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Impact of aflatoxin on seed germination and enzyme activities

A.Janardhan, D.Subramanyam, A.Praveen Kumar, M.Reddi Pradeep, G.Narasimha*

Applied Microbiology Laboratory, Department of Virology, Sri Venkateswara University, Tirupati-517502, Andhra Pradesh, (INDIA)

E-mail : gnsimha123@gmail.com; dr.g.narasimha@gmail.com

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ABSTRACT

A fungal strain *Aspergillus flavas* was isolated from groundnut seeds infected with fungus and it was screened for aflatoxin production, and the effect of aflatoxin on seed germination and seed enzymes were assessed in the present study. Various cereals were soaked with aflatoxin at different time intervals, 3, 6, 12, and 24hrs of incubations and observed seed germinibility and enzyme activities such as protease and phosphatases. The seed germinability was vigorously reduced with increasing in seed incubation time and enzyme activities such as protease and alkaline phosphatases also drastically reduced in aflatoxin treated germinating seeds. Among the cereals used in this study the bean was highly sensitive to aflatoxin produced by *Aspergillus flavas.*

KEYWORDS

Aspergillus flavus;
Aflatoxin;
Seed germination;
Enzymes.

INTRODUCTION

Fungal contamination of harvested seeds and grains is a chronic problem in India, these conditions leads to deteuoration and mycotoxin production in the seeds. Among the mycotoxins, Aflatoxins are carcinogenic and induce neoplasms in the glandular stomach kidney, lung, salivary glands, colon and skin[1].The aflatoxins B1, B2, G1 and G2 produced by the *Aspergillus flavus* and *A.parasiticus*. The *A.parasiticus* mainly by produces the two aflatoxins B1 and B2. The production of aflatoxins in food grains interferes with protein synthesis by incorporation of amino acids into protein resulting in the non-germination of the embryo[2] Some observations show that the aflatoxins affect the plants by inhibition of developing roots in germinating seeds[3,4]. Due to the inhibitory effects of aflatoxin in economically important crop seeds like groundnut and maize the present study were aimed to produce aflatoxin from *Aspergillus flavus* and its impacts on seed germination and their enzyme activities.

MATERIALS AND METHODS

Collection of seeds

The harvested seeds Bean, Red gram, Green gram, Black gram were collected from the agricultural fields in Regional Agricultural Research Station (RARS) Tirupati, Chittoor District Andhra Pradesh, India.

Isolation and identification of fungal strain

The fungal infected groundnut seeds were taken and

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the mycelium developed on the surface were directly transferred with a sterile loop to the Potato Dextrose Agar (PDA) and the plates were incubated at room temperature and examined periodically for the presence of fungal members. The fungal members were stained and observed under the microscope, then transferred to the selective media AFPA for identification[5].

Screening and production of aflatoxins

The fungal isolates were screened for ability to produce aflatoxins with Coconut Cream Agar (CCA) medium[6]. These strains were identified and separated. These fungal spores were inoculated in Sabarouds broth and incubated for 7days. The mycelial mat was separated from the broth and filtered and mixed with organic solvent (ethylacetate) to elute the aflatoxins from the broth. Thus separated aflatoxins were spotted on TLC plates[7].

Effect of aflatoxins on seed germination

The seeds, bean, red gram, green gram and black gram were surface sterilized with 0.1% mercuric chloride for 2min and then washed with sterile distilled water. Thus sterilized seeds were soaked in the fungal filtrate for 3, 6, 12 and 24hrs and a control was maintained with distilled water only. The aflatoxin treated seeds were transferred to petriplates with wetted cotton and allowed for the germination. The plates were maintained at moist condition by frequent spraying of water. After 5days the percentage of seed germination was recorded and the enzyme activities were estimated both in germinating and the non-germinating seeds.

Estimation of seed enzyme activities

Preparation of enzyme extract

For the extraction of enzymes the germinated and non-germinated seeds were separately homogenized in pre chilled (4^0C) condition with 0.05M Tris HCL, pH 7.2. The extract was filtered and centrifuged at 5000rpm for 30min. The supernatant was used as crude extract for the estimation of enzymes.

RESULTS

The fungal strain isolated from the groundnuts was identified as *Aspergillus flavus* based on the microscopic and macroscopic observations and cultured screened on the selective media Aspergillus flavus and parasitiucs (AFPA) and identified as *Aspergillus flavus* based on its colony morphology and its characters as the production of bright orange color on the reverse of the plates. Further the fungal culture was screened on the CCA medium for aflatoxin production which gives fluorescent colonies on the UV, and unique characteristics of aflatoxin producing fungi. For production of aflatoxins the fungal spores was inoculated in the sabourds broth and incubated then filtered with organic solvent and for its detection the crude toxin was spotted on the TLC plates and run with solvent and the fluorescent bands were observed in the UV light (Figure 1).

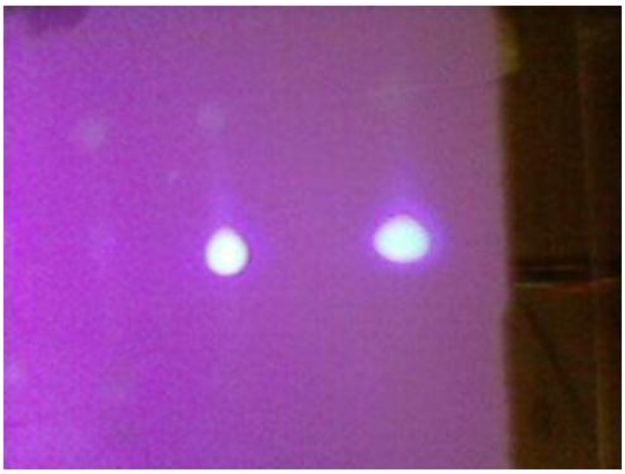

Figure 1 : TLC plate under U.V light showing the fluorescence band comparing with standard

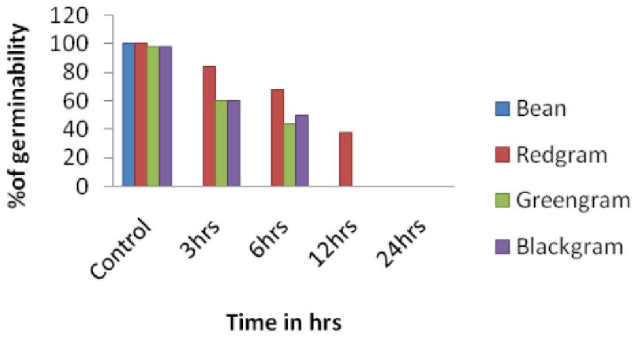

Figure 2 : Percentage of germinability of toxin treated seeds, comparing with the control

Aflatoxin effect on seed germination

The commercial crop seeds like bean, red gram, green gram, black gram were treated with aflatoxins at different time intervals 3-24hrs and were allowed for germination. The germinibility was compared with con-

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trol seeds without toxin treatment and the results were represented in (Figure 2). Among the seeds tested in the present study the bean shows highly sensitive to the aflatoxin and drastic inhibition was observed in the germination in the 3rd hour, where as the red gram was slight resistance and the germinibility was observed even at 12th hour of incubation.

Aflatoxin effect on seed enzymes

The effect of aflatoxins on seed enzymes such as protease and alkaline phosphatase were studied and the represented in TABLE 1 and 2. With increase in the time incubation the enzyme activities gradually decrease in both germinated and non-germinated seeds as the toxin treatment time increases. Among the seeds used in the present study the enzyme activities in green gram were drastically reduced.

Statistical analysis

For the non-germinated seed data, repeated measures Analysis of Variance (ANOVA) technique has been applied to observe the statistical significance of duration and as well as the significance between the enzymes. A p-value < 0.05 is considered to be statistically significant. Mean ± standard error values were presented for each time duration in each enzyme (TABLE 3). The results show that there exists statistical significance between durations of the experiment ($F = 26.593$, $p = 0.000$*) and also between the enzymes ($F = 37.018$, $p = 0.000$*). On performing Duncan's multiple range tests, it was observed that the enzymes (Protease and Alkaline Phosphatase) belong to one group. The line graphs were visualized for each enzyme and their response with respect to the time duration (Figure 3 and Figure 4).

TABLE 1 : Enzyme concentration in non-germinating seeds treated with aflatoxins and control

Samples	Enzyme activities									
	Protease					Alkaline Phosphatase				
	Control	3hrs	6hrs	12hrs	24hrs	Control	3hrs	6hrs	12hrs	24hrs
Bean	111	100	79	61	39	137	117	105	98	80
Red gram	165	126	93	68	25	140	122	111	101	88
Green gram	111	79	57	36	10	139	126	108	99	82
Black gram	100	75	64	50	25	135	119	107	94	81

***The protease activities was measured in liberation of aminoacids per ml per min and alkaline phosphatase was measured in liberation of pNPP per ml per min**

TABLE 2 : Enzyme concentration in germinating seeds, treated with aflatxoins and control

Samples	Enzyme activities									
	Protease					Alkaline Phosphatase				
	Control	3hrs	6hrs	12hrs	24hrs	Control	3hrs	6hrs	12hrs	24hrs
Bean	111	--	--	--	--	137	--	--	--	--
Red gram	165	147	133	108	--	140	129	107	94	--
Green gram	111	97	79	--	--	139	124	105	--	--
Black gram	100	90	79	--	--	135	118	102	--	--

***The protease activities was measured in liberation of aminoacids per ml per min and alkaline phosphatase was measured in liberation of pNPP per ml per min**

For the germinated seed data, Repeated measures ANOVA techniques has been applied to observe the statistical significance between the enzymes at $p < 0.05$ level. Table of Mean and Standard error values are presented (TABLE 4) for the enzymes in three different time durations. The enzymes (Protease and Alkaline Phosphatase) are not significant; whereas the total protein is significantly differing from the enzymes. The line graphs were visualized for each enzyme and their response with respect to the time duration (Figure 5 and Figure 6).

DISCUSSION

In the present study we reported the inhibitory effects of seed germination and two seed enzymes in-

cluding protease and alkaline phosphatase and it was found that the seed germination was significantly decreased by prolonged soaking intervals than the con-

TABLE 3 : Statistical analysis of non-germinated seeds

Enzymes	Duration	MEAN ± S.E
Protease	Control	121.75 ± 14.65
	3hrs	95.00 ± 11.70
	6hrs	73.25 ± 8.02
	12hrs	53.75 ± 6.98
	24hrs	24.75 ± 5.92
Alkaline Phosphatase	Control	137.75 ± 1.11
	3hrs	121.00 ± 1.96
	6hrs	107.75 ± 1.25
	12hrs	98.00 ± 1.47
	24hrs	82.75 ± 1.80

Values are mean ± S.E.M of four individual observations *significant at p<0.05 between control and 12hrs toxin treated group.

TABLE 4 : Statistical analysis of germinated seeds

Enzyme	Duration	Mean	S.E of Mean
Protease	Control	125.3333	20.0859
	3hrs	111.3333	17.9474
	6hrs	97.0000	18.000
Alkaline phosphatase	Control	138	1.5275
	3hrs	123.6667	3.1797
	6hrs	104.6667	1.4529
Enzymes	F-value	Sig. (p-value)	
	41.416	0.000*	

Values are mean ± S.E.M of four individual observations *significant at p<0.05 between control and 12hrs toxin treated group.

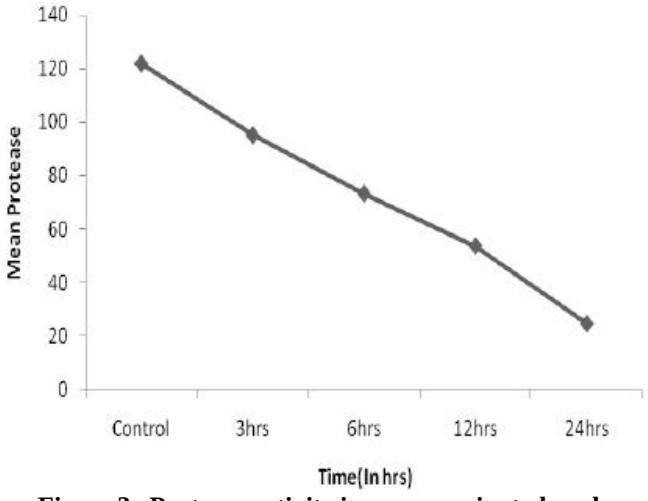
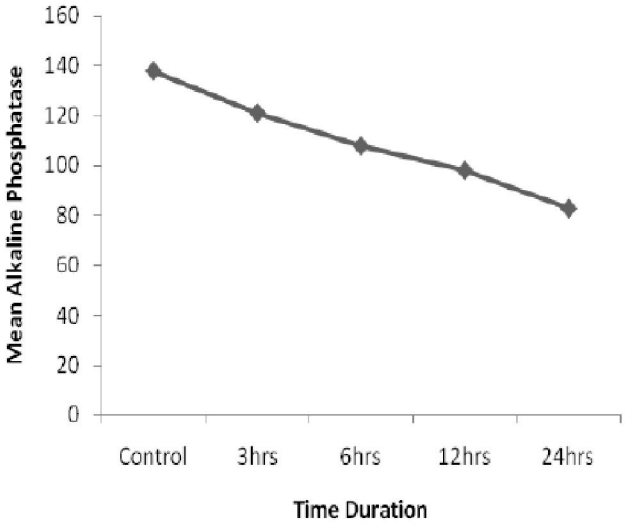


Figure 3 : Protease activity in non-germinated seeds



***The values represented in the figure 3 and 4 are the mean values.**

Figure 4 : Alkaline Phosphatase activity in non-germinated seeds

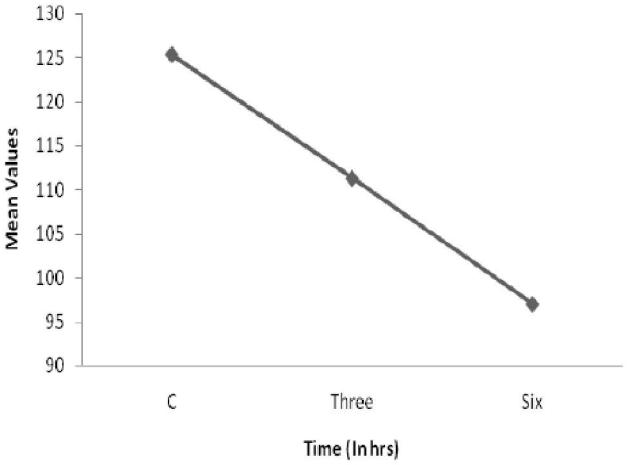
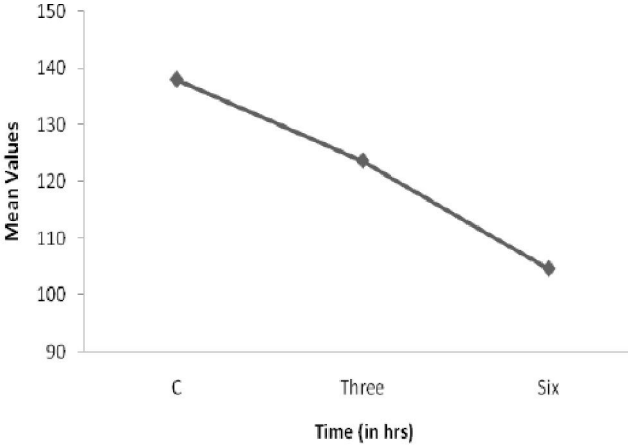


Figure 5 : Protease activity in germinated seeds



***The values represented in the figure 5, and 6 are the mean values**

Figure 6 : Alkaline Phosphatase activity in germinated seed

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trol treatments. Germination failure of different seeds (bean, red gram, green gram and black gram) as a result of aflatoxin has been demonstrated by Schoeuaral and white 1965[8], Joffe 1969[9], Lalithakumari and Govinda swami 1970[10], and Ewaidan 1992[11]. The inhibitory effect of aflatoxin on seed germination suggests that aflatoxin had functioned as anti auxins probably by inhibiting RNA synthesis. Similar reports were given by Cieger and Idlechhos 1968[12], Resiss 1971[13] and Rokesh et al 1994[14].Aflatoxin restricts plant growth by inhibiting seed germination, seedling growth and other physiological process of plants[15,16]. Inhibition of protein synthesis might be attributed to the non-availability of mRNA, where as inhibition of DNA synthesis might be due to the binding of aflatoxin to DNA during replication or due to the inhibition of DNA polymerase[17]. According to the reports of Key *et al* 1964[18] the synthesis of DNA like RNA, assumed to be mRNA is essential for growth and elongation in seedling root tissue of soya bean, corn and radish. In the presence of aflatoxin, the amount of mRNA synthesized would be directly related to the available unbound, aflatoxin free DNA remaining in the cell. Since the molecules of aflatoxin bind with DNA nucleotides in a specific ratio[19]. In the present study the cereals seed germination and seed enzymes and their activities were drastically reduced due to the influence of aflatoxin producted by fungal strain *Aspergillus flavas*, and this is first report of this area.

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