Research Paper :

Eco-friendly management of aflatoxin B_1 at preharvest level in groundnut (*Arachis hypogaea* L.)

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SUMMARY

Biocontrol agents like *Trichoderma viride, Trichoderma harzianum, Trichoderma hamanatum, Bacillus subtilis,* and *Pseudomonas fluorescent* were studied singly and in combination for the management of aflatoxin B_1 in groundnut. Experiments were conducted for continuous three years *i.e. Kharif* 2006 to *Kharif* 2008. Observations were recorded on *A. flavus* population, initial plant population, aflaroot, assessment of kernel infection and aflatoxing B_1 content. Application of these five biocontrol agents in combination resulted in significant reduction in *A. flavus* population and aflatoxin B_1 content in groundnut cultivar GG-20. The combination of *T. viride, B.subtilis* and *P. fluorescent* was most effective in reducing *A. flavus* rhizospheric population, percentage incidence of aflaroot, infection and colonization of kernels and aflatoxin B_1 content. On the basis of the findings, a package can be formulated by combining these three biocontrol agents and could be recommend to use in farmers field on large scale for pre-harvest management of aflatoxin B_1 in groundnut.

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Biocontrol agents possess differing modes of action and all strains of biocontrol agents may not possess all the modes of actions. Efforts were made for different combinations of biocontrol agents to get the desirable results. The detection of biocontrol agents (Gowdu and Balasubramaniam, 1991) helps in assessing the natural load of the biocontrol agent, and thus to plan a strategy to enrich the conditions for the multiplication of these mycoparasites.

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 are colourless, crystalline substances with molecular weight ranging from 312 to 333 and melting point ranging from 237 to 299^{0}C. Among the several aflatoxins, four fractions namely, B_1, B_2, G_1 and G_2, so labelled because of their blue (B) and green (G) fluorescence under ultra violet light, are commonly found in groundnut and its extractions. Aflatoxins M_1 and M_2, products of metabolic hydroxylation of B_1 and B_2, are generally found in milk. Aflatoxins are generally resistant to heat, but decompose at very high temperature and in the presence of moisture.

Aflatoxins are a family of closely related heterocyclic compounds produced by *A. flavus* and *A. parasiticus*. Aflatoxins are highly toxic and teratogenic compounds, contaminate legumes, cereals and oilseeds when stored improperly. Among these aflatoxins B1, a secondary metabolites, is the most potent of all mycotoxins and known to be carcinogenic, hepatotoxic and teratogenic in nature (Groopman and Donshu, 1988). *Aspergillus* spp. moulds are present in the soil and air, gain entry into several crops at different stages. Pseudomonads are known to be inhibitory to large number of fungal, soil-borne plant pathogens, and in this lies their importance organisms for biological control and management of these pathogens (Howell and Stipanovick, 1979; Lynch, 1978;

Thirumalachar and O'Brien, 1977). Many rhizobacteria especially *F. pseudomonads* and some bacilli when applied to seed (seed bacterization) are known to enhance the growth and yield of several plants like potato (Burr *et al*., 1978; Kloepper *et al*.,1980), sugar beets (Suslow and Schroth,1982) wheat (Brown, 1974). The main aim of the study was to evaluated the biocontrol agents for pre-harvest management of aflatoxin B_1 in groundnut.

MATERIALS AND METHODS

Location of trials:

The present study was carried out in experimental field of Directorate of Groundnut Research, Junagadh, Gujarat, during *Kharif* 2006 to *Kharif* 2008.

Biocontrol agents:

In present investigation, the biocontrol agents like *T.iviride, T. harzianum, B. subtilis,* and *P. fluorescent* were used in combinations. All the biocontrol agents were obtained from culture bank of Plant Pathology Department, Directorate of Groundnut Research, Junagad.

Mass production of biocontrol agents:

Mass production of biocontrol agents using low-cost technologies is the basic requirement and in present investigation a bulk quantity of biocontrol agents was produced using a laboratory fermentor (10 litre capacity). Different broths like Potato-dextrose broth (PDB), Glucose-nitrate broth (GNB), Maltose-peptone broth (MPB), Sabouroud-dextrose broth (SDB) and molasses–yeast extract broth (MYB) were tested for mass production of these biocontrol agents in a fermentor. The MYB supported the maximum growth of both *Trichoderma* spp. Considering high cost of yeast extract in MYB, yeast extract was successfully replaced with cheaper nitrogen source *i.e.* soy-flour thus bringing down the cost by about 10 times. The optimum fermentation conditions for obtaining maximum biomass of *Trichoderma* spp. in Molasses-soyflour broth were, (pH 7.0), 72-96 h of fermentation at 28^0C; 40% dissolved oxygen concentration; and a stirring rate of 250 rpm for first 48h and 400 rpm for rest of the period. Silicon (0.1%) was used as an antifoam agent. King's B broth was used for maximum growth of all the bacterial biocontrol agents. The optimum fermentation conditions for obtaining maximum biomass of *bacterial biocontrol agents* in King's B broth were (pH 7.0 to 7.2), 72h of fermentation at 30 to 32^0C; 40% dissolved oxygen concentration; and a stirring rate of 400 rpm and silicon (0.1%) as an antifoam agent.

Sick plots development:

In present investigation, the most virulent and highly toxic strain of *A. flavus* (AF-11-4) was used for sick plots development. Every month 10 kg inoculum of the most virulent and highly toxic strain was multiplied on sorghum grain medium in the laboratory conditions. Thereafter, the inoculums were first mixed with FYM and sand and kept for enriching in natural conditions in a form of heap. The heap was sparingly watered at regular interval to maintain moisture and promote the multiplication of *A. flavus.* After one week, this inoculum was used for soil inoculation in 5x5 M^2 micro-plots. Immediately after soil inoculation, plots were irrigated through sprinkler irrigation system so as to favour growth of *A. flavus.*

Seed treatment:

Healthy seeds of groundnut cultivar GG-20 were treated with slurry prepared by different combinations of biocontrol agents. After seed treatment, seeds were dried for overnight and were sown in 5x5 M^2 micro sick plots in three replications. The seeds without treatment of biocontrol agents were served as control.

Monitoring soil population of *A. flavus*:

For assessment of *A. flavus* soil population, 1 g of soil sample crushed down into the fine powder by crushing it in sterilized mortar and pastel. From this 1 g. fine soil powder was taken in 10 ml. sterile distilled water, then its serial dilution was done so as to obtain 10^3dilution.The 100 µl sample was taken from this dilution and poured on Czapex Dox medium in three replications and incubated for 5 days for the growth of *A. flavus* colonies.

Percentage incidence of aflaroot:

Percentage incidence of aflaroot was recoded after 20 days of sowing in each treatment. This was done by recoding typical symptoms on rotten seeds, seedlings, cotyledon and plumule.

Assessment of kernel infection and colonization:

After harvesting, pods were fully dried, shelled, and clean. One hundred kernels from each sample were surface sterilized with 0.1% $HgCl_2$ for 1 min and washed three times, 2 min each, with sterilized distilled water before plating them onto Czapex Dox Agar (CDA). 30

seeds were placed in three petri plate and incubated at 28 ºC for 4 days in BOD. Number of seeds infected and colonized by typical *A. flavus* colonies was counted and percentage seed infection and colonization was calculated.

Estimation of aflatoxing B_1 by ELISA:

In present study estimation of aflatoxin B_1 was done by indirect ELISA method.

Chemicals and reagents for indirect competitive ELISA:

Aflatioxin B_1 standard, bovine serum albumin (BSA), alkaline phosphates, p-nitrophenyl phosphate disodium, Tween –20 and glutaraldehyde were procured from Sigma Chemical Co., St. Louis, MO, USA. Polystyrene microtitre plates were purchased from Dynatech Lab, Virginia, USA. Other solvents and chemicals used in were of the highest analytical grade.

Treatments detail are as follows : T_1– *T. iviride*, T_2– *T. harzianum,* T_3–*B. subtilis,* T_4–*P. fluorescent,* T_5–*T. iviride +T. harzianum,* T_6–*T.iviride + P. fluorescent + B. subtilis,* T_7–*T.iviride +T. harzianum + B. subtilis+ P. fluorescent,* T_8–Control (without biocontrol agent)

RESULTS AND DISCUSSION

The results obtained from the present investigation as well as relevant discussion have been presented under following heads:

Monitoring soil population of *A. flavus*:

The results indicate that the combination of *T. viride, B. subtilis,* and *P. fluorescent* was most effective in reducing *A. flavus* rhizospheric population. Initial rhizospheric population density of *A. flavus* varied from 1.29 to 3.78 x 10^3 cfu g^{-1} before soil infestation (Table 1), while after soil infestation it varied from 7.08 to 13.27 x 10^3 cfu g^{-1} of soil in different combinations of biocontrol agents. But in control the rhizospheric population density of *A. flavus* increased from 49.27to 55.82 x 10^3 cfu g^{-1} of soil (Table 2).

Table 1 : Initial soil population of *A. flavus* in experimental micro plots before soil infestation

Treatments	Soil population of *A. flavus* x10^3 cfu/g soil *		
	Kharif-2006	*Kharif*-2007	*Kharif*-2008
T_1	1.29	2.57	3.11
T_2	2.34	2.81	3.15
T_3	3.11	3.63	3.45
T_4	2.50	2.39	2.72
T_5	3.45	3.42	3.25
T_6	2.53	3.19	2.72
T_7	3.24	3.78	3.11
S.E.±	0.05	0.09	0.08
C.D. (P=0.05)	0.14	0.26	NS
C.V. %	5.02	8.46	7.85

*Mean of three replications NS=Non-significant

Percentage incidence of aflaroot:

Application of these five biocontrol agents in singly and combination resulted in significant reduction in aflaroot. The combination of *T. viride, B.subtilis,* and *P. fluorescent* was most effective in reducing percentage incidence of aflaroot up to 3.17%. The lowest (3.17) incidence of aflaroot was in combination of *T. viride, B. subtilis* and *P. fluorescent*. Percentage incidence of aflaroot in different treatments ranged from 3.17 to 9.51%

Table 2 : Soil population of *A.flavus* in experimental micro plots after soil infestation at an interval of 30 days

Treatments	Soil population of *A. flavus* x10^3 cfu/g soil *								
	Kharif-2006			*Kharif*-2007			*Kharif*-2008		
	30 DAI	60 DAI	90 DAI	30 DAI	60 DAI	90 DAI	30 DAI	60 DAI	90 DAI
T_1	36.91	26.09	12.17	35.99	25.71	11.02	31.87	25.46	11.27
T_2	32.83	23.00	11.80	32.24	23.43	11.46	30.31	23.40	10.33
T_3	32.00	24.99	12.35	31.70	24.50	10.56	28.99	23.78	10.49
T_4	36.64	25.95	13.27	34.70	24.23	11.83	29.09	23.43	10.26
T_5	38.47	28.63	11.00	35.78	27.55	10.15	35.62	27.75	10.16
T_6	29.71	21.84	9.38	26.39	20.68	8.25	24.24	19.48	7.08
T_7	32.38	25.99	11.45	33.09	25.86	10.24	30.39	25.70	10.51
Control	49.27	54.04	55.84	51.42	55.02	55.78	52.02	54.58	55.82
S.E.±	0.03	0.07	0.07	0.06	0.12	0.09	0.08	0.10	0.10
C.D. (P=0.05)	0.11	0.21	0.22	0.18	0.36	0.27	0.24	0.30	0.31
C.V. %	1.02	2.39	3.58	1.76	4.06	4.63	2.49	3.43	5.49

*-Average of three replications, DAI - days after infestation

Table: 3 Percentage incidence of aflaroot due to artificial loading of *A. flavus*

Treatments	Total plant population*			(%) incidence of aflaroot		
	K-06	K-07	K-08	K-06	K-07	K-08
T_1	527	539	541	8.98	8.66	9.37
T_2	532	542	558	7.33	7.69	7.53
T_3	579	550	574	8.69	8.73	6.97
T_4	551	529	501	8.65	9.51	9.18
T_5	534	558	578	7.49	6.57	5.94
T_6	572	598	578	4.74	3.51	3.17
T_7	559	548	516	7.17	7.18	7.62
Control	443	431	427	13.09	14.15	17.80
S.E.±				0.18	0.18	0.12
C.D. (P=0.05)				0.55	0.56	0.38
C.V. %				4.85	5.01	3.50

K:-*Kharif*, * Average of three replications

while in control it ranged from 13.09 to 17.80 % (Table 3). Typical symptoms due to *Aspergillus flavus* include seed rot, non-emergence of seedling, and infection of cotyledon and plumule. The pathogen was first seen on cotyledon surface and was covered with masses of yellowish green spores. The affected cotyledon showed necrosis of the central tissues leading to reddish-brown lesions. The diagnostic symptoms were reduced first quadrifoliates with pointed tips, and show much variation in shape. The colour of the affected leaves was yellowish-green in comparison with the deep green colour of leaves of healthy plants.

Assessment of kernel infection and colonization:

Kernel infection and colonization studies also revealed predominance seed infection and colonization by *A. flavus* in control treatment over treated treatments. Kernel infection by *A. flavus* varied from 3.11 to 22.40%. In control kernel infection and colonization varied from 39.36 to 47.67%, respectively (Table 4). Here also, the combination of *T.viride, P. fluorescent* and *B. subtilis* showed lowest seed infection and colonization by *A. flavus.*

Estimation of aflatoxin B_1 by ELISA:

The combination of *T. viride, B.subtilis,* and *P. fluorescent* was again most effective in reducing aflatoxin B_1 content. Aflatoxin B_1 content in kernels varied from 2.71 to 235µg/kg. In control aflatoxin B_1 content in kernels varied from 759.97 to 1014.89µg/ kg. The lowest (2.71) aflatoxin B_1 content was in combination of *T. viride, B. subtilis,* and *P. fluorescent* (Table 5).

The results indicate that, for the effective management of pre-harvest aflatoxin B_1, contamination of beneficial biocontrol agents can be used. The combination of *T. viride, B. subtilis,* and *P. fluorescent*

Table 4 : Assessment of % infection and colonization of kernel

Treatments	% Infection and colonization of kernel *					
	Kharif- 2006		*Kharif* - 2007		*Kharif* -2008	
	Infection	Colonization	Infection	Colonization	Infection	Colonization
T_1	11.01	9.53	12.31	7.91	12.22	7.18
T_2	11.31	8.01	12.08	7.41	10.72	7.09
T_3	20.11	12.70	21.56	11.77	22.40	11.35
T_4	8.15	7.16	8.76	7.00	8.43	6.26
T_5	13.56	11.62	14.17	9.93	11.80	10.44
T_6	3.11	1.49	3.37	1.24	3.18	1.12
T_7	7.01	4.72	7.69	4.75	7.23	4.42
Control	47.67	33.14	39.36	25.87	45.87	7.18
S.E. ±	0.06	0.05	0.06	0.06	0.06	0.06
C.D. (P=0.05)	0.18	0.16	0.18	0.18	0.18	0.20
C.V. %	3.27	3.27	3.16	3.99	3.16	4.46

* Average of three replications

Table 5 : Aflatoxin B_1 content in the groundnut

Treatments	Aflatoxin B_1 content (ppb) *		
	Kharif- 2006	*Kharif* - 2007	*Kharif* -2008
T_1	116.09	182.32	148.46
T_2	40.62	39.48	37.92
T_3	18.28	16.89	19.36
T_4	83.28	93.69	65.92
T_5	165.57	235.69	152.10
T_6	2.37	2.87	2.71
T_7	38.92	37.88	40.38
Control	759.97	1014.89	975.37
S.E.±	0.20	0.30	0.50
C.D. (P=0.05)	0.62	0.94	1.54
C.V. %	4.77	6.49	11.87

* Average of three replications

was most effective in reducing *A. flavus* rhizospheric population, percentage incidence of aflaroot, infection and colonization of kernels and aflatoxin B_1 content.

Trichoderma spp. have the ability to inhibit the growth of *A. flavus in vitro* by production of non-volatile antibiotics (Desai *et al*., 2000). Thus, the usage of *T. viride, B. subtilis* and *P. fluorescent* in combination recorded synergism preventing *A. flavus* population from reaching economic levels. The use of combined biocontrol agents also increases the pod yield as compared to control. Combination of *T.viride, P. fluorescent* and *B. subtilis,* improved quality as well quantity of groundnut. Some *Trichoderma* isolates was used in field experiments to evaluate their biocontrol potential against aflatoxin contamination in groundnuts at ICRISAT (Anjaiah *et al.*, 2001). Four isolates of *Bacillus* were evaluated for their efficacy against *A. flavus* and AF135 was effective in reducing soil population and also promoting the growth of groundnut, (Desai *et al.,* 2002). Desai *et al.* (2003) have identified effective strains of *Trichoderma* and *Bacillus* for management of *A. flavus* and thus could be deployed in the integrated management package. Use of biocontrol agents into an integrated aflatoxin contamination management was also suggested by Vijaykrishna Kumar *et al.* (2002).

There is a need for creating awareness among farmers for the adoption of ecofriendly biological method to produce aflatoxin free groundnut. Use of these biocontrol agents with organic matters like FYM, neem cake, castor cake or gypsum in soil will definitely help to reduce aflatoxin B_1 contamination in groundnut and to produce qualitative and quantitative groundnut.

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