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Effect of Co- existing Filamentous Fungi on Growth Inhibition and Aflatoxin production by *Aspergillus parasiticus*

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A B S T R A C T

Aflatoxin belongs to a group of fungal toxins known as mycotoxins. It poses a potential threat to food safety. As aflatoxin is epidemiologically implicated as carcinogen in humans and an environmental contaminant which is widespread in nature, its possible chronic toxicity is therefore of greater concern than acute toxicity. It is a secondary metabolite produced by specific strains of *Aspergillus*. Aflatoxin is classified into a number of subtypes. However, the most important ones are B1, B2, G1 and G2, distinguished by their fluorescence colour under ultraviolet light. In the present investigation the influence of microbial interactions on growth and aflatoxin production by a toxigenic strain of *Aspergillus parasiticus* NRRL2999 *in vitro* was studied. The results clearly indicated that all the fifteen types of interacting fungi were found to inhibit growth and aflatoxins B1, B2, G1 and G2 production by *Aspergillus parasiticus* NRRL2999 strain but the intensity was variable. It is evident that 1ml of culture filtrate of *Alternaria alternata, A. brassicae, Helminthosporium sativum, Chaetomium globosum, Cladosporium herbarum, Fusarium moniliforme, Penicillium citrinum, Mucor mucedo, Rhizopus stolonifer* and *R. nigricans* was found to be highly inhibitory to growth of *A. parasiticus* NRRL2999 that caused 57.47% to 76.55% inhibition. The results confirm that all the fifteen interacting fungi viz. *Aspergillus niger, A. sydowi, Alternaria alternata, A. brassicae, Curvularia lunata, Geotrichum candidum, Monilia sitophila, Helminthosporium sativum, Chaetomium globosum, Cladosporium herbarum, Fusarium moniliforme, Penicillium citrinum, Mucor mucido, Rhizopus nigricans* and *Rhizopus stolonifer* are the best biological agents to inhibit growth and Aflatoxin production ability of *Aspergillus parasiticus* NRRL2999 strain.

Introduction

Aflatoxin belongs to a group of fungal toxins known as mycotoxins. It was discovered some 30 years ago in England following a poisoning outbreak causing 100,000 turkey deaths. Mycotoxins have received considerable attention due to their significance in agricultural loss and human health. Amongst the mycotoxins that are known to cause human diseases, aflatoxins have been studied most. Aflatoxin poses a potential threat to food safety. As aflatoxin is epidemiologically implicated as carcinogen in

humans and an environmental contaminant which is widespread in nature, its possible chronic toxicity is therefore of greater concern than acute toxicity.

Aflatoxin is the secondary metabolite produced by specific strains of *Aspergillus*. These species contaminate various agricultural commodities either before harvest or at post-harvest stages under favourable conditions of temperature and humidity.

Aspergillus is a large genus of mould which grows at an optimal range of temperature of 28-33°C and at the water activity of about 0.83-0.97. The aflatoxigenic moulds, namely *A flavus*, *A parasiticus* and *A nomius* are principally found in soils and decaying vegetation. They occur in warmer parts of the world such as tropical region where temperature and moisture are high. They have a higher affinity of growth in nuts and oilseeds. Aflatoxin is classified into a number of subtypes. However, the most important ones are B1, B2, G1 and G2, distinguished by their fluorescence colour under ultraviolet light. In addition, aflatoxin M1 and M2 are hydroxylated metabolites of aflatoxin B1 and B2. Aflatoxins are odourless, tasteless and colourless. Chemically, they are stable in foods and resistant to degradation under normal cooking procedures. It is difficult to eliminate aflatoxin once it is produced.

Accumulation of aflatoxin is dependent upon weather conditions. Before harvest, the risk for the development of aflatoxin is greatest during major droughts. When soil moisture is below normal and temperatures are high, the number of *Aspergillus* spores in the air increases. These spores infect crops through areas of damage caused by insects, and inclement weather. Once infected, plant stress occurs, the production of aflatoxin is favoured. During post-harvest stage, proliferation of aflatoxin can be exacerbated in susceptible commodities under storage conditions such as hot and humid storage environment.

Human exposure to aflatoxin is principally through ingestion of contaminated foods. Inhalation of the toxins may also occur occasionally due to the occupational exposure. Aflatoxin can cause both acute and chronic toxicity in animals. Effects such as acute liver damage, liver cirrhosis, induction of tumors and teratogenic and other genetic effects are well documented.

The acute toxicity of aflatoxin to humans has been encountered only rarely. Symptoms may include fever, vomiting and jaundice. Acute liver damage can occur which may be fatal in severe cases. There has not been any food poisoning case related to dietary aflatoxin. Long term intake of aflatoxin can be associated with hepatic cancer. Animal studies have showed that hepatocellular liver tumors may develop in animals like rats, hamsters and monkeys after prolonged oral administration. Epidemiological studies supported a positive correlation between the logarithm of aflatoxin ingestion and the occurrence of human primary liver cancer. Studies revealed that the co-existence of hepatitis B virus infection might contribute to higher incidence of liver cancer in aflatoxin exposed populations.

Mycotoxins have detrimental effects, so a number of strategies have been developed to help prevent the growth of mycotoxigenic fungi as well as to decontaminate and/or detoxify mycotoxin contaminated foods and animal feeds. These strategies include the prevention of mycotoxin contamination, detoxification of mycotoxins present in food and feed and inhibition of mycotoxin absorption in the gastrointestinal tract. The prevention of mycotoxin contamination prior to harvest or during post-harvest and storage

is not always possible necessitating decontamination before the use of such materials for food and feed purposes. Therefore various detoxification processes play an important role in helping prevent exposure to the toxic and carcinogenic effect of mycotoxins (Bulent *et al.,* 2006). Detoxification of mycotoxins is typically achieved by removal or elimination of the contaminated commodities or by inactivation of the toxins present in these commodities by physical, chemical, or biological methods (Sinha, 1998). It is generally accepted that mycotoxin levels in food needs to be reduced as low as technologically possible. Many physical, chemical and biological methods have been applied for the removal and biosynthesis of aflatoxins, and also growth of aflatoxigenic moulds inhibition. However, few of these have practical applications (Buser and Abbas, 2002; Luias *et al.,* 2002).

Many methods have been applied in order to control or inhibit mould growth and maize contamination by aflatoxin, although strategies to control growth of mould and mycotoxin contamination has to be considered (Munkvold and Desjardin, 1997), for plants products postharvest methods are also recommended (Bankole and Adebanjo, 2003). The prevention of mycotoxin production includes all phases of food and feed production, because the mould contamination may occur in the field, during storage, as well as in transport. Adequate storage with optimal temperature and humidity of grains and relative humidity and the hygiene in silos may decrease the growth of toxicogenic moulds (Maja *et al.,* 2002).

In order to control aflatoxins contamination, the coexisting fungi have been exploited by some earlier workers viz. roy and Chourasia, 1990; Choudhary and Sinha, 1993; Roy and Kumar, 1997; Shivendra and Awadh, 2001etc. Nanis *et al.,* (2010) have studied the antifungal effect of five *Lactobacillus* strains namely *L. plantarum* ATCC 4008, *L. plantarum* 12006, *Lactobacillus plantarum 299V, L. paracasei* subsp. *paracasei* LMG 13552 and *L.rhamnosus* VT1exhibiting inhibition of growth and aflatoxin producing *Aspergilli.* The mechanism of inhibition of growth and aflatoxin production has been studied by Mehdi Razzaghi-Abyaneh (2011). Rattanaporn Thakaew and Hataichanoke Niamsup (2013) have studied the inhibitory Activity of *Bacillus subtilis* BCC 6327 Metabolites against Growth of Aflatoxigenic Fungi Isolated from Bird Chili Powder.

In the present investigation an attempt has been made to study the influence of microbial interactions on growth and aflatoxin production by a toxigenic strain of *Aspergillus parasiticus in vitro.*

Materials and Methods

The toxigenic strain of *Aspergillus parasiticus* NRRL-2999 was used to assay the growth performance and aflatoxin production in presence of other filamentous fungi. This strain (obtained from Northern regional Research Laboratory, North Carolina, USA had the capacity to produce aflatoxins B1, B2, G1, and G2 and were quantified as 8.34, 6.87, 7.34 and 2.32mg/ml respectively.

Isolation of fungi

The interacting fungi used in the present investigation were isolated from cereal crops viz. wheat, rice and maize and identified by comparing with specimen cultures in the Mycology Department and then confirmed by Commonwealth Mycological Research Institute (CMI), Kew, London. The interacting fungi identified were *Aspergillus niger, A. sydowi, Alternaria alternata, A. brassicae, Curvularia lunata, Geotrichum candidum, Monilia sitiphila,*

Helminthosporium sativum, Chaetomium globosum, Cladosporium herbarum, Fusarium moniliforme, Penicillium citrinum, Mucor mucido, Rhizopus nigricans and *Rhizopus stolonifer.*

Dual culture

Petri dishes containing Potato Dextrose Agar medium (PDA) were seeded equidistantly at four corner with sis days old mycelial inoculum of individual test fungus along with centrally seeded *Aspergillus parasiticus* NRRL2999. The inoculated plates were incubated at 28±2^{0}C for ten days in BOD incubator under alternate cycles of 12hrs. light and dark. After two days of incubation interacting behaviour was observed and recorded by method suggested by Johnson and Curl (1972) as follows:

A - A mutual intermingling of the two organisms.

B - Mutual inhibition on contact

C - Mutual inhibition at a distance

D - Inhibition on contact, the antagonist continues to grow, unchanged or reduced rate through the colony of the inhibited organisms.

E - Inhibition at a distance, the antagonist continues to grow resulting clear zone at an unchanged or reduced rate.

Aflatoxin assay

1 ml culture filtrate, grown in Czapek- dox liquid medium of each of the interacting fungi viz. *Aspergillus niger, A. sydowi, Alternaria alternata, A. brassicae, Curvularia lunata, Geotrichum candidum, Monilia sitophila, Helminthosporium sativum, Chaetomium globosum, Cladosporium herbarum, Fusarium moniliforme, Penicillium citrinum, Mucor mucido, Rhizopus nigricans* and *Rhizopus stolonifer* having growth inhibitory ability in dual culture was supplemented separately in 250ml Erlen Mayer flask containing 50 ml of SMKY liquid medium (Diener and Davis, 1966). Subsequently flasks were inoculated separately with 1 ml spore suspension (10^6 spores/ml) of A. parasiticus NRRL2999 and kept for 10 days under controlled conditions in a BOD incubator.

The culture was then filtered through pre weighed filter paper and extraction was done with chloroform. The dried chloroform extracts were further subjected to Thin Layer Chromatography (TLC) for qualitative and quantitative estimations following the method of Reddy *et al.*, (1970). Confermation of aflatoxins presence was made by trifluoroacetic acid (TFA) and 50% aqueous sulphuric acid (Stach and Pohland, 1975).Amount of Aflatoxins produced was determined by spectrophotometer (CAMAG TLC scanner- II and DU- 64 spectrophotometer).

Determination of growth

After filtration, mycelial mat was dried at 60^{0}C for 24hrs in an oven and weighed to determine the change in dry weight of *A. parasiticus*.

Data were statically analyzed by mean ± S.E. The results obtained have been presented in tables 1, 2 and 3; Figs. 1 and 2.

Results and Discussion

All the fifteen types of interacting fungal associations with *Aspergillus parasiticus* NRRL2999 strain were found to inhibit aflatoxins B1, B2, G1 and G2 production but the intensity was variable (Table 1). In the dual culture of *Aspergillus Parasiticus*

NRRL2999 with *A. sydowi*, *A. niger*, *Geotrichum candidum* and *Monilia sitophila* E type of interaction behaviour was recorded. A clear cut inhibition zone was developed and the growth of A. parasiticus significantly arrested. The inhibition zone was also found to be widened with the increase of incubation period. *Alternaria alternata*, *A. brassicae* and *Helminthosporium sativum* exhibited D type of interacting behaviour with *Aspergillus parasiticus.* Similarly *Curvularia lunata, Chaetomium globosum* and *Penicillium citrinum* exhibited B type and *Cladosporium herbarum, Fusarium moniliforme, Mucor mucedo, Rhizopus stolonifer* and *R. nigricans* A type of interacting behaviour with *A. parasiticus* NRRL2999 (Table 1).

From the results (Table 2: Fig. 1) it is evident that 1ml of culture filtrate of *Alternaria alternata, A. brassicae, Helminthosporium sativum, Chaetomium globosum, Cladosporium herbarum, Fusarium moniliforme, Penicillium citrinum, Mucor mucedo, Rhizopus stolonifer* and *R. nigricans* was found to be highly inhibitory to growth of *A. parasiticus* NRRL2999 that caused 57.47% to 76.55% inhibition (Table 2; Fig. 1).

Growth inhibition of *A. parasiticus* by *Cladosporium herbarum* and *Fusarium moniliforme* was highest, about 76.32- 76.55% followed by *Mucor mucedo, Rhizopus stolonifer* and *R. nigricans* (71.72- 74.48%) and Penicillium citrinum and Chaetomium globosum (67.35- 67.55%). *Aspergillus sydowi, A. niger* and *Curvularia lunata* caused least growth inhibition of *A. parasitica*NRRL2999. *Geotricum candidum* and *Monilia sitophila* caused only about 45% growth inhibition to *A. parasitica*.

Data recorded in table 3 and figure 2 reveals that *Chaetomium globosum, Helminthosporium sativum, Cladosporium herbarum, Fusarium moniliforme, Mucor mucedo, Rhizopus stolonifer* and *R. nigricans* caused maximum inhibition of aflatoxin B1 production by *Aspergillus parasiticus*NRRL2999 (91.84- 98.56%) followed by *Alternaria alternata, A. brassicae, Curvularia lunata* (80.60%, 86.40% and 84.70% respectively) and *Aspergillus sydowi* and *A. niger* (72.6 and73.0% respectively). *Gleotrichum candidum* and *Moinilia sitophila* caused less inhibition og aflatoxin B1 production by *A. parasitica* (Table 3; Fig. 2). Aflatoxin B2 production by *Aspergillus parasiticus* NRRL2999 was strongly inhibited by culture filtrates of *Gleotrichum candidum, Monilia sitophila, Helminthosporium sativum, Chaetomium globosum, Cladosporium herbarum* and *Penicillium citrinum* (65.795- 79%).

Similarly *Alternaria alternata, A. brassicae, Curvularia lunata, Fusarium moniliforme, Mucor mucedo, Rhizopus stolonifer* and *R. nigricans* caused 38.13- 55.54% aflatoxin B2 inhibition.

The culture filtrates of other fungi had less inhibitory action (Table 3; Fig. 2). Aflatoxin G1 production by A. parasiticu was maximaly inhibited by *A. sydowi* (86.105), and A. niger (86.14%), followed by *M. mucedo* and *R. nigricans* (76.15%), *R.stolonifer* (75.74%) and *A. alternata, A. brassicae*, (66.62- 67.98%). *H. sativum, C. globosum, F. moniliforme* and *C. herbarum* caused 60.82- 62.39% inhibition to G1 production. *Fusarium moniliforme* caused 71.98% inhibition of aflatoxin G2 prodiction by *A. parasiticus. Curvularia lunata, G. candidum, M. sitiphila, H. sativum, Chaetomium globosum, Cladosporium herbarum, P. citrinum, M. mucedo, R. stolonifer* and *R. nigricans* caused moderate inhibition of aflatoxin G2 production by A. parasiticus about 55- 70%.

Table.1 Interacting behaviour of co- existing fungi with *Aspergillus parasiticus* in dual culture

Interacting Fungi	Type of interacting behaviour				
	A	B	C	D	E
Aspergillus sydowi	-	-	-	-	+
Aspergillus niger	-	-	-	-	+
Alternaria alternata	-	-	-	+	-
A. brassicae	-	-	-	+	-
Curvularia lunata		+			
Geotrichum candidum	-	-	-	-	+
Monilia sitophila	-	-	-	-	+
Helminthosporium sativum	-	-		+	
Chaetomium globosum	-	+	-	-	-
Cladosporium herbarum	+	-	-	-	-
Fusarium moniliforme	+	-	-	-	-
Penicillium citrinum	-	+	-	-	-
Mucor mucido	+	-	-	-	-
Rhizopus stolonifer	+	-	-	-	-
Rhizopus nigricans	+	-	-	-	-

+ Sign indicates the type of interacting behavior, - Sign indicates no interaction

Table.2 Effect of culture filtrate of interacting fungi on growth of *Aspergillus parasiticus* NRRL 2999

Interacting fungi	**Growth in mg**	**Growth inhibition (%)**
***Aspergillus parasiticus* NRRL 2999 as control**	**2175±2.35**	
Aspergillus sydowi	**1535±1.15**	**29.43**
Aspergillus niger	**1475±1.17**	**30.35**
Alternaria alternata	**885±1.19**	**54.47**
A. brassicae	**850±1.13**	**59.08**
Curvularia lunata	**1435±1.12**	**32.18**
Geotrichum candidum	**1155±1.11**	**45.05**
Monilia sitophila	**1150±1.17**	**45.28**
Helminthosporium sativum	**850±1.16**	**59.08**
Chaetomium globosum	**665±1.11**	**67.58**
Cladosporium herbarum	**470±1.10**	**76.55**
Fusarium moniliforme	**475±1.13**	**76.32**
Penicillium citrinum	**670±1.13**	**67.35**
Mucor mucido	**515±1.25**	**74.48**
Rhizopus stolonifer	**550±1.23**	**72.87**
Rhizopus nigricans	**557±1.17**	**71.72**

Table.3 Effect of culture filtrate of 15 interacting fungi on Aflatoxin production by *Aspergillus parasiticus* NRRL2999 strain

Interacting fungi	**Aflatoxin production by *A. parasiticus* NRRL2999 in presence of 15 interacting fungi**							
	B1	**% inhibition**	**B2**	**% inhibition**	**G1**	**% inhibition**	**G2**	**% inhibition**
***Aspergillus parasiticus*NRRL2999 as control**	**8.34±0.16**		**6.87±0.12**		**7.34±0.16**		**2.32±0.12**	
Aspergillus sydowi	**2.28±0.17**	**72.6**	**5.52±0.16**	**19.65**	**1.02±0.12**	**86.10**	**2.04±0.11**	**12.06**
Aspergillus niger	**2.25±0.14**	**73.0**	**5.35±0.13**	**22.12**	**1.06±0.13**	**85.14**	**2.08±0.12**	**10.34**
Alternaria alternata	**1.62±0.18**	**80.6**	**4.25±0.16**	**38.13**	**2.35±0.14**	**67.98**	**1.75±0.16**	**24.56**
A. brassicae	**1.14±0.16**	**86.4**	**3.75±0.15**	**45.41**	**2.45±0.16**	**66.62**	**1.71±0.11**	**26.29**
Curvularia lunata	**1.28±0.16**	**84.7**	**3.65±0.11**	**46.87**	**3.51±0.15**	**51.36**	**1.05±0.16**	**54.74**
Geotrichum candidum	**5.65±0.17**	**32.25**	**2.21±0.13**	**673.83**	**4.25±0.16**	**42.09**	**0.75±0.16**	**67.67**
Monilia sitophila	**6.85±0.12**	**17.86**	**2.25±0.14**	**67.25**	**4.50±0.12**	**38.69**	**0.70±0.05**	**69.82**
Helminthosporium sativum	**0.68±0.16**	**91.84**	**1.45±0.16**	**78.89**	**2.76±0.16**	**62.39**	**1.03±0.07**	**55.60**
Chaetomium globosum	**0.12±0.14**	**98.56**	**1.75±0.17**	**74.52**	**2.85±0.10**	**61.17**	**1.03±0.05**	**55.60**
Cladosporium herbarum	**0.14±0.16**	**98.32**	**1.77±0.15**	**74.23**	**2.87±0.10**	**60.89**	**1.05±0.06**	**54.74**
Fusarium moniliforme	**0.20±0.16**	**97.60**	**3.26±0.16**	**55.54**	**2.86±0.11**	**61.03**	**0.65±0.06**	**71.98**
Penicillium citrinum	**1.34±0.16**	**83.93**	**2.35±0.13**	**35.79**	**3.25±0.13**	**55.72**	**0.75±0.04**	**67.67**
Mucor mucido	**0.15±0.13**	**98.20**	**3.50±0.16**	**49.09**	**1.75±0.16**	**76.15**	**0.76±0.06**	**67.24**
Rhizopus stolonifer	**0.18±0.12**	**97.84**	**3.45±0.16**	**48.32**	**1.78±0.14**	**75.74**	**0.77±0.06**	**66.80**
Rhizopus nigricans	**0.17±0.11**	**97.96**	**3.43±0.12**	**48.03**	**1.75±0.16**	**76.15**	**0.75±0.07**	**67.67**

Fig.1 Growth inhibition of *Aspergillus parasiticus* NRRL 2999 strain in presence of 15 interacting fungi

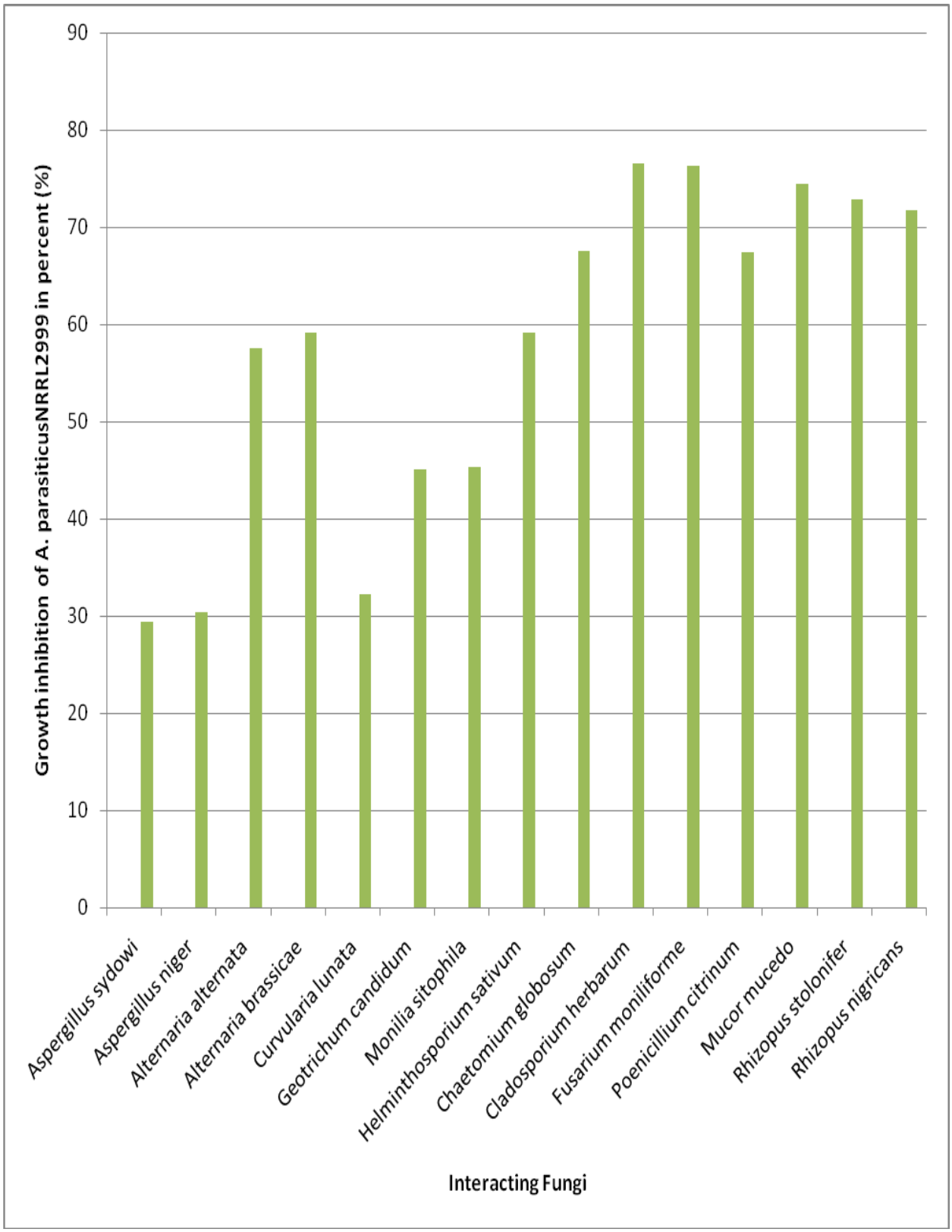
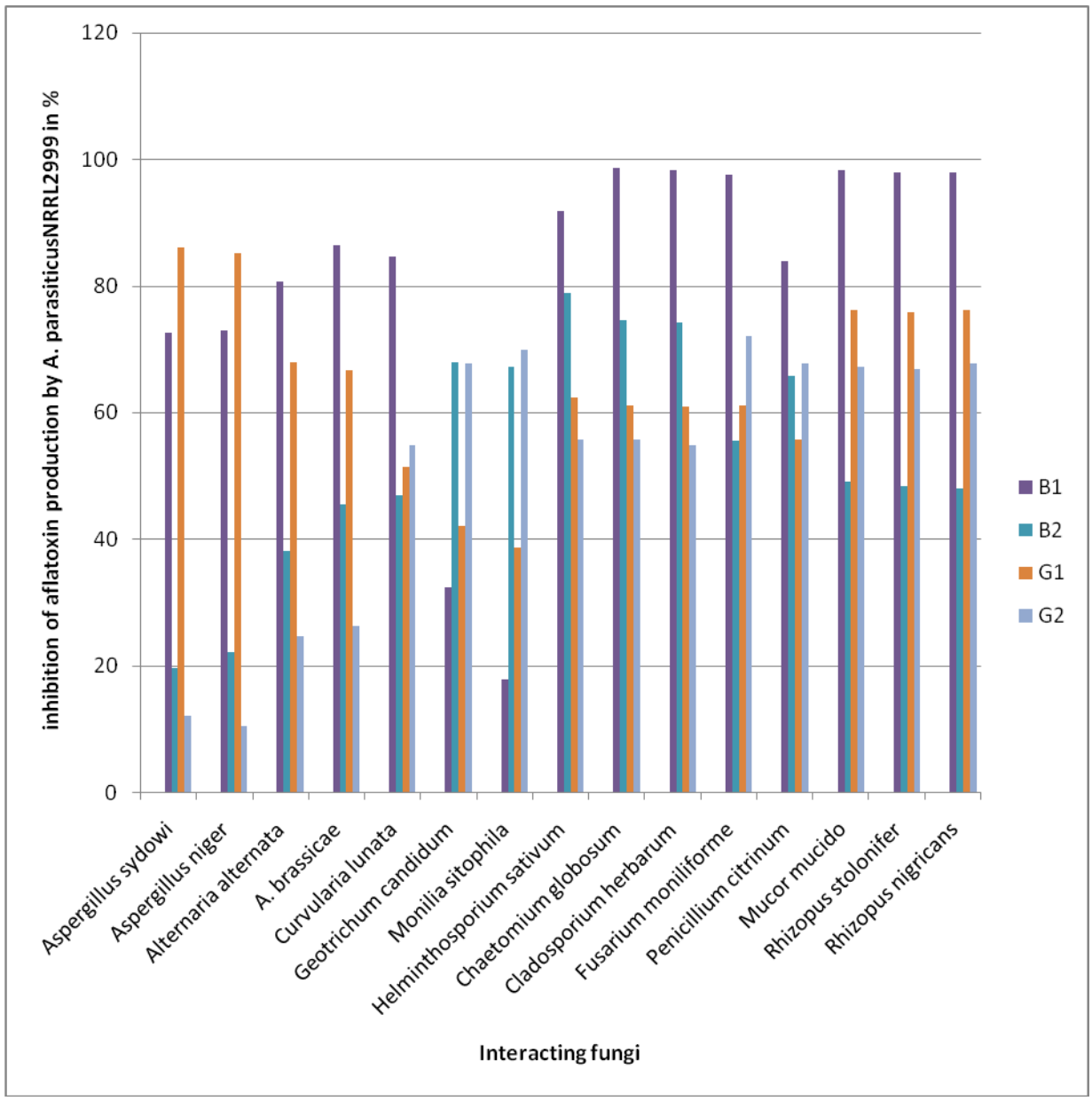


Fig.2 Inhibition of aflatoxin production in percent in presence of interacting fungi



The results confirm that the interacting fungi viz. *Aspergillus niger, A. sydowi, Alternaria alternata, A. brassicae, Curvularia lunata, Geotrichum candidum, Monilia sitophila, Helminthosporium sativum, Chaetomium globosum, Cladosporium herbarum, Fusarium moniliforme, Penicillium citrinum, Mucor mucido, Rhizopus nigricans* and *Rhizopus stolonifer* are the best biological agents to inhibit growth and Aflatoxin production ability of *Aspergillus parasiticus* NRRL2999 strain. The present findings are in agrement with the work of Roy and Kumar (1997), Chehal and Chehal (1993), Shivendra Kumar and Awadh K. Roy (2001) and Amer *et al.*, (1997) who have also noticed similar response of the inhibition. Inhibition of aflatoxin production has been observed in *Aspergillus flavus* when this was co-inoculated with *A. niger* (Tsubouchi *et*

*al.,*1980; Wicklow *et al.,* 1980). Degradation of aflatoxin B1 by *A. niger* and *Corynebacterium rubrum* has also been analysed by adding the C ^{14}- labelled aflatoxinB1 to culture of these microorganisms (Mann and Rehm, 1977). Inhibition of fungal growth and Aflatoxin B1 production in *Aspergillus parasiticus* by *Lactobacillus* strains viz. *L. plantarum* ATCC 4008, *L. plantarum* 12006, *Lactobacillus plantarum 299V, L. paracasei* subsp. *paracasei* LMG 13552 and *L.rhamnosus* VT1has been studied by Gomah Nanis H.*et al.,*(2009).

Similar results have been reported by other investigators. Antifungal activities by *Lactobacillus* species that inhibited both the growth and the aflatoxins production of *A. parasiticus* has been reported by Vanne, *et al.,* (2000) and Onilud, *et al.,* (2005).

Also, Coallier- Ascah and Idziak (1985) found that the inoculation of *A. flavus* spores into a culture of *Streptococcus lactis* in synthetic broth medium resulted in little or no aflatoxin accumulation even though the growth of the fungus was not hindered.

In general, it is suggested that such inhibitory response in *Aspergillus parasiticus* NRRL2999 is possibly due to antibiosis which might be accomplished by secretion of active metabolites by interacting fungi that inhibit growth and aflatoxin production. If, the active substances of these interacting fungi are chemically characterised, that would be a potential measure in the biological control of aflatoxin hazards.

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