontrol efficiency. The aflatoxigenic strains chosen were *Aspergillus flavus* (MTCC 2798) and *Aspergillus parasiticus* (MTCC 2797). Lyophilized form of these cultures were procured from the Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh. The lyophilized cultures were revived and the strains were maintained in Czapek Yeast Extract Agar (CYA). Plates point inoculated with the spores of the fungus were incubated at 25 °C for 7-14 days until full mycelial growth with spores were observed. Subculture was done regularly every 30 days for maintenance.

Evaluation of radial growth in solid media

To discern the radial growth reduction of the aflatoxigenic strains, the three chosen candidate fungi namely *Gliocladium virens* MTCC 3835, *Scopulariopsis canadensis* MTCC 567 and *Penicillium funiculosum* MTCC 2000 were cocultured in various solid media along with *A. parasiticus* and with *A. flavus*. Agar plugs of the candidate fungi and the toxigenic fungi were inoculated in the same petridish 4cm apart. The solid media used were Rose Bengal Chloramphenicol agar, Potato Dextrose agar, Saboraud's Dextrose agar and Czapek Yeast extract agar to check for medium independent antagonism (Nielson and Sorenson, 1997).

Simultaneous co culture in liquid media

Simultaneous co culture in liquid media was done, where equal amount of spores (10^5/ mL) of the aflatoxigenic and the antagonist fungi were inoculated in Czapek Yeast extract broth, incubated at 28 °C under stationary conditions in dark for 7 days. During this coculture, time course inhibition of the aflatoxin B1 concentrations, pH of the medium, and mycelial dry weights were determined at each 24 hour interval of the 7 day culture period (Shantha, 1999). Control cultures of *A. flavus* and *A. parasiticus* were also maintained for comparison. The changes in the morphology of the spores was also observed. All the experiments were performed in triplicates.

The culture filtrates were subjected to extraction of aflatoxin by the method of Setamou *et al* (1997). Quantitation of aflatoxin B1 was done by Thin layer chromatography using crystalline aflatoxin B1 (Sigma, USA) as standard, visualized in Alpha Digidoc under UV light (365 nm). The pH of the media was checked on each day of the culture period using a Cyber scan 510 pH recorder.

The mycelial dry weights were determined by drying at 80°C in a petridish for 48 hr to constant weight before weighing (Singh and Singh, 2002). The morphology of the spores of control toxigenic fungi were compared with the spores from cocultures during the seventh day of the culture period.

Coculture in corn kernels

To validate the results of coculture, corn kernels (COH 1 variety) were chosen as a biological substrate and inoculated with spores of both antagonist and aflatoxigenic fungi. About 5 g of sample was surface sterilized with 1% sodium hypochlorite solution and inoculated with spore mixtures and incubated in petridishes with wet cotton for 14 days at 28 °C (Sanchez *et al*., 2005). The aflatoxinB1 levels were monitored during a 14 day culture period and estimated by TLC with crystalline Aflatoxin B1 standard (Sigma).

RESULTS

Radial growth inhibition in solid media

A reduction in radial growth was evidenced in all the four types of media namely Rose Bengal Chloramphenicol agar, Potato Dextrose agar, Saboraud's Dextrose agar and Czapek Yeast extract agarby all the antagonistic fungi, but a higher reduction percentage of 80.1±0.1% was obtained in Czapek Yeast extract medium for *A. parasiticus* by *G. virens*, when compared with *S. canadensis* and *P. funiculosum*. The same trend was evidenced in coculture with *A. flavus*, where *G. virens* showed a maximum inhibition of 77±0.15% (Table 1) in Czapek media than the other candidates. Further, the hyphae of the gluconic acid producing *G. Virens* did not intermingle with either of the toxigenic fungi (Fig. 1a and b), suggesting that antagonism exists between them due to explicit nutrient competition.

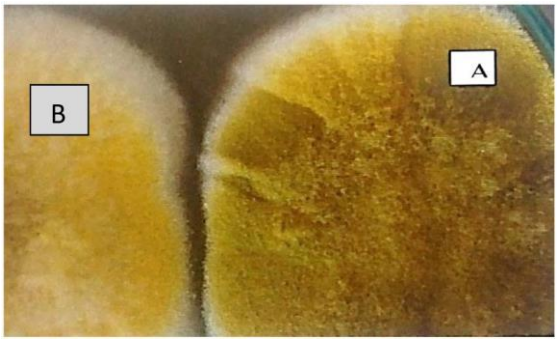
Simultaneous coculture

Reductions in aflatoxin B1 levels: In simultaneous co-culture, the ability of *A. parasiticus* to produce aflatoxin was conspicuously reduced when cultured along with *G. virens* and the percentage of aflatoxin reduction in the medium was seen to reach a maximum of 78.6% on the seventh day of the co-

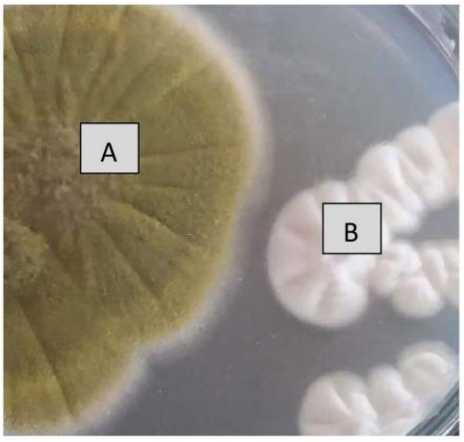
Table 1. Evaluation of radial growth of *A. flavus* and *A. parasiticus* during coculturewith candidate fungi in various media

Media	Percentage growth reduction of *A. parasiticus* during coculture with			Percentage growth reduction of *A. flavus* during coculture with		
	S. canadensis	*G. virens*	*P. funiculosum*	*S. canadensis*	*G. virens*	*P. funiculosum*
RBCA	25±0.1	75±0.35	4.3±0.7	20±0.2	68.9±0.76	10±0.1
PDA	41±0.02	70.2±0.7	7.6±0.85	43±0.07	73.1±0.1	15±0.05
SDA	40±0.13	66.6±0.08	9.1±0.11	32±0.01	75.2±0.11	14.8±0.06
CYA	35.2±0.3	80±0.1	7.4±0.56	35±0.1	77±0.15	13.7±0.07

Values are means of triplicates ± standard deviation



(a) A- Aspergillus parasiticus, B- *Gliocladium virens*



(b) A- Aspergillus flavus B- *Gliocladium virens*

Pict. 1. Hyphal interaction between *Aspergillus parasiticus* and *Gliocladium virens*

culture (Table 2). The reductions were quite low with *S. canadensis* (31.5%) and *Penicillium funiculosum* (6.7%). Similar reductions were observed for *A. flavus* with a maximum reduction level of 65.7% (Table 3) obtained during culturewith *G. virens*compared to the reductions obtained with *S. canadensis* and *P. funiculosum* which were 34% and 11.6% respectively.

Reductions in pH: pH measurements recorded during simultaneous co-culture indicated a gradual decrease to 3.2 from an initial pH value of 6.2 when *A. parasiticus* was cultured with *G. virens*.The reduction was 46.6 % and was much higher than those obtained for *S. canadensis* (10%) and *P. funiculosum* (16.6%). Similarly, reductions in pH from 6.2 to 4.0 were also observed with cocultures of *A. flavus* with *G. virens* accounting to 35.4% reduction, which was comparatively higher than those obtained for *S. canadensis* (19%) and *P. funiculosum* (15%).

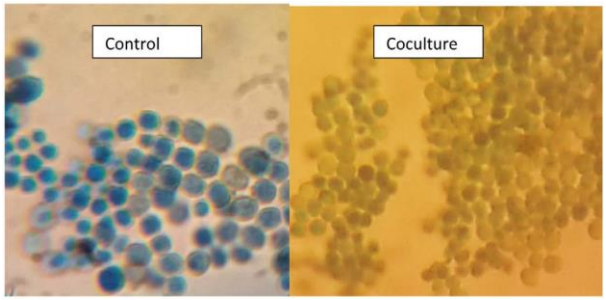
Reductions in mycelial growth: The mycelial growth of *A. parasiticus* also showed a pronounced reduction in dry weights by 62.5% (Figure 1) in the co-culture with *G. virens*, when compared to the other two co-cultures during the seventh day of simultaneous culture. This trend was also found for *A. flavus* dry weights (Figure 2) when cocultured

Table 2. Aflatoxin B 1 levels of *A. parasiticus* during simultaneous coculture with *G. virens*

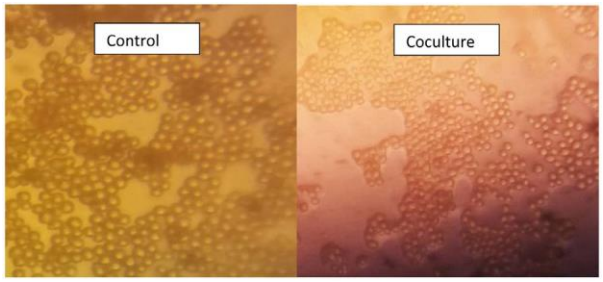
S. No	Day of coculture	Concentration of aflatoxin (µg/100mL media)		Percentage of aflatoxin reduction by
		Control	Co-cultures	*A. parasiticus*
1	First	10.2±1.2	10±1.1	0
2	Second	80.8±1.1	60.8±0.9	24.7
3	Third	220.4±1.2	140.8±0.6	36.1
4	Fourth	380.5±1.4	156.4±0.4	58.9
5	Fifth	520.9±0.2	160±0.2	69.3
6	Sixth	640.4±0.9	160±0.2	75.0
7	Seventh	760.3±0.1	162±0.7	78.6

Values are means of triplicates ± standard deviation

with *G. virens* which was more pronounced than the dry weights observed for other candidate fungi with *A. flavus*.



(a) Spores of *A. parasiticus*



(b) Spores of *A. flavus*

Pic. 2. Comparison of spore morphology of *A. parasiticus* and *A. flavus* in control and in coculture with *G. virens*

Spore morphology: The morphology of spores from the cocultures of *A. flavus* and *A. parasiticus* with *G. virens*appeared to be shrunken and dark when compared to 7 day old control cultures. The spores were damaged extensively, disintegrated and non-viable (Picture 2)

Simultaneous coculture in corn kernels

About 95.5% reductions in toxin levels of *A. parasiticus* was obtained with *G. virens* in simultaneous culture in corn kernels as substrate (Table 4). Cocultures with *S. canadensis* and *P. funiculosum* showed reductions of 26.1% and 5.9% which were not marked when compared to those obtained with *G. virens*. Similarly, *A. flavus* also showed reductions of about 65.9% when cocultured with *G. virens* which was higher than those observed with *S. canadensis* and *P. funiculosum*.

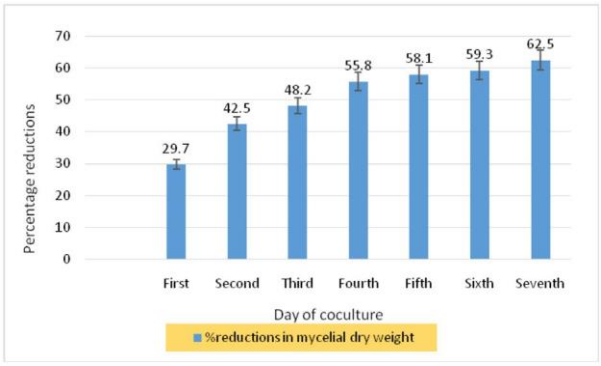


Fig. 1. Mycelial dry weight of *A. parasiticus* during simultaneous co-culture with *G. virens*

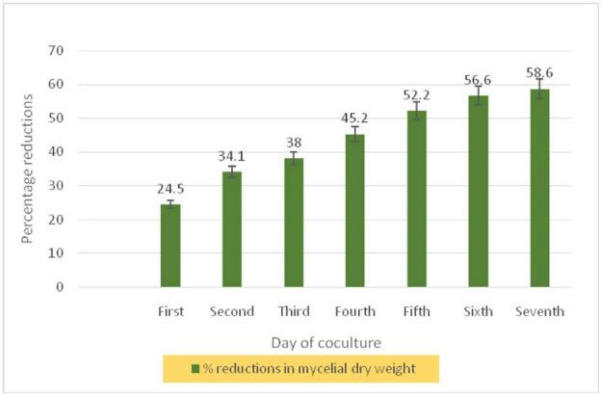


Fig. 2. Mycelial dry weight of *A. flavus* during simultaneous co-culture with *G. virens*

DISCUSSION

Many coculture studies support the inhibitory behavior of typical biocontrol strains. Growth

Table 3. Aflatoxin B 1 levels of *A. flavus* during simultaneous coculture with *G. virens*

S. No.	Day of coculture	Concentration of aflatoxin (µg/100mL media)		ercentage of aflatoxin reduction by *A. flavus*
		Control	Co-cultures	
1	First	10.2±1.2	10±0.6	0
2	Second	80.8±1.1	60±0.9	25
3	Third	220.4±1.2	160±0.6	27.2
4	Fourth	380.5±1.4	220±0.5	42.1
5	Fifth	520.9±0.2	240±0.2	53.8
6	Sixth	640.4±0.9	250±0.2	60.9
7	Seventh	760.3±0.1	260±0.7	65.7

Values are means of triplicates ± standard deviation

Table 4. Aflatoxin B 1 levels of *A. parasiticus* during simultaneous coculture in corn with *G. virens*

S. No	Day of co-culture	Concentration of aflatoxin (mg/100 mL media)		Percentage of aflatoxin reduction by *A. parasiticus*
		Control	Co-cultures	
1	Second	80±5.5	30±8.7	62.5
2	Fourth	240±3.6	70±8.9	70.8
3	Sixth	480±4.9	130±10.1	72.9
4	Eighth	790±5.3	200±7.7	74.6
5	Tenth	840±5.4	150±8.6	82.1
6	Twelfth	860±10.8	80±10.5	90.6
7	Fourteenth	880±10.2	40±4.3	95.5

Values are means of triplicates ± standard deviation

Table 5. Aflatoxin B 1 levels of *A. flavus* during simultaneous coculturein corn with*G. virens*

S. No	Day of co-culture	Concentration of aflatoxin (mg/100mL media)		Percentage of aflatoxin reduction by *A. flavus*
		Control	Co-cultures	
1	Second	80±5.5	70±8.7	12.5
2	Fourth	240±3.6	190±8.3	20.8
3	Sixth	480±4.9	390±9.1	37.5
4	Eighth	790±5.3	300±3.7	58.2
5	Tenth	840±5.4	330±8.7	61.9
6	Twelfth	860±10.8	310±10.1	63.9
7	Fourteenth	880±10.2	300±4.6	65.9

Values are means of triplicates ± standard deviation

inhibitions of about 72% have been reported in several dual culture studies with *Trichodermaviride* and *Macrophominaphaseolina*, indicating the production of antimicrobial metabolites by the antagonistic fungi (Kavitha and Nelson, 2013). Several coculture inhibitions of *Trichoderma* sp with *Pythium*, *Rhizoctoniasolani* and *Colletotrichumcapsici* have also been studied as a preliminary basis to establish their antagonistic activity (Anita *et al*., 2012)

The metabolic rate of a toxigenic culture will be at a maximum during the seventh day at which time, the aflatoxin levels reach a peak (Singh and Singh, 2002). This supports the view that, the reduction in aflatoxin production in coculture with *G. virens* might be due to lower metabolic rates, thus providing meager opportunity for the *A. parasiticus* and *A. flavus*to prepare for secondary metabolism.

Aflatoxin production has been modeled in *A. parasiticus* by Molina and Giannuzzi (2002), where the results suggest that a higher aflatoxin production was observed at pH 5.9 and toxin levels decreased as the pH decreased to 5.5.

Further, *G. virens*, *Scopulariopsissp* and *P. funiculosum* have been found to produce gluconic acid in significant amounts (Ramachandran *et al*, 2006). Hence, reductions in pH of the media may be attributed to the production of gluconic acid, which in turn affect aflatoxin biosynthesis (Howell, 2003). The spore wall of the toxigenic fungi has undergone considerable damage due to the candidate fungi which can be correlated with the inhibition of growth and mycelia formation in toxigenic fungi. This indicates that *G. virens* proves to be a competing strain for both *A. parasiticus* and *A. flavus* among the three chosen candidate fungi.

CONCLUSION

Thus, it can be suggested that the type of hyphal interaction involved between the fungi might be a direct interaction due to some secondary metabolite produced by *G. virens* and the main cause for antagonism may be attributed to antibiosis and competition for nutrients. The reduction in aflatoxin production might be attributed to lower metabolic rates. Further, sporulation and decrease in mycelial dry weights can also be associated directly with decreased levels of aflatoxin in the simultaneous culture. Since *Gliocladiumvirens* has already been known to have an aggressive mode of growth and produces several antibiotics such as 'Gliovirin' and

'Gliotoxin', it might serve as an effective biocontrol agent against *A. parasiticus*.

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