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**SHORT COMMUNICATION**

# Evaluation of bacteria and *Trichoderma* for biocontrol of pre-harvest seed infection by *Aspergillus flavus* in groundnut

V. ANJAIAH[1], R. P. THAKUR[1], & N. KOEDAM[2]

[1]*International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Andhra Pradesh, India, and* [2]*Laboratory of Microbial Interactions, Vrije Universiteit Brussel, Brussels, Belgium*

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**Abstract**
Pre- and post harvest aflatoxin contamination of groundnut caused by *Aspergillus flavus* is a major problem in the semi-arid tropics. Fluorescent *Pseudomonas*, *Bacillus* and *Trichoderma* spp. potentially antagonistic to *A. flavus* were isolated from the geocarposphere (pod-zone) of groundnut and used successfully for the control of pre-harvest groundnut seed infection by *A. flavus*. In greenhouse and field experiments, inoculation of selected antagonistic strains on groundnut resulted in significant reduction of seed infection by *A. flavus*, and it also reduced >50% of the *A. flavus* populations (as cfu) in the geocarposphere of groundnut.

**Keywords:** *Aflatoxin*, Arachis, Aspergillus flavus, *biocontrol*, *fluorescent* Pseudomonas, Bacillus, Trichoderma *spp.*

Groundnut (*Arachis hypogaea* L.) is a major oilseed crop widely grown in tropical and subtropical regions of the world, and is an important source of protein. Aflatoxin contamination of groundnut by the mould fungi *Aspergillus flavus* and *A. parasiticus*, both pre- and post-harvest, is a serious problem and has a tremendous impact on the global groundnut industry as well as poseing public health risks (Will et al. 1994). Aflatoxins have been detected in a wide range of commodities, including groundnut, maize and cotton, used for both human and animal consumption (Doyle et al. 1982).

Despite considerable research efforts around the world, stable genetic resistance/tolerance in groundnut genotypes against seed infection or to aflatoxin contamination by *A. flavus* have not been identified (Anderson et al. 1995) owing to multiple genes conferring resistance to seed colonization, post-harvest infection and aflatoxin production (Burow et al. 1997). Chemical control methods are ineffective, and are not eco-friendly as they increase environmental and health hazards. Biological control by the use of non-toxigenic strains of *A. flavus* to counteract toxin-producing strains in

Correspondence: Vanamala Anjaiah, Department of Botany, University of Delhi, Delhi – 110 007, AP, India. Fax: 91 40 30713074. E-mail: a_vanamala@yahoo.com

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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 (pod-zone) bacterial strains were used to reduce groundnut pod colonization by *A. flavus* (Mickler et al. 1995). Since the geocarposphere microbial community is the last barrier for *A. flavus* prior to pod colonization, native and competitive antagonistic microorganisms, bacteria and *Trichoderma* were isolated from this pod-zone region and evaluated for biocontrol of *A. flavus* in developing pods and seeds of groundnut.

Bacterial strains antagonistic to *A. flavus* were isolated from the geocarposphere of groundnut grown in ICRISAT fields using a double layer technique. In this technique, conidial suspension of *A. flavus* 11-4 [$10^8$ colony forming units (cfu) $mL^{-1}$] was plated on glucose casamino acid yeast extract agar (15 mL/plate as ground layer) medium (GCY; Anjaiah et al. 1998). The geocarposphere soil along with the pods of groundnut was suspended, diluted serially ten-fold with sterile water, spread on to a plate (top layer) and incubated at 28°C for 3–4 days. The potential antagonists of *A. flavus* selected by this method were further tested using a dual-culture plate technique (Anjaiah et al. 1998). The selected bacterial strains were characterized by their morphological traits based on their colony morphology, Gram strain and spore staining; physiological traits based on their growth on different media (P-isolation agar [Hi-media, India], King's B and Casamino acid agar medium [CAA; Anjaiah et al. 1998]); sporulation on nutrient agar (NA) medium by heat killing the vegetative cells. Further, fluorescent *Pseudomonas* strains were characterized for molecular traits using multiplex PCR amplification for the presence of the outer membrane lipoprotein genes *opr*I and *opr*L (De Vos et al. 1993).

Toxigenic *A. flavus* 11-4 used in the study was selected as described earlier based on its aggressive colonization of groundnut seeds and its ability to produce aflatoxins (Thakur et al. 2000). *Trichoderma* spp. were selected based on our earlier *in vitro* characterization (Desai et al. 2000). The selected isolates of *Trichoderma* spp. were characterized for the presence of chitinase gene(s) by PCR amplification method using degenerated primers designed in the conserved regions of a chitinase sequence using 20-mer oligonucleotide primers (Tchit primer I: 5′-ACT TCC AAG CAG ATG GCA CT-3′; and Tchit primer II: 5′-AGA TGG GCA TAC CAA GAA CG-3′). The amplification reactions were carried out by using the following conditions: 94°C for 4 min (one cycle), 92°C for 60 s (denaturation), 52°C for 45 s (annealing), 72°C for 60 s (extension) for 30 cycles and final extension at 72°C for 5 min (one cycle).

Pot culture and field experiments were conducted to evaluate the selected antagonists for their effectiveness in suppressing *A. flavus* infection in susceptible groundnut cv. JL 24. In greenhouse experiments, seeds were grown in pots of *A. flavus* infested soils ($10^4$–$10^5$ cfu $g^{-1}$ soil) with three replicates/treatment, and pots were arranged in a randomized complete block design. The antagonists, eight *Trichoderma* spp. and one isolate of *Pseudomonas* and *Bacillus*, were applied both at sowing as seed dressing and at the peg formation stage as a soil drench. A field experiment was conducted during the rainy season in Alfisol at ICRISAT Patancheru, India, in a complete randomized block design with four replicates per treatment. Each plot consisted of four rows of 4 m long, with rows 75 cm apart and plants spaced at 10 cm within the row. *A. flavus* 11-4 inoculum was produced on autoclaved pearl millet seeds by incubating at 28°C for 7 days in the dark and mixed with farm yard manure. Inoculum was added to the furrow before sowing the seed to give a minimum

population of $10^4$ cfu $g^{-1}$ of soil (top 5 cm soil) and also applied at flowering (40 days after seedling emergence) to small furrows made at both sides of the plants. The antagonists were applied twice as seed dressings at sowing, and also as a soil application with farmyard manure as a carrier at peg formation stage. The same amount of farmyard manure was added to the control plots. Soil moisture stress was imposed from 80 days of planting until crop maturity. In the field experiment, *A. flavus* infection was monitored in developing pods 80 days after sowing as described by Mehan et al. (1987). *A. flavus* population in the geocarposphere (Pit et al. 1983) and seed infection by *A. flavus* (Mehan et al. 1987) at harvest in both greenhouse and field experiment was measured.

Data were subjected to analysis of variance (ANOVA) using GENSTAT statistical package (Rothamsted Experiment Station, Herpenden, Herts, UK). Log transformation of *A. flavus* population data was carried out to obtain homogenous variance. Treatment means were compared using standard statistical procedures. All results were tested for significance at the 5% level of probability.

In the double layer technique, *A. flavus* grew through the agar medium and covered the whole surface in 3–4 days. Antagonistic bacteria grew on the upper surface layer and inhibited the growth of *A. flavus* in their vicinity. Further, the promising 12 antagonistic bacterial strains were selected in dual culture plate method based on *in vitro* antagonism against *A. flavus*. Morphological characterization of bacterial strains revealed that eight of the 12 strains were non-sporulating Gram-negative rods. Growth on different media, siderophore production and fluorescence under UV on P-isolation agar (*Pseudomonas* specific media), Kings' B and CAA indicated that these eight strains were fluorescent pseudomonads. Further, multiplex PCR amplification of the outer membrane lipoprotein genes showed amplification of the *opr*I gene for the same eight strains and no amplification for the *opr*L gene (data not shown). This confirmed that all these eight strains were rRNA group I fluorescent pseudomonads (De Vos et al. 1993). Since there was no amplification of the *opr*L gene, the results suggested that there were no *P. aeruginosa* strains. The other four bacterial strains were found to be aerobic sporulating Gram-positive bacilli. The presence of endospores in these bacteria was identified by spore staining and further confirmed by heating killing the vegetative cells. The endospore present in the heat-killed vegetative cells suspension formed colonies on NA, which confirmed as *Bacillus* spp. *Bacillus* spp. also showed amylase, protease, lipase and cellulase activity (data not shown). All these bacterial strains were inoculated on groundnut plants and confirmed as non-pathogenic as there were no adverse effects on plant growth and total biomass of groundnut plants. *Trichoderma* strains were assessed for the presence of chitinase gene, a trait often contributing to their biocontrol ability. The presumed chitinase gene fragment of 700 bp was amplified in the majority of the tested *Trichoderma* spp. Eight species of *Trichoderma* that showed the chitinase gene were selected for further evaluation.

Pot culture and field experiments revealed that the groundnut seeds treated with bacteria or *Trichoderma* spp. were equally effective in suppressing the *A. flavus* population in the geocarposphere and subsequent infection in groundnut seeds (Tables I and II). In pot culture experiments, *Bacillus* sp. 52 and *T. logibrachiatum* (T-16) reduced >50% of *A. flavus* infection both in seeds as well as in developing pods, whereas *Pseudomonas* sp. 135 and the six *Trichoderma* spp. (T-13, T-16, T-17, T-23, T-25 and T-28) reduced >50% of *A. flavus* infection only in developing pods

Table I. Effect of antagonistic bacteria and *Trichoderma* spp. on reduction of *A. flavus* populations in the geocarposphere, developing pod and seed infection in groundnut grown in *A. flavus* infested soils in the greenhouse.

| Treatment | *A. flavus* population in geocarposphere at harvest (cfu $\times 1000$ g$^{-1}$ soil)[1] | Infection at harvest (%) | |
|---|---|---|---|
| | | Developing pod[2] | Seed[3] |
| *T. harzianum* (T-13) | 62 (81) | 24 (55) | 45 (26) |
| *T. longibrachiatum* (T-16) | 16 (95) | 26 (51) | 29 (52) |
| *T. viride* (T-17) | 210 (37) | 25 (53) | 53 (13) |
| *T. auroviride* (T-18) | 170 (49) | 48 (9) | 59 (3) |
| *T. viride* (T-20) | 28 (92) | 31 (42) | 44 (28) |
| *T. harzianum* (T-23) | 66 (80) | 26 (51) | 33 (46) |
| *T. viride* (T-25) | 27 (92) | 24 (55) | 45 (26) |
| *Trichoderma* sp. (T-28) | 41 (88) | 14 (74) | 45 (26) |
| *Pseudomonas* sp. 135 | 76 (77) | 12 (77) | 34 (44) |
| *Bacillus* sp. 52 | 82 (75) | 19 (64) | 27 (56) |
| *A. flavus*-infested control | 333 (0) | 53 (0) | 61 (0) |
| SEM (+/−) | 33.2 | 9.2 | 4.3 |
| LSD | 94.3 | 26.1 | 12.2 |

[1]Percent of reduction over control given in parenthesis. [2]Mean of 30–40 pegs/treatment from three replicates; percent of reduction over control in parenthesis. [3]Mean of 50 seeds/treatment from three replicates; percent of reduction over control in parenthesis.

(Table I). However, two bacterial species (*Pseudomonas* sp. 135, *Bacillus* sp. 52) and six *Trichoderma* spp. (T-13, T-16, T-20, T-23, T-25 and T-28) reduced >75% of *A. flavus* population in the geocarposphere regions (Table I). In field experiments, the reduction in *A. flavus* population ranged from 56 to 81% in the geocaposphere of

Table II. Effect of antagonistic bacteria and *Trichoderma* spp. on reduction of *A. flavus* populations in the geocarposphere, developing pod and seed infection in groundnut grown in *A. flavus* infested soils under field conditions.

| Treatment | *A. flavus* population in geocarposphere at harvest (cfu $\times 1000$ g$^{-1}$ soil)[1] | Developing pod infection (%)[2] (80 DAS) | Seed infection (%)[3] (at harvest) |
|---|---|---|---|
| *T. harzianum* (T-13) | 182 (62) | 14 (56) | 19 (44) |
| *T. longibrachiatum* (T-16) | 100 (79) | 12 (63) | 11 (68) |
| *T. viride* (T-17) | 160 (67) | 16 (50) | 18 (47) |
| *T. viride* (T-25) | 150 (69) | 18 (44) | 15 (56) |
| *T. harzianum* (T-23) | 125 (74) | 13 (59) | 18 (47) |
| *Trichoderma* sp. (T-28) | 120 (75) | 15 (53) | 21 (38) |
| *Pseudomonas* sp. 135 | 118 (75) | 13 (59) | 12 (65) |
| *Bacillus* sp. 52 | 90 (81) | 13 (59) | 17 (50) |
| *P. fluorescens* Pf2 | 210 (56) | 14 (56) | 26 (24) |
| Control (No biocontrol agents) | 480 (0) | 32 (0) | 34 (0) |
| SEM (+/−) | 52.90 | 2.35 | 4.61 |
| LSD | 154.0 | 6.80 | 6.53 |

DAS, days after sowing. [1]Percent of reduction over control given in parenthesis. [2]Mean of 30–40 pegs/treatment from three replicates; percent of reduction over control in parenthesis. [3]Mean of 50 seeds/treatment from three replicates; percent of reduction over control in parenthesis.

groundnut in plots inoculated with antagonists (Table II). The highest reduction of *A. flavus* infection was recorded with *T. longibrachiatum* (T-16) and *Pseudomonas* sp. 135, both in seeds and developing pods. However, *Pseudomonas* sp. 135, *Bacillus* sp. 52, *T. longibrachiatum* (T-16) and *Trichoderma* sp. (T-28) reduced the *A. flavus* population >75% in the geocarposphere and >50% of *A. flavus* infection in developing pods compared to the control (Table II). The selected antagonistic strains performed consistently well in both glasshouse and field experiments.

The process of invasion of groundnut pods by *A. flavus* infection to seeds and subsequent production of aflatoxin is quite complex and different (Dorner et al. 1998) from any root or seedling disease problem where biological control has been used successfully (Handelsman & Stabb 1996). In the case of the groundnut–*A. flavus* system, the biocontrol agent has to be active for almost 4 months and it has to compete successfully with *A. flavus* together with other microorganisms for nutrient and growth. In the present studies, the antagonists (bacteria and fungi) isolated from the geocarposphere of native soils were successful in competing with a highly toxigenic strain of *A. flavus* 11-4, and gave control of pre-harvest seed infection by *A. flavus* in groundnut both in field and greenhouse experiments. All these antagonists are easy to apply in the field, and have been used for control of several soil-borne pathogens in many crops and considered as effective biological control agents against soil-borne plant pathogens (Handelsman & Stabb 1996). The use of native biocontrol agents in the integrated management of aflatoxin contamination in groundnut may provide greater potential for control of *A. flavus* population, seed infection and subsequent aflatoxin production.

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