African Journal of Biotechnology Vol. 9(27), pp. 4247-4250, 5 July, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Biological control of *Aspergillus flavus* growth and subsequent aflatoxin B_1 production in sorghum grains

K.R.N. Reddy[1*], C.R. Raghavender[2], B.N. Reddy[2] and B. Salleh[1]

[1]School of Biological Sciences, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia,
[2]Department of Botany, Osmania University, Hyderabad, India.

Accepted 7 April, 2010

Sorghum grains suffer from severe infection and colonization by several toxigenic fungi and subsequent production of mycotoxins, posing a threat to human and animal health. Among all the mycotoxins, aflatoxins represent one of the most important toxicants considered as important constraint of grain quality and sorghum production. Various physical and chemical methods of reducing mycotoxins have been recommended, but only few have been accepted for practical use. Biodegradation of aflatoxins, deploying microbes is an attractive strategy for mycotoxin management. This study, therefore explored the potential use of certain biocontrol agents for the reduction of growth of toxigenic *Aspergillus flavus* and subsequent aflatoxin B_1 (AFB1) production in sorghum. Among all the biocontrol agents tested, culture filtrate of *Rhodococcus erythropolis* completely inhibited the *A. flavus* growth and AFB1 production at 25 mlkg^{-1} concentration. The other biocontrol agents, *Bacillus subtilis*, *Pseudomonas fluorescens* and *Trichoderma viride* showed 72, 74 and 65% inhibition of *A. flavus* growth whereas 54, 62.6 and 39% reduction of AFB1 at 200 mlkg^{-1} of sorghum grains, respectively.

Key words: Sorghum, *Aspergillus flavus*, AFB1, biological control.

INTRODUCTION

Mycotoxin contamination and grain mold of sorghum are considered as the most important constraints of grain quality and production, globally. Sorghum grains suffer from infection and colonization by several fungi during panicle and grain developmental stages (Waliyar et al., 2008). Several species of *Aspergillus*, *Alternaria*, *Cladosporium*, *Diplodia*, *Fusarium*, *Curvularia*, *Phoma* and *Penicillium* are among the prevalent grain mold pathogens in sorghum (Bandopadyay et al., 2000). Infection by grain mold fungi and mold development are highly influenced by relative humidity and temperature. Furthermore, humid and warm conditions during flowering and grain development stages favour infection and mold development, whereas dry conditions prevent it (Thakur et al., 2006). Mycotoxin contamination is regarded as unavoidable. In other words, it is not possible to use current agronomic and manufacturing practices to entirely prevent their occurrence during cultivation, harvesting, storage and processing operations. Regulatory bodies in several countries carried out this risk assessment in order to establish regulatory guidelines aimed at protecting public health (López-García and Park, 1998; FAO, 2004; van Egmond and Jonker, 2005). Acute and chronic harmful effects of mycotoxins on human and animal health are serious problems in developing nations. Among all the mycotoxins, aflatoxin B_1 (AFB1) is considered as the most dangerous toxic metabolite because of its hepatotoxic, teratogenic, immunosuppressive and mutagenic nature. The International Agency for Research on Cancer has classified AFB1 as a probable human carcinogen (IARC, 1993).

The global occurrence of mycotoxins is considered a

*Corresponding author. E-mail: drkrnreddy@gmail.com. Tel: +604-6534001. Fax: +604- 6565125.

Abbreviations: AFB1, Aflatoxin B_1; **ELISA,** enzyme-linked immunosorbent assay; **IgG-ALP,** immunoglobulin G -alkaline phosphatase ; **BSA,** bovine serum albumin; **PBST,** phosphate buffered saline tween-20.

major risk factor, and according to the Food and Agricultural Organization (FAO), 25% of the world's commodities are annually affected by known mycotoxins (Schatzmayr et al., 2006). Mycotoxin contamination is a serious concern that occurs in the field before harvest or during storage, despite efforts of prevention (Lillehoj, 1983). Therefore, it is important to find practical, cost effective and non-toxic methods. Various methods have been developed by deploying various microbes to degrade these mycotoxins. Detoxification of mycotoxin-contaminated food and feed by microorganisms has been amply documented in the literature (Bhatnagar et al., 1991; Bata and Lásztity, 1999; Galtier, 1999; Karlovsky, 1999; Mishra and Chitrangada, 2003; Dorner, 2004; Shetty and Jespersen, 2006). This present study assessed the effects of certain microbes on growth and aflatoxin production by *Aspergillus flavus* infecting sorghum grains.

MATERIALS AND METHODS

Preparation of biocontrol agents

The bacterium, *Rhodococcus erythropolis* (MTCC 1526) was cultivated in standard 1 broth (Goodfellow, 1986) comprising 1.5% (w/v) peptone, 0.3% (w/v) yeast extract, 0.6% (w/v) sodium chloride and 0.1% (w/v) glucose for 48 h at 30°C in a shaker and the culture was centrifuged at 10,000 g for 10 min to get cell-free filtrate. *Pseudomonas fluorescens* culture (OU-593) was grown in King's B broth for 4 days at 28°C under shaker and the cultures were centrifuged at 10,000 g for 10 min to get cell-free filtrate (Reddy et al., 2007). The bacterium, *Bacillus substilis* (MTCC 121) was grown in nutrient broth for 48 h at 28°C in a shaker and the culture was centrifuged at 10,000 g for 10 min to get cell-free filtrate. The fungus, *Trichoderma viride* (MTCC 800) was grown on potato dextrose broth for seven days at 28°C under shaker and the culture filtrate was collected aseptically by filtration using Whatman No 41 to get cell-free filtrate (Choudary et al., 2007).

Efficacy of biocontrol agents on the growth of *A. flavus*

The stored seeds of sorghum line (GM-8 2006) which showed a natural infection of *A. flavus* (100%) were used. These naturally infected sorghum grains were treated with culture filtrates of *R. erythropolis* at a concentration of 5, 10 and 25 $mlkg^{-1}$ of sorghum seeds. Similarly, the culture filtrates of *B. subtilis*, *P. fluorescens* and *T. viride* at a concentration of 100, 150 and 200 $mlkg^{-1}$ of sorghum grains were treated. Deploying the agar plate technique of the international seed testing association (1966), 400 sorghum grains were treated with these culture filtrates and plated on a one-half strength potato dextrose agar medium containing Rose Bengal at a concentration of 50 ppm (Cotty, 1994). The plates were then incubated for 5 days at room temperature, and the incidence of *A. flavus* colonies was counted and percent inhibition calculated. For each treatment of biocontrol agent, 16 replications were maintained; whereas sterile broth used for the growth of biocontrol agents served as control.

Preparation of *A. flavus* spore suspension

Stock culture of aflatoxin B_1 producing *A. flavus* (MTCC 2798) was maintained on potato dextrose agar slants at 4°C and was harvested by adding 10 ml of sterile distilled water to get the final concentration to 10^{12} spore/ml.

Testing of biocontrol agents on AFB1 produced by *A. flavus* on sorghum grains

The healthy sorghum lines (SGMR 12-3-1) were treated with all 4-biocontrol agents at the above-mentioned concentrations and subsequently, these 20 g sorghum grains were inoculated with 1 ml spore suspension of *A. flavus* and incubated at 25°C for 5 days. Furthermore, the extraction of AFB1 from each of these samples was done with 0.5% KCl (100 ml) in 70% methanol and blended in a waring blender. This extraction was followed by agitating the mixture for 30 min using a rotary shaker and then filtered through a Whatman No. 41 paper. This filtrate was diluted 10-folds with phosphate-buffered saline containing 0.05% tween-20 and 0.2% bovine serum albumin (PBST-BSA) prior to subjecting these samples to enzyme-linked immunosorbent assay (ELISA) (Reddy et al., 2009).

Materials for ELISA and procedure for the AFB1 detection by ELISA

AFB1-BSA conjugate, goat anti-rabbit immunoglobulin G -alkaline phosphatase (IgG-ALP) conjugate, *ρ*-nitrophenyl phosphate and bovine serum albumin (BSA) purchased from Sigma (St Louis, USA) and microtitre plates (Maxi-sorp F96) were from Nunc (Nalge Nunc International, Denmark). All other chemicals were reagent grade and/or chemically pure. Highly specific polyclonal antibodies for AFB1 that did not cross-react with other aflatoxins in indirect competitive ELISA procedure (Thirumala-Devi et al., 1999) were purchased from International Crop Research Institute for the Semi-Arid Tropics, Patancheru, India.

The detection of AFB1 from sorghum grains was estimated by deploying the method of Reddy et al. (2009). ELISA microtitre wells were coated with 100 $ngml^{-1}$ of AFB1-BSA in sodium carbonate buffer [pH 9.6; 150 µl/well] and left overnight at 4°C. These plates were then washed in PBST, added with 0.2% BSA and allowed to stand at 37°C for 1 h. ELISA plates were again washed with PBST and added with 100 µl AFB1 standards ranging from 25 ng to 10 $pgml^{-1}$. Pre-incubation was carried out with 50 µl antiserum diluted in PBST-BSA (1: 6000) and held for 45 min at 37°C. Filtrate samples extracted from sorghum grains with aqueous methanol-KCl as described earlier were added to wells at 1: 10 dilution in PBST-BSA. Goat anti-rabbit immunoglobulins conjugated to alkaline phosphatase were used at a 1: 4000 dilution to detect rabbit antibodies attached to AFB1-BSA. *ρ*-Nitrophenyl phosphate was used as a substrate at 0.5 mg/ml. Absorbance was recorded at 405 nm with an ELISA plate reader (Biorad-680) after incubation at 28°C for 45 - 60 min. Standard curves were obtained by plotting log_{10} values of AFB1 dilutions at A_{405}. AFB1 ($ngml^{-1}$) in samples was determined from the standard curves as: AFB1 μgkg^{-1} of sorghum grain = [aflatoxin ($ngml^{-1}$) in sample x buffer (ml) x extraction solvent (ml)]/sample weight (g). To recover the AFB1, 20 g of uninfected and healthy sorghum grains were mixed with pure AFB1 in a concentration range of 5 to 100 μgkg^{-1} and these samples were extracted and assayed as unknown samples (Reddy et al., 2009).

RESULTS AND DISCUSSION

Effect of biocontrol agents on the growth of *A. flavus* and AFB1 production

B. subtilis inhibited the growth of *A. flavus* (72%) leading

Table 1. Effect of biocontrol agents on growth of *A. flavus* and AFB1 production.

Culture filtrate and concentration (mlkg^{-1})	*A.flavus*		AFB1 (µgg^{-1} of seed)	Reduction over control (%)
	Colonies after treatment	Inhibition over untreated control (%)		
B. subtilis				
100	52	48	3.21	15.5
150	41	59	2.40	36.8
200	28	72	1.75	54.0
P. fluorescens				
100	42	58	3.02	20.5
150	31	69	2.65	30.2
200	26	74	1.42	62.6
R. erythropolis				
5	65	35	2.58	32.1
10	13	87	1.02	73.1
25	0	100	0	100
T. viride				
100	100	0	3.78	0.05
150	49	51	3.52	0.07
200	35	65	2.32	39.0
Control	100	0	3.80	0
CD (P > 0.05)	3.5	-	1.06	-
CV (%)	1.9	-	0.52	-

to the reduction of AFB1 (54%) at a concentration of 200 mlkg^{-1} of sorghum grains. *P. fluorescens* culture filtrate showed a 74% inhibition of *A. flavus* growth and 62.6% AFB1 reduction. *R. erythropolis* showed a percent inhibition of *A. flavus* growth and AFB1 reduction. Similarly, *T. viride* showed an inhibition of 65% on *A. flavus* growth and 39% AFB1 reduction at 200 mlkg^{-1} of sorghum grain (Table 1). It has been stated in literature that *Bacillus* spp., *Pseudomonas* spp., *Rhodococcus* spp. and *Trichoderma* spp. are able to inhibit mycotoxigenic fungi and degrade mycotoxins including AFB1 (Etcheverry et al., 1998; Chaurasia, 1995; Podile and Prakash, 1996; Munimbazi and Bullerman, 1998; Reddy et al., 2004; Stander et al., 2000; Reddy et al., 2009; Alberts et al., 2006; Teniola et al., 2005; Mann and Rehm, 1976; Kumar and Popat, 2007).

Recovery of AFB1 from sorghum samples

The sensitivity and correctness of the extraction procedure was confirmed by supplementing pure AFB1 to ground sorghum grain and extracted in 70% methanol-KCl. Recoveries from sorghum samples estimated by ELISA were greater than 95%.

CONCLUSION

In summary, these microbiologicals showed a considerable inhibition of *A. flavus* growth and AFB1 reduction. The inhibitory activity was likely due to the extracellular nature of the metabolites produced by these microbes in the growth medium. Therefore, the potential use of these microbial metabolites to control mycotoxigenic fungi should be thoroughly investigated since many chemical fungicides are being taken out from the commercial market.

ACKNOWLEDGEMENTS

Dr. K.R.N. Reddy acknowledges the Universiti Sains Malaysia, Penang, Malaysia for providing a postdoctoral fellowship. We are grateful for the research grants 1001/PBIOLOGI/811009 and 1001/PBIOLOGI/811147 provided by USM.

REFERENCES

Alberts JF, Engelbrecht Y, Steyn PS, Holzäpfel WH, Vanzyl WH (2006). Biological degradation of aflatoxin B_1 by *Rhodococcus erythropolis*

cultures. Int. J. Food Microbiol. 109: 121-126.
Bandopadyay R, Butler DR, Chandrashekar A, Reddy RK, Navi SS (2000). Biology, epidemiology and management of sorghum grain mold. In: Chandrashekar A, Bandyopadhyay R, Hall AJ, editors. Technical and institutional options for sorghum grain mold management: Proceedings of an international consultation. International Crops Research Institute for Semi-Arid Tropics, Patancheru 502324, Andhra Pradesh, Indian. pp. 34-71.
Bata A, Lásztity R (1999). Detoxification of mycotoxin-contaminated food and feed by microorganisms. Trends Food Sci. Technol. 10: 223-228.
Bhatnagar D, Lillehoj EB, Bennett JW (1991). Biological detoxification of mycotoxins. In: Smith JE, Henderson, RS editors. Mycotoxins and animal foods. CRC Press, Boston, MA: pp. 816-826.
Chaurasia HK (1995). Kernel infection and aflatoxin production in peanut (*Arachis hypogaea* L) by *Aspergillus flavus* in presence of geocarpospheric bacteria. J. Food Sci. Technol. 32(6): 459-464.
Choudary DA, Reddy KRN, Reddy MS (2007). Antifungal activity and genetic variability of *Trichoderma harzianum* isolates. J. Mycol. Plant Pathol. 37(2): 295-300.
Cotty PJ (1994). Comparison of four media for the isolation of *Aspergillus flavus* group fungi. Mycopathologia, 125(3): 57-62.
Dorner JW (2004). Biological control of aflatoxin contamination of crops. J Toxicol - Toxin Rev. 23(2&3): 425-450.
Etcheverry M, Cavaglieri L, Chulze S (1998). Microbial interaction of *Aspergillus parasiticus* and *Bacillus subtilis* with *Alternaria alternata*. Production of alternariol, alternariol monomethylether and tenuazonic acid on sunflower seeds. Mycotoxin Res. 14(1): 2-8.
FAO (2004). Worldwide regulations for mycotoxins for 2003. A compendium. Food and Nutrition Paper No. 81. Rome, Italy. Food Agric. Organ. United Nations.
Galtier P (1999). Biotransformation and fate of mycotoxins. J. Toxicol. Toxin Rev. 18: 295-312.
Goodfellow M (1986). Genus *Rhodococcus*. In: Sneath PHA, Mair NS, Sharpe ME & Holt JG, editors. Bergeys Manual of Systemic Bacteriology, Vol. 2. The Williams and Wilkins Co., Baltimore, USA, pp. 1472-1481.
IARC (1993). Some naturally occurring substances: Food items and constituents, heterocyclicaromatic amines and mycotoxins. IARC monographs on the evaluation of carcinogenic risks to humans. International Agency for Research on Cancer, Geneva, 56: 489-521.
ISTA (1966). International rules for seed testing. Proc. Int. Seed Test Assoc. 31: 49-85.
Karlovsky P (1999). Biological detoxification of fungal toxins and its use in plant breeding, feed and food production. Nat. Toxins, 7: 1-23.
Kumar GDS, Popat MN (2007). Knowledge and adoption of aflatoxin management practices in groundnut farming in Junagadh, Gujarat, India. SAT e-Journal 3(1): 1-2.
Lillehoj EB (1983). Effect of environmental and cultural factors on aflatoxin contamination of developing corn kernels. In: Diener UL, Asquith RL, Dickens JW, editors. Aflatoxin and *Aspergillus flavus* in corn. Department of Research Information, Alabama Agricultural Experiment Station, Auburn University, Auburn, USA, pp. 27-34.
López-García R, Park DL (1998). Effectiveness of postharvest procedures in management of mycotoxin hazards. In: Sinha KK, Bhatnagar D, editors. Mycotoxins in Agricultural and Food Safety, Marcel Dekker, New York, USA, pp. 407-433.
Mann R, Rehm HJ (1976). Degradation products from aflatoxin B1 by *Corynebacterium rubrum*, *Aspergillus niger*, *Trichoderma viride* and *Mucor ambiguus*. Eur. J. Appl. Microbiol. 2: 297-306.
Mishra HN, Chitrangada D (2003). A review on biological control and metabolism of aflatoxin. Crit. Rev. Food Sci. Nutr. 43(3): 245-264.
Munimbazi C, Bullerman LB (1998). Inhibition of aflatoxin production of *A. parasiticus* NRRL 2999 by *Bacillus pumilus.* Mycopathologia 140:163-169.
Podile AR, Prakash AP (1996). Lysis and biological control of *A. niger* by *Bacillus substilis* AF1. Can. J. Microbiol. 42: 533-538.
Reddy CS, Reddy KRN, Kumar RN, Laha GS, Muralidharan K (2004). Exploration of aflatoxin contamination and its management in rice. J. Mycol. plant Pathol. 34(3): 816-820.
Reddy KRN, Choudary DA, Reddy MS (2007). Antifungal metabolites of *Pseudomonas fluorescens* isolated from rhizosphere of rice crop. J. Mycol. Plant Pathol. 37(2): 280-284.
Reddy KRN, Reddy CS, Muralidharan K (2009). Potential of botanicals and biocontrol agents on growth and aflatoxin production by *Aspergillus flavus* infecting rice grains. Food Control, 20(2): 173-178.
Schatzmayr G, Zehner F, Täubel M, Schatzmayr D, Klimitsch A, Loibner AP, Binder EM (2006). Microbiologicals for deactivating mycotoxins. Mol. Nutr. Food Res. 50: 543-551.
Shetty PH, Jespersen L (2006). *Saccharomyces cerevisiae* and lactic acid bacteria as potential mycotoxin decontaminating agents. Trends Food Sci. Technol. 17: 48-55.
Stander MA, Bornscheuer UT, Henke E, Steyn PS (2000). Screening of commercial hydrolases for the degradation of ochratoxin A. J. Agric. Food Chem. 48(11): 5736-5739.
Teniola OD, Addo PA, Brost IM, Farber P, Jany KD, Alberts JF, Vanzyl WH, Steyn PS, Holzäpfel WH (2005). Degradation of aflatoxin B_1 by cell free extracts of *Rhodococcus erythropolis* and *Mycobacterium fluoranthenivorans* sp. Int. J. Food Microbiol. 105: 111-117.
Thakur RP, Reddy BVS, Indira S, Rao VP, Navi SS, Yang XB, Ramesh S (2006). Sorghum grain mold. Information Bulletin no. 72, Int. Crops Res. Institute for Semi-Arid Tropics, Patancheru 502324, Indian: p. 32.
Thirumala-Devi K, Mayo MA, Reddy KLN, Delfosse P, Reddy G, Reddy SV, Reddy DVR (1999). Production and characterization of monoclonal antibodies for aflatoxin B_1. Lett. Appl. Microbiol. 29: 284-288.
van Egmond HP, Jonker MA (2005). Worldwide regulations on aflatoxins. In: Abbas HK, editor. Aflatoxin and Food Safety, CRC Press, USA, pp. 77-94.
Waliyar F, Reddy CR, Alur AS, Reddy SV, Reddy BVS, Reddy AR, Rai KN, Gowda CLL (2008). Management of grain mold and mycotoxins in sorghum. International Crops Research Institute for Semi-Arid Tropics, Patancheru 502 324, Indian, p.32.