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Influence of biocontrol agents on population density of *Aspergillus flavus* and kernel infection in groundnut

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ABSTRACT: Aflatoxin contamination of groundnut, caused by *Aspergillus flavus* (Af) group of fungi, is a major problem in the rainfed agriculture in the semi-arid tropics. Biological control could be one of the components of integrated management to reduce pre-harvest kernel infection in the field. Six *Trichoderma* and three *Pseudomonas* strains that were identified as highly antagonistic to Af 11-4 (a highly toxigenic strain) *in vitro*, were evaluated in field to determine their biocontrol potential. The antagonists were applied as seed dressing and soil application at flowering in Af-sick plots. All the antagonists significantly reduced seed infection in all three field experiments. Two *T. viride* (Tv 17 and Tv 23), one *T. harzianum* (Th 23), and one *Pseudomonas* (Pf 2) isolates provided greater protection to seed infection by Af 11-4 than others. The reduced seed contamination occurred due to significant reduction in Af population in the rhizosphere of groundnut.

Key words: Groundnut, aflatoxin, *Aspergillus flavus*, *Trichoderma*, *Pseudomonas*, biocontrol

Aflatoxin contamination of groundnut (*Arachis hypogaea* L.) kernels by *Aspergillus flavus* Link ex Fries and *A. parasiticus* Speare, seriously affects the quality of the produce, both food and feed (Cotty *et al.*, 1994). It has tremendous impact on the global groundnut industry, on consumers, and poses health risks to humans and cattle. Aflatoxins, a group of secondary metabolites, produced by *A. flavus* group of fungi, are known to be highly carcinogenic and immunotoxic and cause growth retardation in animals (Hall and Wild, 1994) and young children (Gong *et al.*, 2002). Among several forms of aflatoxins known, aflatoxin B_1 is the most toxic and also most commonly found in several food and feed commodities (Bryden, 1991; Cullen and Newberne, 1994; Urano *et al.*, 1992). Factors governing preharvest kernel infection and aflatoxin production are several and highly interactive (Dorner *et al.*, 1989). Despite considerable research efforts around the world, stable genetic resistance to seed infection in groundnut by *A. flavus* has not been identified (Anderson *et al.*, 1995; Mehan *et al.*, 1987; Upadhyaya *et al.*, 1997; Thakur, 2000). A number of other approaches have been advocated and tested for reduction of aflatoxins in groundnuts, such as improved farm management techniques and post-harvest procedures involving drying and storage. One of the promising approaches is biological control by competitive exclusion of aflatoxigenic aspergilli in the integrated management of aflatoxin contamination in groundnut. The use of non-toxigenic strains of *A. flavus* to counteract toxin-producing strains in the environment by competitive exclusion has been demonstrated on corn (Brown *et al.*, 1991), cottonseed (Cotty, 1990) and groundnut (Dorner *et al.*, 1992, 1998). In another strategy, geocarposphere bacterial strains were used to reduce groundnut pod colonization by *A. flavus* (Mickler *et al.*, 1995). In earlier studies (Anjaiah and Thakur, 2000; Desai *et al.*, 2000) several isolates of *Trichoderma* and *Pseudomonas* were characterized for their antagonism against *A. flavus* and for their biocontrol potential. In this study, we used selected isolates of *Trichoderma* spp. and *Pseudomonas fluorescens* both as seed dressing

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and soil application to determine their effects on population dynamics of aflatoxigenic population of *A. flavus* in the geocarposphere and subsequently on infection of groundnut kernels.

MATERIALS AND METHODS

Biocontrol agents (BCAs)

Six isolates of *Trichoderma spp.* and three of *Pseudomonas fluorescens* that were highly antagonistic to a highly toxigenic strain of *A. flavus* (Af 11-4) and nonpathogenic to groundnut plants (Anjaiah and Thakur, 2000) were used as potential BCAs. These were: *Trichoderma harzianum* (Th 13), *T. longibrachiatum* (Tl 16), *T. viride* (Tv 17), *T. viride* (Tv 20), *T. harzianum* (Th 23), *Trichoderma sp* (T 28), *Pseudomonas fluorescens* (Pf 2), *P. fluorescens* (Pf 76), and *P. fluorescens* (Pf 153).

Field experiment

Ten treatments (nine BCAs and one control) were used in a randomized complete block design with four replications. Each plot consisted of 2 rows of 4 m long, rows 60 cm apart and plants at 10 cm within a row. The experiment was conducted using a groundnut cultivar JL 24 in three environments, two at Patancheru (rainy and postrainy seasons) and one at Anantapur (rainy season) during 2000-2001.

Inoculum preparation and soil application of *A. flavus*

Inoculum of *A. flavus* (Af 11-4) was multiplied on autoclaved pearl millet grain by incubating for 7 days at 28ºC in dark. This inoculum (10g infested grain row^{-1}) was applied at flowering (40 days after seedling emergence) in the furrows adjacent to the plants, covered with a thin layer of soil, and irrigated using sprinklers.

Trichoderma inoculum and seed treatment

Autoclaved pearl millet grains were used as a substrate to multiply *Trichoderma* isolates for 10 days at 28ºC in dark. The infested millet grains with *Trichoderma* isolates were mixed with sterile distilled water, stirred for 4-5 min to detach the spores, and filtered through double-layered muslin cloth to obtain spore suspension. Two hundred grams of groundnut seeds were coated with 100 ml aqueous spore suspension (1×10^9 spores ml^{-1}) by adding 1 ml of 0.5% carboxy methyl cellulose (CMC) as sticker and 20 g of bentonite powder as filler for seed dressing.

Pseudomonas inoculum and seed treatment

The cultures were grown in Luria Bertani (LB) broth for 72 h in a 125 rpm shaker incubator at 28ºC in dark. Bacterial cell pellet (centrifuged at 5000 rpm) was suspended in sterile distilled water to obtain the aqueous cell suspension (1×10^9 cfu ml^{-1}). Two hundred grams of groundnut seeds were coated with 100 ml of aqueous cell suspension (1×10^9 cfu ml^{-1}) by adding 1 ml of 0.5% carboxy methyl cellulose (CMC) as sticker and 20 g of bentonite powder as filler for seed dressing. Seeds dressed with CMC and bentonite without BCAs served as control.

Soil application of biocontrol agents

Trichoderma cultures multiplied on pearl millet grains, as above, were mixed with farm-yard manure (100 ml spore suspension in 900 g autoclaved FYM), and applied in furrow (20 g per row) adjacent to the plants at the peg-formation stage (60 days after seedling emergence). Similarly, the bacterial cultures multiplied on LB broth were applied. Farm-yard manure without BCAs was applied in control plots.

Soil assay for *A. flavus* population

Three random samples (10 g soil per sample) from each plot were collected and mixed thoroughly to make a composite sample per plot. Two samplings were done, one at sowing and another at harvesting from geocarposphere. Aqueous soil suspension (10g soil + 90 ml sterile distilled water) prepared from each composite sample was serially diluted to 10^{-3} and 10^{-4}, and a 500 µl soil suspension was spread on AFPA (*A. flavus* and *A. parasiticus* specific medium) plates (2 plates per dilution) and incubated for 4 days at 28ºC in dark. Typical Af-colonies were counted and colony density per gram soil determined according to the dilution factors.

Assay for kernel infection by *A. flavus*

One hundred kernels from each plot surfacesterilized with 1% NaOCl for 3 min and washed with two changes of sterile distilled water were plated on Czapek Dox Agar (CDA) supplemented with rose Bengal and streptomycin. Kernel infection data recorded 4 days after incubation at 28ºC.

Data analysis

Analysis of variance (ANOVA) was done for individual experiments to find the treatment means. Since there was no significant difference between experiments for treatment means, pooled ANOVA was done to determine significant differences among treatments across three experiments.

RESULTS AND DISCUSSION

Significant (P<0.001) difference in *A. flavus* population was recorded with several treatments over control (Table 1). The lowest population of *A. flavus* (8.3×10^3 cfu g^{-1} soil) was recorded in soil samples from the plots applied with T 28 compared with the control where no BCA was applied (32.1×10^3 cfu g^{-1} soil). Mean reduction in Af population across three experiments varied from 17% (Pf 153) to 73% (T28) (Table 2).

Effect of BCA treatments on kernel infection was highly significant (P<0.001) in the three experiments (Table 1). The mean kernel infection in BCA treated plots varied from 12% (Pf 153) to 25% (Th 13) compared with 44% in the control (Table 2). Treatment with isolate Pf 153 appeared marginally superior over that with Th 13, while others were not significantly different. All the isolates of *Trichoderma* and *P. fluorescens* were highly effective in reducing kernel infection. Highest reduction (73%) in kernel infection was recorded with Pf 153 followed by Tv 20 (65%), Th 23 (63%) and the least with T 28 (40%) (Table 2). At Patancheru higher kernel infection occurred during the postrainy than the rainy season experiment.

There was no significant effect of BCA application on kernel yield (Table 1). Yield gains of 1 to 23% over control were recorded in some treatments, with highest being with Th 23 (Table 2). Kernel yield in some treatments were higher at Anantapur than at Patancheru, and again it was higher during the dry season experiment than in the rainy season. There was also visible over all better plant growth in BCA treated plots than in the control plots.

The process of invasion and infection of groundnut kernels by *A. flavus* and subsequent production of aflatoxin is quite complex and different from any root or seedling disease problem where biological control method has been used successfully (Dorner *et al.*, 1989; Handelsman and Stabb, 1996). In the case of the groundnut-*A. flavus* system, the biocontrol agent has to be active for at least two months during the peg formation to pod maturity and it has to compete successfully with *A. flavus* and other microorganisms for nutrient and growth. In this study, all the antagonists were successful in competing with a highly toxigenic strain of *A. flavus* 11-4 in all the three field experiments.

One isolate each of *T. viride* (Tv 20), *T. harzianum* (Th 13), *Trichoderma* sp. (T 28) and *P. fluorescens* (Pf 2) provided greater protection to kernel infection by *A. flavus* than others. These isolates could be used in combination to enhance their effectiveness. Also, use of bacterial strains as seed dressing and soil application of *Trichoderma* at flowering could provide a better control of *A. flavus* population in rhizosphere and protected the pods and kernels from invasion. Fluorescent pseudomonads are prominent inhabitants in the rhizosphere of many crop plants, and these have frequently been considered as effective biological control agents against soil-borne plant pathogens because of their rapid and aggressive colonization on plant roots (Anderson *et al.*, 1988).

Table 1. Analysis of variance for Af-population ($\times 10^3$), kernel infection (%) and kernel yield (g $plot^{-1}$)

Source of variation	df	MS: Af-population	Kernel infection	Kernel yield
Replication	3	149.2	22.7	3674.0
Experiment (E)	2	112.9	75.9	218197.0***
Treatments (T)	9	539.3***	1064.5***	4778.0
E × T	18	214.87	329.34***	4771.0
Residual	87	85.42	71.83	4353.0

***Significant at P<0.001.

Table 2. Effect of biocontrol agents as seed treatment and soil application on population density of *Aspergillus flavus* (Af 11-4), kernel infection and kernel yield in three field experiments during 2000-2001

Treatment	Af-population ($\times 10^3$ cfu (gm^{-1} soil)^a	Reduction in Af-population over control	Kernel infection (%)^a	Reduction (%) in kernel infection over control	Kernel yield (g plot^{-1})^a	Kernel yield (%) over control
T. harzianum (Th 13)	14.9	51	25	41	248	116
T. longibrachiatum (Tl 16)	10.8	61	22	50	217	101
T. viride (Tv 17)	17.2	29	13	59	213	100
T. viride (Tv 20)	11.8	67	14	65	210	98
T. harzianum (Th 23)	14.7	42	14	63	263	123
Trichoderma sp. (T 28)	8.3	73	24	40	218	102
P. fluorescens (Pf 2)	12.3	55	20	55	209	98
P. fluorescens (Pf 76)	15.3	41	17	56	247	115
P. fluorescens (Pf 153)	20.9	17	12	73	246	115
No BCA – Control	32.1	-	44	-	214	100
LSD (P<0.05)	8.40	-	12	-	93	-

[a]Mean of 3 field experiments: Patancheru July-November 2000 ; January-May 2001 and Anantapur July-November 2000. Note that each treatment was used as seed dressing and soil application.

It has been demonstrated that fluorescent pseudomonads and *Trichoderma* not only have direct effects on the pathogen but also induce systemic resistance in plants (Van Loon *et al.*, 1998). The reduced kernel contamination occurred due to reduction in *A. flavus* population density in the rhizosphere of groundnut indicating the competitive survival ability and growth potential of BCAs over *A. flavus*. Considerable increase in kernel yields in some treatments and no adverse effects on plant growth in general suggests the safe use of these BCAs in groundnut crop. Integration of BCAs with genetic and cultural management practices could be explored to develop an integrated management package for aflatoxin contamination in groundnut. The advantages of using native biocontrol agents include minimal disturbance of the ecosystem, positive consumer perception and acceptance, and the potential for readily transferable technology.

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