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Control of aflatoxin biosynthesis in peanut with geocarposphere bacteria : a biotechnological approach for sustainable development

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ABSTRACT

Roots and pods of field grown peanut were sampled at three developmental stages and a total of seven bacterial strains and one toxigenic Aspergillus flavus isolates were isolated from the geocarposphere. The biocontrol potential of each bacterial strains was tested against growth and Afl-B_1 production potential of toxigenic A. flavus. In green house experiments, co-inoculation with toxigenic A. flavus and geocarposphere bacteria in root regions of 1 to 2 week old peanut plants resulted in lower synthesis of Afl-B_1 in the peanut kernels at maturity. Of the seven bacterial strains tested, four strains showed reduction in aflatoxins production in varying extents. Pre- inoculation of bacterial strains (1-day earlier) resulted in greater inhibition of aflatoxin accumulation than those bacterial strains introduced 1-day after inoculation of toxigenic A. flavus strain. Bacillus megaterium showed maximum inhibition of aflatoxin biosynthsis, as compared to remaining three potential bacterial strains.

***Keywords*:** Aflatoxin, bacteria, control, geocarposphere, peanut

Aflatoxins, the toxic metabolities of *Aspergillus flavus* Link and *A. parasiticus Speare*, pose serious health hazards as potent carcinogens to humans and domestic animals, because of their frequent occurrence in agricultural commodities including cereals and spices (Chourasia, 2001; Chourasia *et al*., 2008; Mandal *et al.*, 2010). Soil surrounding the peanut pod, *i.e.*, the geocarposphere contains higher populations of microorganisms than buffer soil (Kloepper and Bowen, 1991).

In recent past, a wide range of chemicals and bio-agents have been tested against pre- and post-harvest aflatoxin contamination of peanut kernels (Bowen and Backman, 1989). However, paucity of potential bio-competitive agents is striking particularly among geocarposphere bacteria (Dorner *et al.*, 1990; Chourasia, 1995). It was hypothesized that geocarposphere bacteria would be ideal candidates for protecting the developing peanut pods against aflatoxigenic fungi. The present study was aimed to study the biocontrol potential of geocarposphere bacteria against aflatoxin biosynthesis in peanut.

MATERIAL AND METHODS

Sampling procedures

Three samples of different stages *i.e.* emergence of peg, swelling of peg, and full-size pod with visible seed cotyledon were used. Six replicate plants were randomly selected on each sampling date. Six replicate soil samples were also collected from nonplanted buffer zones and shaken in 10 ml 0.2 M phosphate buffer pH 7.0 (PB). Peg and pod samples were macerated with sterile mortars and pestles and all samples were plated on tryptic soy agar for bacterial collection. Plates for bacteria were incubated at 28^0C for 24-96 hr and colonies were enumerated using, a laser colony counter with bacterial enumeration software (Spiral System Instruments, Bethesda, Maryland). Pure culture of fungi were incubated for 7 days at 25^0C and *A. flavus* was identified under a Nikon stereomicroscope by following the manual of Barnett and Hunter (1972). Bacterial strains were identified with the help of bacterial enumeration software system and physiological characterization. The identification of bacterial strains and *A. flavus* was made by sending the pure culture to IMTECH, Chandigarh as reference.

Inoculum preparation

A. flavus was grown on PDA and stored at 4^0C. Spores were harvested by flushing 5 days-old cultures in a sterile 0.01 per cent solution of Triton X-100. The spore concentration was standardized to $3x10^6$ spores/ml by using an improved Neubauer hemocytometer with suitable dilutions. All bacteria under study were grown on nutrient agar slopes at 28^0C and the suspension of 10^5 cells/ml was used as the inoculum potential.

Antibiosis test of bacteria against *A. flavus*

Organisms were inoculated individually or in pairs on PAF (Pseudomonas Agar Fluoroscent) and TSA (Tryptic Soya Agar) culture media separately. In case of dual cultures, colony diameters and the gap between the colonies were measured daily from 2 to 15 days and microbial interactions on culture media were classified by the method of Johnson and Curl (1972).

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Screening of bacteria in the assays

Seed assay

Surface-disinfested and dried peanut seeds (5 subsamples per bacterial strain) were soaked in each bacterial suspension for 5 minutes and then placed on water agar. Controls consisted of seeds soaked in sterile water. After incubation for 24 hours at 28^0C, seeds were inoculated with 20 ml of a log 6 conidial suspension of *A. flavus* and incubated at 30^0C. Controls of no bacteria with and without *A. flavus* were included. Data on fungal growth were collected.

Root-radicle assay

Bacterial suspensions were prepared as described above, and surface-disinfested and dried seeds with emerged radicles were soaked in each suspension for 5 minutes. After 24 hr incubation at 28^0C, 20 ml of a log 6 conidial suspension of *A. flavus* were inoculated on the radicles 1.0 cm from the seed. Inoculated seeds were incubated and data on fungal growth, number of root branches, final root length, and time to complete radicle coverage with mycelial growth were recorded.

Inoculation of bacteria / toxigenic *A. flavus* in the root-regions of peanut plant under greenhouse condition

Sound mature peanut kernels were surface-disinfested by agitating in 20 per cent household bleach and drying at 25^0C for 1 hr in open petridishes in a laminar flow hood. Surface disinfested kernels were germinated in 3 pot^{-1} containing promix, moistened and autoclaved twice before use. At 7 days after emergence of plant from promix (at two leaves stage), root region of each plant was inoculated by known inoculum of bacteria and toxigenic *A. flavus* strains with an adjustable pipette having a disposable tip. In co-inoculation experiment, root region was inoculated simultaneously with toxigenic *A. flavus* and selected bacterial strain. In pre-inoculation experiment, bacterial strain was inoculated 1-day earlier inoculation of toxigenic *A. flavus* strain. Similarly, in post-inoculation experiment, bacterial strain was inoculated 1-day after inoculation of toxigenic *A. flavus* strain. In all the tests, peanut pods were harvested at maturity (14 weeks after inoculation) and dried at 60^0C for 48 hr. Dried kernels were kept at room temperature in sealed plastic bags. All the experiments were performed twice, with six replicates for each treatment in each experiment.

Aflatoxin extraction and quantification

Aflatoxin was extracted by reversed phase high pressure liquid chromatography (Stubblefield and Shotwell, 1977). The mobile phase consisted of HPLC grade acetonitrile : tetrahydrofuran : water (10 : 6 : 84, v/v/v), adjusted to pH 3.9 with acetic acid. The flow rate was 2 ml min^{-1}.

RESULTS AND DISCUSSION

Table 1: Effect of *A. flavus* inoculum density on fungal development on seeds.

Inoculum density of *A. flavus*	Hrs* to first appearance of fungus	Hrs* to complete coverage of seeds by the fungus
Log 6 spores ml^{-1}	60.62	97.25
Log 5 spores ml^{-1}	80.50	108.50
Log 4 spores ml^{-1}	82.50	109.50
Log 3 spores ml^{-1}	85.50	109.75
Log 2 spores ml^{-1}	89.16	110.16
50 spores ml^{-1}	92.23	115.66
25 spores ml^{-1}	93.20	117.20

** Mean value of eight replicates*

Seed assay

The concentration of *A. flavus* inoculum had a significant effect on fungal growth in both the seed and root-radicle assays. Increasing the inoculum concentration of *A. flavus* from 25 spores. ml^{-1} to log 6 spores/ml resulted in a 20 hr difference in time to complete coverage of seeds by mycelia (Table 1).

An inoculum concentration of log 6 conidia per seed was selected for routine screening, since this gave complete coverage of seeds by mycelia within 4 days, which was deemed a good time frame for screening. It could therefore be inferred that the complete coverage of the seed by mycelia was dependent of the amount of inoculum. In these studies, the cultures developing from dilute inocula hardly sporulated even after a prolonged incubation, however sporulation was much faster and more profuse in the cultures developing from greater inoculum levels, suggesting the involvement of mycelia differentiation in aflatoxin production.

Root-radicle assay

More effects of inoculum concentration were noted in the root-radicle assay (Table-2) than the seed assay. Inoculum concentrations of 25 spores/ml and 50 spores ml^{-1} were ineffective in the development of fungus on the root-radicle, and log 6 conidia per radical was selected as the rate for routine screening. With this rate, mycelia were first apparent approximately 3 days after inoculation. results of screening biocontrol agents in the root-radicle assay reveal that some bacteria completely prevent fungal growth, while others allow limited growth but prevent complete mycelia coverage of inoculated radicles. In addition, pronounced root growth enhancement was also noticed with some bacterial strains.

Table 2: Effect of *A. flavus* inoculum density on fungal development on root-radicles.

Inoculum density of *A. flavus*	Hrs* to first appearance of fungus	Hrs* to complete coverage of seeds by the fungus
Log 6 spores ml^{-1}	51.80	72.00
Log 5 spores ml^{-1}	54.57	75.00
Log 4 spores ml^{-1}	62.00	81.20
Log 3 spores ml^{-1}	76.00	99.60
Log 2 spores ml^{-1}	82.00	114.00
50 spores ml^{-1}	—	—
25 spores ml^{-1}	—	—

** Mean value of eight replicates*

In these assay experiments with 7 geocarposphere bacteria with known biocontrol activity against toxigenic *A. flavus* on peanut, 4 were found effective against growth and sporulation of *A. flavus.*

Antibiosis test of bacteria against *A. flavus*

All seven bacterial strains mentioned above, were screened for antibiosis towards *A. flavus* using two different media (PAF and TSA). Two bacterial strains showed a minimum inhibitory effect (0 to 1 mm), while one bacterial strain had maximum effect (5 to 10 mm) on PAF medium. However, on TSA medium, maximum zones of inhibition (10 to 12 mm) were observed with four bacterial strains (Table 3). Thus TSA medium appeared to be a suitable medium for testing antibiotic effect of different types of bacteria on *A. flavus*.

Table 3: No. of bacterial strains exhibiting antibiosis against *A. flavus on culture media.**

	Zone of Inhibition (mm)				
	0 – 1	1 – 3	3 – 5	5 – 10	10+
PAF	2	2	2	1	0
TSA	0	0	1	2	4

** Based on evaluation of 7 bacterial strains from geocarposhere of peanut plants.*

Inoculation of bacteria / toxigenic *A. flavus* in the root-regions of peanut plant under greenhouse condition

In co-inoculation experiments with toxigenic *Aspergillus flavus* and geocarposphere bacteria resulted in lower synthesis of Afl-B_1 in the peanut kernels as compared to control. Of seven bacterial strains tested, four strains showed reduction in aflatoxins production in varying extents (Table 4). One-day earlier inoculation of bacterial strains resulted greater reduction in aflatoxin accumulation. *Bacillus megaterium* showed maximum inhibition of aflatoxin biosynthsis, as compared to remaining three potential bacterial strains.

In greenhouse experiments, significant level of Afl-B_1 (48.20 mg g^{-1}) was produced in peanut kernels when toxigenic strains of *A. flavus* was inoculated alone. A mixed type of results in Afl-B_1 production potential of *A. flavus* (i.e. highest, lowest and moderate level) were found in three different co-inoculation experiments with bacteria. When *A. flavus* was inoculated simultaneously with each of the 7 bacterial strains, Afl-B_1 production increased in case of all bacterial strains except *B. megaterium*. Interestingly, a 5-fold increase in Afl.-B_1 level (230.90 mg g^{-1}) was found when *A. flavus* was co-inoculated with *B. laterosporous*. Similarly, 2-and 1.5-fold increase in Afl.-B_1 level were noticed with *C. cartae* and *P. rubiacearum,* respectively. In pre-inoculation experiment, when bacterial strains were inoculated 1-day earlier of *A. flavus* inoculation, 4 bacterial strains showed greater reduction in aflatoxin production in peanuts. The maximum reduction or negligible amount of Afl-B_1 (3.47 mg g^{-1}) in peanut kernels was found with *B. megaterium*. Similar reduction in Afl-B1 level was recorded with *B. laterosporous* (9.12 mg g^{-1}), *P. aurofaciens* (15.5 mg g^{-1}) and *X. maltophila* (19.17 mg g^{-1}). In post-inoculation experiment i.e. when bacterial strains were inoculated 1-day after inoculation of *A. flavus*, Afl-B_1 level was not much reduced with all 7-bacterial strains.

The present results of co-inoculation experiments indicate that almost all geocoposphere bacteria, except *B. megatorium* stimulated the overall level of Afl-B_1 production by *A. flavus*. The 5-fold, 2-fold and 1.5-fold increase in Afl-B_1 production with *B. laterosporous*, *C. cartae* and *P. rubiacearum*, respectively is might be due to production of certain metabolites by these bacteria which altered the substrate, enhancing the growth of *A. flavus* or its ability to produce aflatoxin or both. It is possible that these bacterial strains could here enzymatically changed the substrates, making them more favourable for aflatoxin biosynthesis. Similarly, compounds from these organisms could have been released, leading to enhanced growth or aflatoxin production or both. It is also possible that these bacterial strains could attached to the hyphae of *A. flavus* and travelled with hyphae into the tisue, thereby altering not only the substrate but also the fungal wall membrane structure, changing its diffusibility or increasing the rate at which metabolites, such as the aflatoxins, can leak through the membrane.

The results of pre-inoculation experiments show that out of 7 bacterial strains, two strains viz; *B. megaterium* and *B. laterosporous* reduced Afl-B_1 synthesis by 90-100 per cent. The reduction in aflatoxin biosynthesis might be due to the nutritional competition, altering the levels of O_2 or CO_2 between the bacterial and toxigenic *A. flavus* strains, thereby exhibiting pronounced changes

Table 4 : Effect of geocarposphere bacteria on Afl-B_1 production in peanut kernels by toxigenic *A. flavus* strain.

Organisms	Afl-B_1 content* of peanut kernels (mg g^{-1})			
	Inoculated alone	Co-inoculated Au-32 and bacteria	Inoculated bacteria 1- day earlier Au-32	Inoculated bacteria 1- day after Au-32
Toxigenic *A. flavus* (Au-32) - control	48.20	—	—	—
A.F./*Bacillus megaterium*	—	36.60	3.47	30.50
A.F./*B. laterosporus*	—	230.90	9.12	35.10
A.F./*Cellulomonas cartae*	—	98.65	40.07	42.19
A.F. /*Flavobacterium odoratum*	—	51.10	35.40	40.10
A.F. /*Phyllobacterium rubiacearum*	—	75.65	37.16	40.80
A.F. /*Pseudomonas aurofaciens*	—	57.15	15.50	37.20
A.F. /*Xanthomonas maltophila*	—	50.45	19.17	38.15

** Mean value of eight replicates*

in growth, sporulation and toxin production. The degradation, detoxification and absorption of toxin by bacterial strains can cause reduction in aflatoxin level. Bacterial cells can degrade the aflatoxin produced by *A. flavus*, possibly via production of cell wall lytic enzymes.

The results suggest the potential of bacteria in inhibition of pre-harvest aflatoxin biosynthesis in developing peanuts. Detailed investigations are desirable to bring the advantages of this greenhouse experiment to a field level and to evaluate the practicability of the approach. These biocontrol agents could be exploited for sustainable development in peanut.

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