

Seed Treatment Studies Against *Aspergillus flavus* Infection Under Controlled Conditions

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ABSTRACT

An experiment was conducted to know the effect of seed treatments on plant growth and pre harvest aflatoxin contamination using three bio control agents and two fungicides in groundnut cv. JL 24. Seeds treated with *T. harzianum* was superior in recording higher seed germination, plant height and yield followed by *T. viride* and *P. fluorescens*. The fungicides carbendazim followed by mancozeb were also found effective in improving seed germination, plant height and yield over untreated and pathogen treated seeds. Aflatoxin contamination were detected in pathogen treated and untreated seeds which is recorded below permissible level whereas contamination was not observed in the seeds treated with *T. harzianum*, *T. viride* and *P. fluorescens*.

Key words: Groundnut, Biocontrol agents, *A. flavus*, Aflatoxin.

INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is commonly known one of the most important oil seed crop produced in the world. The oilseeds are second most important crop after food grains in terms of area under cultivation, production and value in the agricultural economy of India. To minimize the gap between the demand and supply of oil seeds, intensive efforts are being made to increase their production. 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 posing public health risks²¹.

Aflatoxin contamination is a major problem in the nutritional and confectionery quality of groundnut, which can occur both before and after harvest, and during storage. The components of resistance are also complex pre and post harvest resistance, dry seed resistance and resistance to aflatoxin production. Aflatoxins have been detected in a wide range of commodities, including groundnut, maize and cotton, used for both human and animal consumption⁸.

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Aflatoxins are colourless, crystalline substances with molecular weight ranging from 312 to 333 and melting point ranging from 237 to 299°C. Among the several aflatoxins, four fractions namely, B₁, B₂, G₁, and G₂, so labelled because of their blue (B) and green (G) fluorescence under ultra violet light, are commonly found in groundnut and its extractions. 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 aflatoxins¹⁹.

As ever-increasing population and urbanization cannot allow increase in the land area under the cultivation of oilseeds anymore due to the pressure on land. Hence, there is an urgent need to improve the yield per unit area. To achieve this objective, agricultural scientists have laid more emphasis on bioagents like *Trichoderma viride*, *Trichoderma harzianum* and *Pseudomonas fluorescens*. Biological control, by means of using naturally occurring antagonistic microorganisms, is now widely recognised as environmentally safe and sustainable alternative to the use of synthetic chemical fungicides in controlling plant pathogens. Several biocontrol agents have been reported to control aflatoxin contamination in groundnut in Africa⁴. *Trichoderma* spp. was selected based on our earlier in vitro characterization⁵. *T. viride* has also been recognized as potent biological agent to control plant diseases by producing antibiotics and cell walls degrading enzymes that can kill the pathogen¹⁸. *P. fluorescens* commonly known as mycorrhiza helper bacteria are found to be associated with mycorrhiza to promote the symbiosis between fungus and plant by stimulating fungal growth or protecting the fungus against other fungal competitors. It acts as a systemic bio-control agent against various fungal and bacterial diseases by producing a number of secondary metabolites including antibiotics, siderophores and hydrogen cyanide⁹. Fungicides has been also found effective in preventing the growth of *A. flavus*³, chemical control is neither practical

nor economical. Moreover, the use of fungicides may pose threat to the environment by polluting the ecosystem and may lead to the development of tolerance in the target organisms. Furthermore, the usage of fungicides produces negative impact on nodulation of legumes by nitrogen fixing beneficial bacteria¹². Therefore, efforts have been directed towards the development of environmentally sound and sustainable strategies for prevention of aflatoxin contamination in groundnut.

Keeping in view the above information, present investigation was undertaken to investigate the efficacy of bioagents and fungicides alone i.e. *T. viride*, *T. harzianum*, *P. fluorescens*, carbendazim and mancozeb to find out the best treatment having the maximum capability of enhancing the growth and reducing the pre harvest infection of *A. hypogaea* in pot experiment under glasshouse conditions.

MATERIALS AND METHODS

Seed treatment:

Healthy seeds of groundnut cultivar JL 24 (susceptible) were surface sterilized using 0.01 % clorax solution for one min. Again seeds were washed in three changes of sterile distilled water and placed on dry blotter paper to remove the excess moisture. Later the seeds were dipped in pathogen solution containing *A. flavus* @ 10⁹ conidia/ml solution for about one minute. Again seeds were treated with slurry prepared by different biocontrol agents. After seed treatment, seeds were dried for overnight and were sown in four replications. The seeds without treatment of biocontrol agents were served as control.

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, with three replicates/treatment, and pots were arranged in a complete randomized design. The antagonists, *Trichoderma* spp, *Pseudomonas* and fungicides were applied at ICRISAT Patancheru, India.

Preparation of *A. flavus* culture:

Toxin producing aflatoxigenic strain of *A. flavus* (Af 11 - 4) fungal culture was grown on potato dextrose agar (PDA) media. PDA plates were kept in BOD incubator for 7 days at $25 \pm 2^{\circ}\text{C}$. Greenish fungal sporulation was observed at 7 days after incubation.

Potato Dextrose Agar (PDA) medium

PDA medium was prepared by using the following ingredients

Potatoes	-	200 g
Dextrose	-	20 g
Agar	-	20 g
Distilled Water	-	1000 ml
pH	-	6.8

Peeled potato pieces were boiled in 500 ml of distilled water in a 1000 ml beaker till the pieces got softened and the extract were collected in a beaker by sieving through a double layered muslin cloth. Agar - agar was melted in another 500 ml of distilled water in 1000 ml beaker into which 20g dextrose was added. The final volume of the medium was made up to 1000 ml by adding sterile distilled water. The pH of the medium was adjusted to 6.8 with 0.1 N NaOH or 0.1 N HCl as the case may be with the pH meter. The medium was sterilized in an autoclave at 15 psi for 15 minutes.

Efficacy of Bioagents against *A. flavus* in groundnut

Bioagents and fungicides were tested against *A. flavus* along with suitable controls (Un inoculated and Inoculated) in groundnut. Pots with a size of seven inches in diameter and capacity to hold 3 - 5 kg of sterilized soil with a mixture of soil, sand and FYM was placed in the proportion of 2:1:1. All the treatments were replicated four times.

Observations**Germination (%)**

The number of seeds germinated in each treatment was counted on seventh day after sowing. Four replications were maintained for each treatment.

Plant height (cm)

The plant height was recorded in cm from the base of the plant at 15, 30 and 45 DAS.

Harvesting

The crop was harvested at maturity, threshed and sun-dried for 3 days.

Yield (g)

Pod weight in grams was recorded in different treatments with replication wise.

Aflatoxin levels

Pods after harvest were manually shelled and seeds were assessed for aflatoxin by following the indirect competitive ELISA¹⁶.

Estimation of seed quality by using ELISA (Enzyme Linked Immunosorbant Assay)

The effect of *A. flavus* on seed quality i.e. aflatoxin levels in the groundnut samples were estimated by using indirect competitive method of ELISA (Enzyme-linked immunosorbent assay)¹⁶. In this the basic principle of Enzyme-linked immunosorbent assays (ELISA) lies in immobilizing the antigen onto a solid surface or capturing antigen by specific antibodies and probing with specific immunoglobulins carrying an enzyme label. The enzyme retained in the case of positive reaction is detected by adding suitable substrate. The enzyme converts substrate to a product which is recognized by its colour. ELISA tests rely on evaluation of a visible antigen-antibody precipitate.

Groundnut seed (100 g) was grinded into powder using a blender. The seed powder was titrated in 70% methanol (v/v-70 ml absolute methanol in 30 ml distilled water) containing 0.5 % KCl (proportion used in 100 ml for 20 g seed) in a blender until the seed powder was thoroughly ground. The extract was transferred to a conical flask and shaken for 30 min at 300 rpm in the mechanical shaker. The extract was filtered through Whatman No. 1 filter paper. To estimate lower levels of AFB₁ (<10 $\mu\text{g Kg}^{-1}$), prior to ELISA, a simple liquid cleanup and concentration (5:1) procedure was adopted. Twenty ml of methanol extract, 10 ml of distilled water and 20 ml of chloroform were mixed in a separating funnel and used for cleanup. After vigorous shaking for one min, the lower chloroform layer was collected and evaporated to near desiccation in water bath at 60°C. To

the residue, four ml of PBS - Tween containing 7 % methanol was added and used for analysis by ELISA.

AFB₁ - BSA conjugate was prepared in carbonate coating buffer at 100 ng/ml concentrations and 170 µl of the diluted AFB₁ - BSA is dispensed to each well of ELISA plate. The plate was incubated in a refrigerator overnight at 37° C for at least one and half-hour.

The plates were washed in three changes of PBS – Tween allowing 3 min gap between for each wash (To inhibit non-specific binding of antibodies and thus give false positive reaction). BSA (0.2 %) was prepared in PBS - Tween was added in 170 µl per each well of ELISA plate and incubated at 37°C for 1h. The plates were washed in three changes of PBS - Tween allowing 3 min between each wash.

Statistical analysis

The data obtained in various laboratory/glasshouse experiments were statistically analyzed by using Completely Randomized Design (CRD) as suggested by Gomez and Gomez¹⁰. The data pertaining to percentage were angular transformed wherever necessary.

RESULTS AND DISCUSSION

Effect of seed treatments on germination, plant height and yield under glass house conditions

The germination percentage was significantly improved in all the seed treatments as compared to untreated seeds. Among the seed treatments, groundnut seeds treated with *T. harzianum* recorded higher seed germination (96 %) followed by seed treatment with *T. viride* (91 %) and it was found on par with seeds treated with *P. fluorescens* (88.2 %). The other seed treatments viz., carbendazim (81 %) and mancozeb (73.5 %) were also found effective in increasing the seed germination over untreated seeds (65 %) and pathogen treated seeds (54.5 %) (Table 1).

Significant differences in plant height due to different seed treatments in groundnut seeds were observed when compared with

untreated and pathogen treated seeds. However, seed treatment with *T. harzianum* recorded higher plant height (4.75, 12.9 and 14.1 cm) followed by seed treatment with *T. viride* (4.1, 11.5 and 13.5 cm) and it was found on par with seeds treated with *P. fluorescens* (3.4, 10.2 and 10.8) at 15, 30 and 45 DAS. The other seed treatments, carbendazim (2.7, 8.0 and 9.2 cm) and mancozeb (2.7, 6.9 and 8.4 cm) were also found effective in increasing plant height over untreated seeds (2.6, 6.6 and 7.3 cm) and pathogen treated seeds (2.5, 5.6, 6.4 cm) at @ 15, 30 and 45 DAS (Table 3.1).

Yield per plant was significantly improved in all the seed treatments as compared to treated and untreated seeds. Among the seed treatments, groundnut seeds treated with *T. harzianum* recorded higher pod yield per plant (4.6) followed by seed treatment with *T. viride* (4.2) and it was found on par with seeds treated with *P. fluorescens* (4.1). Seeds treated with carbendazim (3.6) and mancozeb (3.3) were also found effective in increasing pod yield over untreated seeds (2.9) and pathogen treated seeds (2.5) (Table 1).

Divakara *et al*⁷, who reported that seed treatment with talc based powder formulations of antagonist rhizobacteria and *Trichoderma* sp. improved crop yield and reduced aflatoxin production in sorghum and ensuring high economic returns. Dey *et al*⁶, also observed that improvement in the growth, yield and nutrient uptake in groundnut cultivar JL 24 in pots prior inoculated with PGPR isolates. Raju *et al*¹⁴, reported that formulations of *P. fluorescens* were effective in reducing *F. moniliforme* infection and also increasing the germination, vigour index and field emergence.

Estimation of aflatoxin content in the harvested seeds of groundnut cv. JL 24

Groundnut seeds of cv. JL 24 treated with bioagents and fungicides were evaluated under glasshouse conditions. The aflatoxin levels through ELISA method in the harvested produce of different seed treatments were assessed. The presence of aflatoxin was detected in pathogen treated seeds (1.38µg/kg)

and untreated seed (0.69 μ g/kg) which is below permissible levels (Table 2).

T. harzianum inhibited the growth of *A. flavus* to an extent of 70% followed by *T. viride* (68.9 %) ¹. The beneficial effects of seed treatments with bioagents and fungicides in minimizing the pre harvest infection in groundnut was reported earlier by Mixon *et al* ¹¹. who stated that treatment of groundnut pods with *T. harzianum* reduced the aflatoxin contamination. The present results were also in agreement with Sanskrit *et al* ¹⁷., who reported that *P. fluorescens* showed inhibitory activity against *A. flavus*. Use of biocontrol agents in aflatoxin management was also suggested by Vijay Krishna Kumar *et al* ²⁰. The present results are similar with Rathod *et al* ¹⁵., who

found that carbendazim was found effective against storage rot of groundnut caused by *A. flavus*. Seed treatments with carbendazim and mancozeb + carbendazim were found effective in reducing the population densities of *A. flavus* ²². Alemayehu *et al* ²., also reported that the total aflatoxin levels in *Aspergillus flavus* positive samples of groundnut seed varied between 15 and 11865 μ g/kg. Antagonistic activities of three *Trichoderma* species, i.e. *T. viride*, *T. harzianum* and *Trichoderma* sp against seven pathogenic fungi, namely *Aspergillus niger*, *A. flavus*, *Phytophthora* sp., *Fusarium oxysporum*, *Rhizoctonia solani*, *Penicillium notatum* and *Alternaria solani* ¹³.

Table 1: Estimation of Aflatoxin content (μ g kg⁻¹) in the harvested produce of groundnut cv. JL 24 treated with bioagents and fungicides

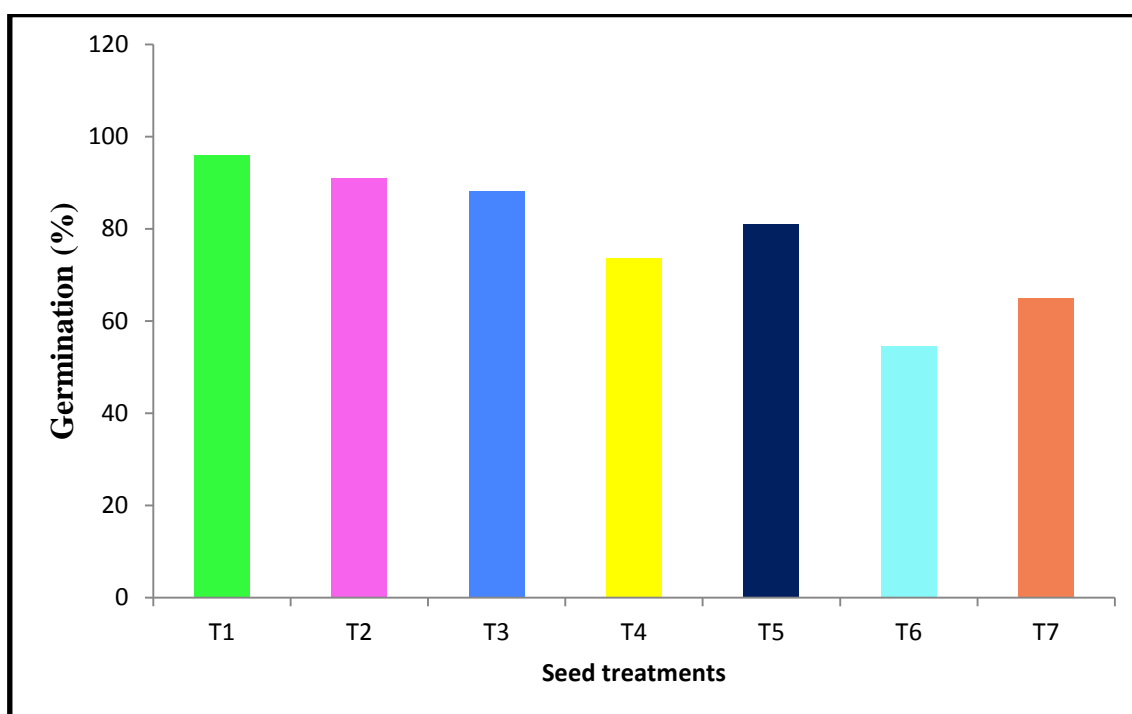
S. No	Treatments	Dosage (g kg ⁻¹)	Aflatoxin content (μ g kg ⁻¹)
1	<i>Trichoderma harzianum</i>	10	0 (1.00)
2	<i>Trichoderma viride</i>	10	0 (1.00)
3	<i>Pseudomonas fluorescens</i>	10	0 (1.00)
4	Mancozeb	2.5	0 (1.00)
5	Carbendazim	2	0 (1.00)
6	Inoculated control/Treated seeds		1.38 (1.29)
7	Uninoculated control/untreated seeds		0.69 (1.53)
	SEm \pm		0.12
	CD at 5 %		0.04

Figures in the parenthesis are square root transformed values.
Each value is means of four replications

Table 2: Evaluation of seed treatments with bioagents and fungicides against *A. flavus* under glasshouse conditions

S. No	Treatments	Dosage (g/kg)	Germination (%)	Plant height 15 DAS (cm)	Plant height 30 DAS (cm)	Plant height 45 DAS (cm)	Pod yield per plant (g)
1	<i>Trichoderma harzianum</i>	10	96.0 (79.7)	4.75	12.9	14.1	4.60
2	<i>Trichoderma viride</i>	10	91.0 (73.4)	4.10	11.5	13.5	4.20
3	<i>Pseudomonas flourescens</i>	10	88.2 (69.9)	3.40	10.2	10.8	4.10
4	Mancozeb	2.5	73.5 (59.0)	2.72	6.92	8.43	3.37
5	Carbendazim	2.0	81.0 (64.1)	2.77	8.02	9.21	3.65
6	Inoculated control/Treated seeds		54.5 (47.5)	2.57	5.65	6.41	2.55
7	Uninoculated control/untreated seeds		65.0 (53.7)	2.67	6.62	7.37	2.92
	SEm ±		1.49	0.34	0.80	0.78	0.09
	CD at 5 %		4.43	1.01	2.36	2.31	0.27

Figures in parenthesis are angular transformed values. Each value is mean of four replications.

**Fig. 1: Evaluation of seed treatments with bioagents and fungicides against *A. flavus* on germination**

T₁ - *Trichoderma harzianum*

T₆ - Inoculated control/Treated seeds

T₂ - *Trichoderma viride*

T₇ - Uninoculated control/untreated seeds

T₃ - *Pseudomonas flourescens*

T₄ - Mancozeb

T₅ - Carbendazim

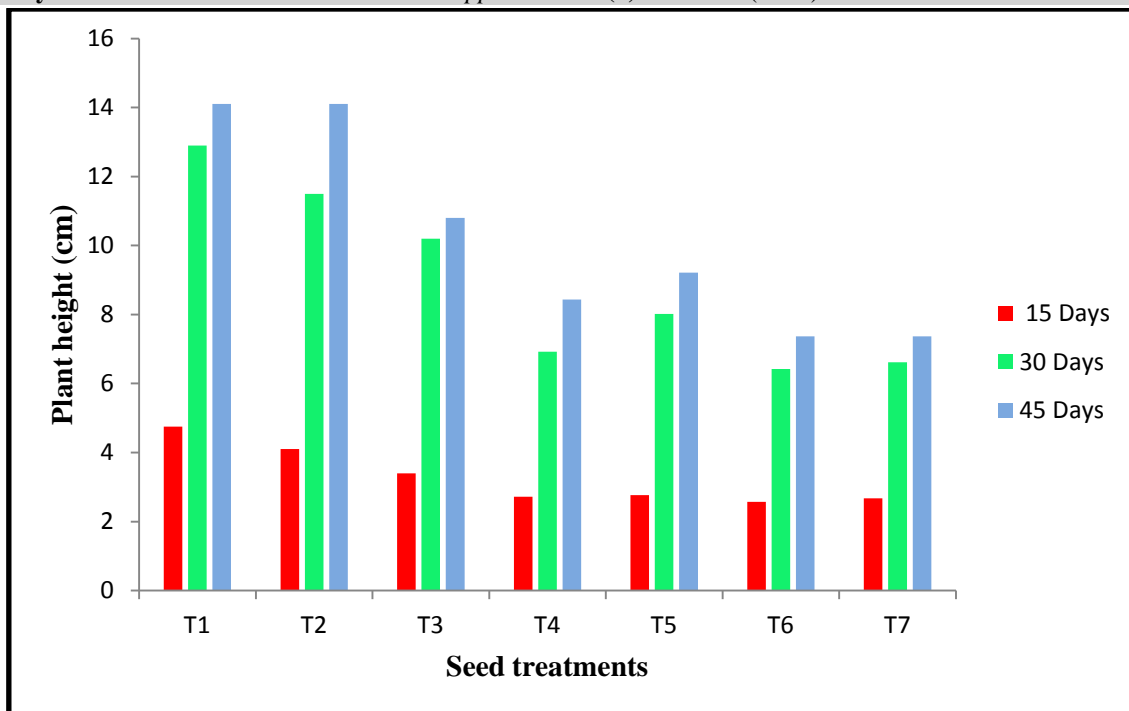


Fig. 2: Evaluation of seed treatments with bioagents and fungicides against *A. flavus* on plant height

- T₁ - *Trichoderma harzianum*
- T₂ - *Trichoderma viride*
- T₃ - *Pseudomonas flourescens*
- T₄ - Mancozeb
- T₅ - Carbendazim
- T₆ - Inoculated control/Treated seeds
- T₇ - Uninoculated control/untreated seeds

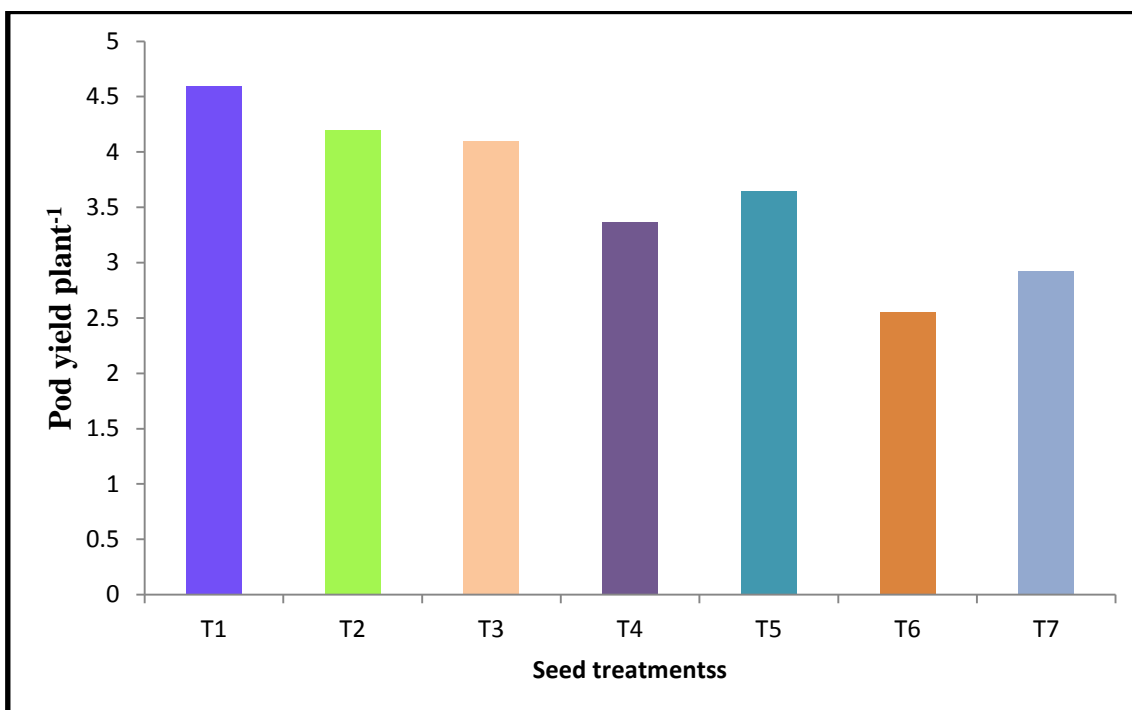


Fig. 3: Evaluation of seed treatments with bioagents and fungicides against *A. flavus* on yield

- T₁ - *Trichoderma harzianum*
- T₂ - *Trichoderma viride*
- T₃ - *Pseudomonas flourescens*
- T₄ - Mancozeb
- T₅ - Carbendazim
- T₆ - Inoculated control/Treated seeds
- T₇ - Uninoculated control/untreated seeds

CONCLUSION

Studies were conducted that concept of biological control was effective in reducing the pre harvest aflatoxin contamination and also helps in improving the plant growth. There is a need for creating awareness among farmers for the adoption of eco friendly biological method to produce aflatoxin free groundnut. By using this biocontrol with plant products like neem/castor cake will definitely help to reduce pre harvest infection in groundnut and to produce qualitative and quantitative groundnut and it can also be recommended to the farmers after proper confirmation. Further studies are also needed with combination of treatments in reducing aflatoxin contamination.

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REFERENCES

1. Ajitkumar, K., Naik, M.K. and Savita, A.S., Reduction of aflatoxin contamination in chilli using eco-friendly management practices. *J. of Pt Dis. Sci.*, **6(2)**: 153 – 156 (2011).
2. Alemayehu, C., Abdi, M., Amare, A. and Helge, S., Natural occurrence of aflatoxins in groundnut (*Arachis hypogaea* L.) from eastern Ethiopia. *Food Con.*, **30**: 602-605 (2012).
3. Breneman, T.B., Wilson, D.M. and Beaver, R.W., Effects of diniconazole on *Aspergillus* population and aflatoxin production in peanut under irrigated and non irrigated conditions. *Plant Dis.*, **77**: 608-612 (1993).
4. Chiuraise, N., Yobo, K.S. and Laing, M.D., Seed treatment with *Trichoderma harzianum* strain kd formulation reduced aflatoxin contamination in groundnuts. *J. of Pt. Dis Pro.*, **122**: 74-80 (2015).
5. Desai, S., Thakur, R.P., Rao, V.P. and Anjaiah V., Characterization of isolates of *Trichoderma* for biocontrol potential against *Aspergillus flavus* infection in groundnut. *International Arachis Newsletter.*, **20**: 57-59 (2000).
6. Dey, R., Pal, K.K., Bhatt, D.M. and Chauhan, S.M., Growth promotion and yield enhancement ofpeanut (*Arachis hypogaea* L.) by application of plant growth-promoting rhizobacteria. *Microl Res.*, **159**: 371-394 (2004).
7. Divakara, S.T., Aiyaz, M., Hariprasad, M., Nayaka, S.C. and Niranjana, S.R., *Aspergillus flavus* infection and aflatoxincontamination in sorghum seeds and their biological management. *Archives of Phytopath and Pt Pro.*, **47(17)**: 2141-2156 (2014).
8. Doyle, M.P., Applebaum, R.S., Brackett, R.E. and Marth, E.H., Physical, chemical and biological degradation of mycotoxins in foods and agricultural commodities. *Journal of Food Pro.*, 45-64 (1982).
9. Frey-Klett, P., Garbaye, J. and Tarkka, M., The mycorrhiza helper bacteria revisited. *New Phytol.*, **176**: 22–36 (2007).
10. Gomez, K.A. and Gomez, A.A., Statistical procedures for agricultural research (second edition) John Wiley and sons, New York (1984).
11. Mixon, A.C., Bell, D.K. and Wilson, D.M., Effect of Chemical and Biological agents on the incidence of *Aspergillus* and aflatoxin contamination of peanut seed. *Phytopath.*, **74**: 1440-1444 (1984).
12. Muthomi, J.W., Otieno, P.E., Cheminingwa, G.N., Nderitu, J.H., Wagachaja, J.M., Effect of legume root rot pathogens and fungicide seed treatment on nodulation and biomass accumulation. *J.I of Biol. Sci.*, **7**: 1163-1170 (2007).
13. Patale, S.S., Mukadam, D.S., Management of plant pathogenic fungi by using *Trichoderma*. *Biosci. Discov.*, **2(1)**: 36 37 (2011).
14. Raju, N.S., Niranjana, S.R., Janardhana, G.R., Prakash, H.S., Shetty, H.S. and Mathur, S.B., Improvement of seed quality and field emergence of *Fusarium*

- moniliforme* infected sorghum seeds using biological agents. *J.I of the sci of Food and Agri.*, **79**: 206-212 (1999).
15. Rathod, L.R., Jadhav, M.D., kanse, D.S., Patil, D.P., Gulhane, S.D. and Deshmukh, P.S., Effects of fungicides on seed borne pathogen of groundnut. *Inter. J. of Advanced Biotech and Res.*, **1(1)**: 17-20 (2010).
 16. Reddy, S.V., Kiran Mayi, D., Uma Reddy, M., Thirumala Devi, K. and Reddy, D.V.R., Aflatoxin B₁ in different grades of chillies (*Capsicum annum*) as determined by indirect competitive-ELISA. *Food Add and Conta.*, **18**: 553-558 (2001).
 17. Sanskriti, R., Vijayalakshmi., Neelam, R., Ezilrani, P. and Christopher, J.G., Biocontrol of *Aspergillus flavus* in groundnut. *Life Sci Archives.*, **1(1)**: 28-34 (2015).
 18. Schuster, A. and Schmoll, M., Biology and biotechnology of *Trichoderma*. *Appl. Microbiol. Biotech.*, **87**: 787-799 (2010).
 19. Thakur, R.P., Rao, V.P., Reddy, S.V. and Ferguson, M., Evaluation of wild *Arachis* germplasm accessions for in vitro seed colonization and aflatoxin production by *Aspergillus flavus*. *Inter.l Arachis Newsletter.*, **20**: 44-46 (2000).
 20. Vijay Krishna Kumar, K., Desai, S., Rao, V.P., Nur, H.A. and Thakur, R.P., Evaluation of an integrated aflatoxin contamination package to reduce pre-harvest seed infection by *Aspergillus flavus* in groundnut. *Inter. Arachis Newsletter.*, **22**: 42-44 (2002).
 21. Will, M.E., Holbrook, C.C. and Wilson, D.M., Evaluation of field inoculation techniques for screening peanut genotypes for reaction to pre-harvest *A. flavus* group infection and aflatoxin contamination. *Peanut Sci.*, **21**: 122-125 (1994).
 22. Yitayih, G., Ayalew, A. and Dechassa, N., Management of Aflatoxigenic fungi in groundnut production in Eastern Ethiopia. *East African J. of Sci.*, **7(2)**: 85-98 (2013).