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Pseudomonas fluorescens Pf7: A Potential Biocontrol Agent against *Aspergillus flavus* Induced Aflatoxin Contamination in Groundnut

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Authors' contributions

This work was carried out in collaboration among all authors. Authors KVKK and HS designed the study, performed the statistical analysis and wrote the protocol. Author MRT performed the study, managed the literature searches and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aflatoxin contamination is a qualitative problem in groundnut (*Arachis hypogaea* L.) occurring at both pre-and post-harvest stages. These aflatoxins are secondary metabolites produced by *Aspergillus flavus* and *A. parasiticus* and have carcinogenic, hepatotoxic, teratogenic and immuno-suppressive effects. Use of plant growth-promoting rhizobacteria (PGPR) is a viable and sustainable option in managing aflatoxin problem in groundnut. Our present study is aimed at identifying a plant growth-promoting rhizobacteria (PGPR) strain with superior antagonistic abilities on *A. flavus* infection, aflatoxin contamination and to determine its mode of action. Ten native *P. fluorescens* isolates were isolated from groundnut rhizosphere and screened against *A. flavus* by dual culture and *in vitro* seed colonization (IVSC) assays. In dual culture and IVSC studies, *Pf7* exhibited higher degree of antagonism on *A. flavus* (54% inhibition), inhibited its colonization and reduced aflatoxin contamination (27.8 µg kg^{-1}) in kernels.

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1. INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is an important grain legume and oilseed crop with huge revenue potential. Groundnut production all over the world is hampered by several biotic stresses that result in severe yield reduction [1,2]. The important biotic stress in groundnut cultivation is aflatoxin contamination which occurs at both pre-and post-harvest stages of the crop. Aflatoxins are a group of 20 secondary metabolites produced by *Aspergillus flavus* Link ex Fries and *Aspergillus parasiticus* Speare [3,4]. It is a qualitative problem affecting grain quality and trade [5].

Several management strategies have been attempted to minimize the aflatoxin problem. Important of them are development of resistant lines [6], development of transgenics or enhancing host plant resistance [7,8]. Strong sources of genetic resistance are however not available in the cultivable germplasm of groundnut. Of different management strategies, biological control of aflatoxin producing *A. flavus* is a viable option and is sustainable over long run. Of different biocontrol agents, use of plant growth-promoting rhizobacteria (PGPR) is gaining momentum. Several PGPR genera have been reported to suppress *A. flavus* besides producing plant growth-promoting effects [9]. Of different PGPR, *Pseudomonas* is one of the widely used genuses against major plant pathogens in groundnut [10]. Earlier reports indicated the use of PGPR in groundnut for controlling soil and foliar diseases besides yield enhancement [9].

Identification of a superior PGPR isolate with high degree of antagonism against *A. flavus* is necessary prior to conducting of greenhouse and field studies. In view of this, screening of the *P. fluorescens* isolate against *A. flavus* under *in vitro* and *in vivo* conditions is a pre-requisite. In particular, the extent of inhibition of *A. flavus* infection by a PGPR isolate on groundnut seed need to be ascertained through *in vitro* seed colonization assays [11]. In addition to inhibition of pathogen growth and multiplication, the PGPR isolates also contribute to increased yields. Plant growth-promoting effects and enhancement of pod yields by *P. fluorescens* have been reported in groundnut [9]. The present study therefore focused on documenting the effectiveness of elite PGPR isolate against *A. flavus* through dual culture studies and *in vitro* seed colonization assay.

2. MATERIALS AND METHODS

The present investigation was carried out with the facilities available International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, India.

2.1 Isolation of *Pseudomonas fluorescens*

Soil samples were collected from groundnut fields at ICRISAT, Patancheru, Telangana**,** India. Serial dilution method was followed [12] to isolate PGPR (*P. fluorescens*). The bacteriological tests for confirming the *P. fluorescens* isolates were conducted as per laboratory guide for "Identification of Plant Pathogenic Bacteria" published by the American Phytopathological Society [13]. Ten isolates of *P. fluorescens* were isolated and designated as *Pf*1 through (to) *Pf*10. These PGPR isolates were then maintained on nutrient agar for further studies.

2.2 Dual Culture Studies

Ten *P. fluorescens* strains were used in the present study. The antagonistic activity of *P. fluorescens* on *A. flavus* was tested by dual culture technique [14]. The toxigenic strain of *A. flavus,* AFT5b identified in our studies was used in the present study [15]. PGPR isolates were streaked at one side of Petri dish (one cm away from the edge) containing PDA. A mycelial disc from seven days old PDA culture of *A. flavus* was placed at the opposite side of Petri dishes perpendicular to the bacterial streak and incubated at 28±2ºC for seven days. Petri dishes with PDA inoculated with fungal discs alone served as control. Altogether, there were 10 treatments plus a control. Three replications were maintained for each treatment. Observations on radial growth of test fungus were recorded and per cent inhibition was calculated by using the formula proposed by Vincent (1927) [16].

Per cent inhibition (I) = 100(C-T)/C

where,

C= radial growth of *A. flavus* in control
T= radial growth of *A. flavus* in treatment

The current experiment was executed in a Completely Randomized Block Design (CRD), and the data were analyzed using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA) and the treatment means were differentiated by a least significant difference (LSD) at P=0.05 using PROC- GLM.

2.3 *In vitro* Seed Colonization Assay

The efficacy of *P. fluorescens* strains in reducing aflatoxin production by *A. flavus* was studied by using the procedure of *in vitro* seed colonization (IVSC) according to Thakur et al. (2000) [11]. Multi-well plates were used for this purpose. Healthy and undamaged groundnut kernels (JL24) were surface sterilized and then dipped in PGPR inoculum at 1 $x10^9$ CFU ml^{-1} for one minute. Kernels dipped in sterile distilled water serves as control. Later, the seeds were sprayed with an aflatoxigenic *A. flavus* strain- AFT5b at1 $x10^8$ CFU $ml^{-1}$and then the multi-well plates were kept in plastic trays with wetted blotting papers to provide moisture. Later the plastic trays with multi-well plates were incubated for one week in dark at 28ºC. There were altogether 11 treatments including control. Each treatment was replicated thrice. After incubation, the seeds were rated for colonization severity by *A. flavus* on severity scale of 1-4 [11] (Table 1). The experiment was executed in a completely randomized design (CRD). The data pertaining to the IVSC results were analyzed using a non-parametric approach. Kruskal-Wallis test was used for converting the measured observations and ranks were assigned. The treatments means were differentiated based on Wilcoxon ranks. Further, the same seeds used for IVSC experimentation, were later used for estimating aflatoxin content through indirect ELISA [17].

The indirect ELISA experiment was executed in a Completely Randomized Design (CRD). The data pertaining to aflatoxin levels obtained by ELISA were square root transformed and analyzed using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA) and the treatment means were differentiated by a least significant difference (LSD) at P=0.05 using PROC- GLM.

3. RESULTS

Ten *Pseudomonas fluorescens* (*Pf*) strains were isolated by serial dilution technique from the soil samples collected from groundnut fields of ICRISAT, Patancheru. These ten strains were used in the present study to test the efficacy of *P. fluorescens* in reducing the *A. flavus* infection and aflatoxin contamination in groundnut kernels. The toxigenic *A. flavus* strain AFT5b, isolated from groundnut kernels collected from Karimnagar district (Bachu Veera Mallaiah & Sons Oil Mill) of Telangana was used as test fungus in the present study.

3.1 Dual Culture Studies

The *in vitro* efficacy of *P. fluorescens* in reducing the mycelial growth of *A. flavus* was studied using dual culture technique and the results are presented in Table 2. There was a significant difference among the treatments evaluated (P<0.0001). In general, all the *Pf* strains under study have shown inhibition on *A. flavus*. Of different treatments, highest inhibition of *A. flavus* was obtained with *Pf*7 (54.8%) (Fig. 1), followed by *Pf*2 (48.7%) and *Pf*6 (48.2%). However, no significant differences were observed among these three strains. Next best inhibitions of test fungus were obtained with *Pf*4 (46.2%) and *Pf*9 (44.6%) with no significant differences between them. Further, these two strains were statistically at par with *Pf*2 and *Pf*6. For the remaining *Pf* strains, the per cent inhibition was up to 35.9 (*Pf*8). The inhibitions of *A. flavus* by *Pf*1, *Pf*10 and *Pf*3 were about 31.7%, 32.3% and 33.3% respectively. Least inhibition of *A. flavus* was obtained with *Pf*5 (28.2%).

3.2 *In vitro* Seed Colonization Assay (IVSC)

3.2.1 Colonization severity

Of different *P. fluorescens* (*Pf*) strains evaluated in IVSC, the colonization severity of *A. flavus* was significantly reduced over control when seeds were treated with bioagents, *Pf*2, *Pf*6, *Pf*7 and *Pf*9. Of these, seed treatment with *Pf*7 has resulted in least colonization severity of

Table 1. *Aspergillus flavus* seed colonization severity scale on groundnut kernels

Scale	Description
1	<5% seed surface colonized with scanty mycelial growth and scanty sporulation
2	5-25% seed surface colonized with good mycelial growth and scanty sporulation
3	26–50% seed surface colonized with good mycelial growth and good sporulation
4	>50% seed surface colonized with heavy sporulation

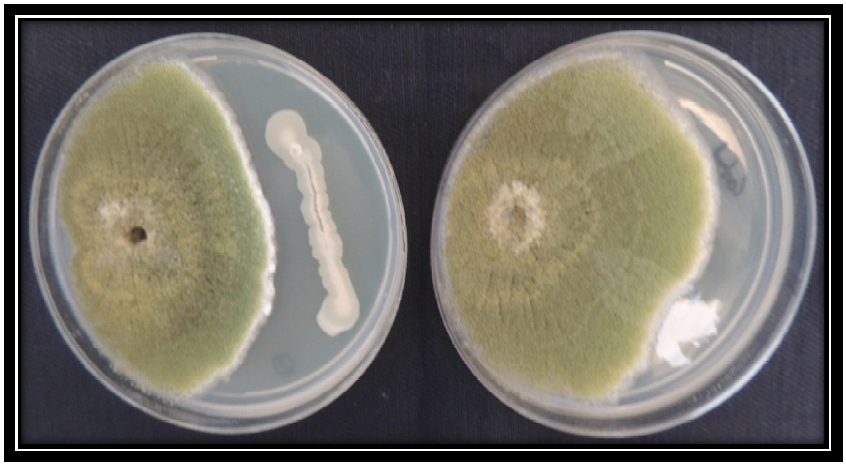

Fig. 1. *In vitro* efficacy of *Pseudomonas fluorescens* (*Pf7*) in reducing the mycelial growth of *Aspergillus flavus* in dual culture studies

Table 2. *In vitro* efficacy of *Pseudomonas fluorescens* isolates in inhibiting the radial growth of *Aspergillus flavus* in dual culture studies

***Pseudomonas fluorescens* isolates**	**% inhibition of *A. flavus* growth over control**
Pf1	31.76[c]
Pf2	48.7[ab]
Pf3	33.33[c]
Pf4	46.2[b]
Pf5	28.2[c]
Pf6	48.2[ab]
Pf7	54.8[a]
Pf8	35.93[c]
Pf9	44.63[b]
Pf10	32.33[c]
LSD (5%) = 7.87; CV = 23.73	

Means with the same letter are not significantly different

A. flavus (6.2 wilcoxon score). This is followed by *Pf*2 &*Pf*9 (9.3 wilcoxon score each) and *Pf*6 (13.5 wilcoxon score). The remaining six *Pf* strains (*Pf*s1, 3, 4, 5, 8 and *Pf*10) have not shown significantly less colonization severity over control. The seeds in control have recorded maximum colonization severity by *A. flavus* (32 wilcoxon score). The difference in colonization severity over control was highest with *Pf7* (25.8), followed by *Pf*2 & *Pf*9 (22.7) and *Pf*6 (18.5). Overall, the *Pf* strains, *Pf7*, *Pf*2, *Pf*9 and *Pf*6 were effective in reducing *A. flavus* infection on groundnut seeds (Table 3).

3.2.2 Aflatoxin contamination

The data on kernel aflatoxin levels among different treatments were non-significant at P=0.05% (Pr=0.24). Further, the Type I and Type III error sum of squares also had shown non-significance. However, based on the toxin levels in kernels in various treatments, the results were summarized as follows. Of different treatments, the aflatoxin content was least in seeds treated with *Pf7* (27.8 µg kg^{-1}). The efficacy of *Pf7* was significantly superior over other *Pf* strains. This was followed by seeds treated with *Pf*1 (754.7 µg kg^{-1}), *Pf*8 (1051.6 µg kg^{-1}) and *Pf*2 (1151.9 µg kg^{-1}) with no significant differences among them. For the remaining *Pf* strains (*Pf*s3, 4, 5, 6, 9 and *Pf*10), the aflatoxin content ranged from 1218.6 to 1512.7 µg kg^{-1}. The performances of these six *Pf* strains were not significantly superior over control. Seeds in control have recorded highest aflatoxin content of 1521.1 µg kg^{-1} (Table 4). Overall, the PGPR strain, *Pf7* was highly effective in reducing kernel aflatoxin contamination in groundnut through IVSC assays.

Table 3. Efficacy of *Pseudomonas fluorescens* isolates in reducing *Aspergillus flavus* infection on groundnut seeds through *in vitro* seed colonization assay (IVSC)

Pseudomonas fluorescens isolates	Mean score	Difference(TRT-Control)
*Pf*1	20.8 (2.7)^{ns}	11.167
*Pf*2	9.3 (1.7)^{s}	22.667
*Pf*3	20.8 (2.7)^{ns}	11.167
*Pf*4	20.8 (2.7)^{ns}	11.167
*Pf*5	20.8 (2.7)^{ns}	11.167
*Pf*6	13.5 (2.0)^{s}	18.5
Pf7	6.2 (1.3)^{s}	25.833
*Pf*8	16.7 (2.3)^{ns}	15.333
*Pf*9	9.3 (1.7)^{s}	22.667
*Pf*10	16.7 (2.3)^{ns}	15.333
Control	32.0 (4.0)	

(LSD: 15.47) (ns- Non Sisgnificant;s- Significant), Scores for variables are Wilcoxon scores estimated by non-parametric Kruskal-Wallis test, Values in parentheses are means of original colonization on severity scale of 1-4 Groundnut seeds (CV JL 24) were treated with Pf strains at 1 x10^{9} CFU/ml, followed by A. flavus at 1x10^{8}spores/ml , Observations were recorded at one week after incubation

Table 4. Efficacy of *Pseudomonas fluorescens* isolates in inhibiting the aflatoxin production by *Aspergillus flavus* in groundnut by *in vitro* seed colonization (IVSC) assay

Pseudomonas fluorescens isolates	Kernel aflatoxin content (µg kg^{-1})*
*Pf*1	754.7^{ab} (23.1)
*Pf*2	1151.9^{ab} (29.0)
*Pf*3	1512.7^{a} (38.8)
*Pf*4	1442.2^{a} (37.7)
*Pf*5	1347.8^{a} (36.1)
*Pf*6	1335.7^{a} (29.8)
Pf7	27.8^{b}(5.1)
*Pf*8	1051.6^{a}(31.7)
*Pf*9	1218.6^{a} (30.8)
*Pf*10	1247.5^{a} (34.1)
Control	1521.1^{a} (38.9)
LSD at 5% =1185.4 (23.9)	

*Groundnut seeds (CV JL 24) were treated with Pf strains at 1 x10^{9} CFU/ml, followed by A. flavus at 1 x 10^{8}spores/ml, Observations were recorded at one week after incubation, *Aflatoxin content did not differ significantly at 0.05%., Values in the parenthesis were square root transformed. Means with the same letter are not significantly different*

4. DISCUSSION

PGPR are one of the commonly used antagonists in managing soilborne diseases of several crops [18]. Of different PGPR, *P. fluorescens* is widely used in controlling several plant pathogens [19]. In combating aflatoxin problem in groundnut, PGPR are experimentally tried with limited success. In our studies, the *Pf7* strain was found to be superior among other *P. fluorescens* strains. Plant growth-promotion by PGPR is due to direct and indirect mechanisms [20]. Direct mechanisms involve either facilitating resource acquisition (nitrogen, phosphorus and essential minerals) or modulating plant hormone levels. Indirect mechanisms of plant growth-promotion are by decreasing the inhibitory effects of various pathogens on plant growth and development [21].

In our present study, the *Pf7* strain exhibited superior activity in inhibiting mycelial growth of *A. flavus*, its colonization on groundnut seeds and aflatoxin production. Antifungal activity of *P. fluorescens* is due to the production of siderophores [22,23]; HCN [24]; competition for space and nutrients and also by production of antibiotics [25]. A wide range of antifungal metabolites (antibiotics) are produced by *P. fluorescens* strains against plant pathogens. For example, certain strains of *P. fluorescens* produce 2, 4-diacetylphloroglucinol (2,4-DAPG) that has antifungal and antihelminthic activity [25]. Similarly, reports on the production of other

antibiotics by *P. fluorescens* are also available [26]. In our studies, an inhibition zone between test fungus (*A. flavus*) and *Pf7* appeared in dual culture studies.

Further the *Pf7* strain also showed significant effect on groundnut seeds in reducing colonization of *A. flavus* in an IVSC assay. IVSC assays were earlier used in groundnut to assess the resistance among germplasm to *A. flavus* infection (11). Inhibition of *A. flavus* in IVSC in the present study by *Pf7* is attributed to both antibiosis and hyper parasitism. Reduction in aflatoxin content in *Pf7* treated seeds in IVSC assay is also attributed to the fact that the bio-agent occupied the groundnut spermoplane (seed surface) and thereby prevented the significant invasion of *A. flavus* and subsequent aflatoxin production. Overall, *Pf7* was highly effective in reducing aflatoxin contamination.

5. CONCLUSION

The PGPR (*P. fluorescens*) strain to be a good candidate bio-agent at field level, besides being inhibitory to soil *A. flavus* populations, it is desirable to possess certain growth-promoting and specific pathogen inhibitory traits. Characterization of the identified PGPR strain is therefore necessary to understand the exact trait possessed and its role in plant growth-promotion, pod yield enhancement besides reducing *A. flavus* populations in soil and also the aflatoxin contamination. In this context, it is essential to characterize *Pf7*, identify the potential antibiotic produced, investigate its efficacy under greenhouse and field conditions against pre-harvest aflatoxin contamination.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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