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USE OF AMMONIA GAS IN THE CONTROL OF *ASPERGILLUS FLAVUS* INFECTION AND AFLATOXIN PRODUCTION IN SUNFLOWER SEED

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Abstract

Sunflower seeds inoculated with *Aspergillus flavus* reduced seed germination with greater production of aflatoxin during storage. Use of ammonia gas although reduced seed germination but infection of *A. flavus* decreased with consequent reduction in aflatoxin production. Use of ammonia gas for 15 minutes was found optimum for significant supression of the production of aflatoxin B_1 and 5 minutes for aflatoxin B_2.

Introduction

Sunflower (*Helianthus annuus* L.), an important oil seed crop is cultivated over 25, 899 hectares in Pakistan (Anon., 1990). The seeds contain 32-45% oil which is a rich source of polyunsaturated fatty acid used for human consumption. Sunflower seed cake is used in poultry feed. Sunflower seeds are also consumed roasted and a coffee substitute is prepared from roasted seed (Sastri, 1959); Ambasta *et al.*, 1986). Of the mold fungi associated with sunflower seed, *Aspergillus flavus* was found to be predominant (Dawar & Ghaffar, 1991b), where 54% samples of sunflower seeds were contaminated with aflatoxin B_1 (Dawar & Ghaffar, 1991a). The fungus is known to produce mycotoxins where aflatoxin B_1 and B_2 are carcinogenic, damage liver, reduce growth rate and milk production in animals and man (Goldblatt, 1969). Ammonification is reported to be an effective method for detoxifying animal feed stuff (Cole, 1989). Experiments were therefore carried out on the use of ammonia gas for prevention and control of *A. flavus* infection and subsequent aflatoxin production in sunflower seed during storage.

Materials and Methods

Moisture content of seed was determined by oven dry method. Moisture content of the seed was adjusted at 15% by adding the required amount of sterilized distilled water to the seed. The seeds were mixed thoroughly and kept in a refrigerator at about 4-5°C for 24 h with frequent mixing to facilitate uniform distribution of moisture throughout the seed (Lutey & Christensen, 1963). Aflatoxin producing strain of *A. flavus* culture (KUMH 38) isolated for sunflower seed was used. Fifty g of seed sample with 15% moisture content were inoculated with 2 ml suspension of *A. flavus* @ 8.5×10^7 conidia/ml. Inoculated seeds were placed in a 30 cm diameter 625 mm sieve lined by a tissue paper. The sieve was put on a wooden stand at the bottom of which a 20 cm diam., Petri plate containing 25 ml of Ammonium hydroxide was placed which was covered with a bell jar to produce a minichamber for the treatment of seeds with ammonia gas. Seeds treated with ammonia gas for 10, 20, 30 and 40 minutes were placed in plastic bags. A cotton plug was placed at the mouth of each plastic bag and the mouth was tied up with a rubber band. The bags were stored at 30°C in an incubator. Three replicates of each treatment were used for the detection of *A. flavus* infection and aflatoxin production after 0, 15, 30 and 60 days interval. Infection of seed by *A. flavus* was assessed by using Blotter method (Anon., 1976), whereas aflatoxin production was estimated by AOAC method (Anon, 1975).

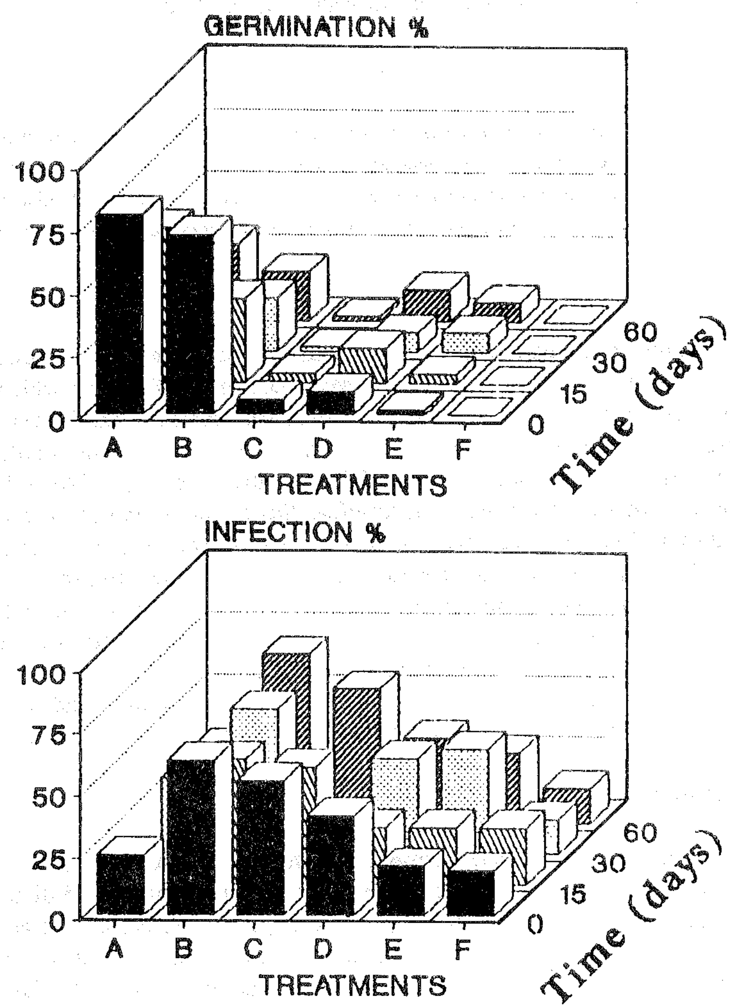

Fig.1. Effect of ammonia gas treatment on germination of sunflower seed and seed-borne infection by *Aspergillus flavus* during storage.

A = Control. B = Inoculated with *A. flavus*. C = Inoculated with *A. flavus* + NH_3 gas treatment for 10 min. D = Inoculated with *A. flavus* + NH_3 gas treatment for 20 min. E = Inoculated with *A. flavus* + NH_3 gas treatment for 30 min. F = Inoculated with *A. flavus* + NH_3 gas treatement for 40 min.

In another experiment sunflower seeds were ground in a grinder and inoculated with a toxigenic strain of *A. flavus* @ $6x10^7$ conidia/ml. Uninoculated seeds served as control. The seeds were treated with ammonia gas for 5,10,15,20 and 30 minutes and after 10 days of incubation at 30°C production of aflatoxin was detected by the method described above. Data were subjected to Factorial Analysis of variance (FANOVA) following Gomez & Gomez (1984).

Results and Discussion

Inoculation of seeds with *A. flavus* showed a gradual decline in seed germination as compared to control ($p<0.001$). Treatment of seeds with ammonia gas significantly reduced seed germination ($p<0.001$) and none of the seeds showed germination when exposed to ammonia gas for 40 minutes (Fig.1). A change in seed colour from black to green was also observed.

Seed infection by *A. flavus* was greater in inoculated seeds than in uninoculated control. Seeds inoculated with *A. flavus* when exposed to ammonia gas showed a significant reduction in infection by *A. flavus* with gradual increase in treatment time ($p<o.ool$). However, none of the treatments showed complete elimination of *A. flavus* infection after 15,30 & 60 days of storage (Fig.1). Pongsawart & Chinanwate (1991) reported that *A. flavus* and *A. parasiticus* used @ 10 spores/g was completely inhibited by ammonium benzoate in the control of aflatoxin production on peanut and maize. In the present study, a higher number of spores/g of seed used for inoculation could be the reason for the failure of ammonia gas in complete elimination of *A. flavus* infection. It is interesting to note that ammonia gas released from the decomposing oats and barley tissues in soil was also found lethal to soilborne fungi (Lewis, 1976). It might also be possible that the spores of *A. flavus* used for inoculation were killed by ammonia gas treatment and *A. flavus* infection observed in the inoculated seeds treated with ammonia gas was internally seed borne since non-inoculated seeds also showed infection by *A. flavus*.

Production of aflatoxin B_1 in uninoculated control was zero at 0-days and in traces at subsequent intervals. Seeds inoculated with toxigenic strain showed higher production of aflatoxin B_1 at different interval of storage. No aflatoxin production was observed at 0-days in seed treated with ammonia gas. Amount of aflatoxin B_1 and B_2 significantly decreased with the increase in time ($p<0.001$). Production of aflatoxin B_1 was observed in ammonia gas treated seeds also after 60 days period which approached to the tolerance limit of 20 μg/kg (Anon, 1977) whereas production of aflatoxin B_2 was either zero or in traces (Table 1). It may be mentioned that Ammonium hydroxide showed 60% reduction in aflatoxin production in peanut and maize (Napaporn *et al.*, 1991).

In another experiment, sunflower seeds substrate was inoculated with spore suspension of *A. flavus* and then treated with ammonia gas for 5, 10, 15, 20 and 30 minutes. A 5 minutes treatment was not effective whereas treatment for 10 minutes reduced the production of aflatoxin B_1 very close to the tolerance limit of 20 μg/Kg whereas treatment for 15 minutes or more completely prevented the production of aflatoxin B1. Use of ammonia gas treatment for 5 minutes or more significantly suppressed the production

Table 1. Effect of ammonia gas treatment on production of aflatoxins in sunflower seeds during storage.

Treatment		Aflatoxin B_1 ($\mu g/kg$)				Aflatoxin B_2 ($\mu g/kg$)			
	Days	0	15	30	60	0	15	30	60
Control		0	Traces	Traces	Traces	0	0	0	0
Inoculated with toxigenic strain of *A. flavus* and treated with ammonia gas for									
0 minute		73	20	54	73	36	0	9	0
10 minute		0	Traces	36	37	0	0	0	0
20 minute		0	0	6	15	0	0	Traces	0
30 minute		0	Traces	4	29	0	0	Traces	0
40 minute		0	0	Traces	29	0	0	Traces	0

of aflatoxin B_2 (Table 2). No significant difference in aflatoxin production was observed in treatment where seed substrate were treated with ammonia gas. There are reports that NH_4OH solution sprayed on contaminated poultry feed reduced the aflatoxin content (Mahalingam *et al.*, 1990). Aflatoxin contamination was effectively detoxified by NH_4OH on copra (Mercado *et al.*, 1991) and in contaminated grains or feed (Bennett *et al.*, 1980; Young, 1986). Since sunflower seed is an important source of edible oil and its seed cake is used in poultry feed, the sunflower seeds contaminated with aflatoxin thus poses a potential threat for the life of human being and poultry birds. The present studies would therefore suggest that ammonia gas treatment of sunflower seed could be used to reduce aflatoxin production during storage.

Table 2. Detoxification of aflatoxins by ammonia gas treatment on sunflower seed substrate.

	Aflatoxin B_1 $\mu g/kg$	Aflatoxin B_2 $\mu g/kg$
Control (uninoculated)	0	0
Inoculated with *A. flavus* and treated with ammonia gas for		
0 minutes	80	36
5 minutes	73	0
10 minutes	22	Traces
15 minutes	0	0
20 minutes	0	0
30 minutes	0	0

References

Ambasta, S.P., K. Ramachandra, K. Kashyapa and R. Chan. 1986. *The useful plant of India*. C.S.I.R.I Publication and Information Directorate, Hillside Road, New Delhi. 918 pp.

Anonymous. 1975. *AOAC methods*. 12th ed. Chapter 26.

Anonymous. 1976. *International rules of seed testing*. Proc. Int. Seed Tst . Assoc., 4: 3-49.

Anonymous. 1977. FAO Document No. Myc-4C. Joint FAO/WHO/UNSP Conference on Mycotoxins, Nairobi (Kenya), pp 19-21.

Anonymous. 1990. *Agriculture Statistics of Pakistan*, 1989-1990. Ministry of food, Agriculture and Co-operatives, Food and Agriculture Division (Economic Wing. Govt. of Pakistan Islamabad, 316 pp.

Bennett, G.A., O.L. Shotwell and C.W. Hesseltine. 1980. Destruction of Zearelenone in contaminated Corn. *Journal of the American Oil Chemists Society*, 57: 245-247.

Cole, R.J. 1989. Technology of aflatoxin contamination. *In: Mycotoxins and phycotoxins*. (Eds.) S. Natori, K, Hashimoto and Y. Ueno. Elsevier, Science Publisher, 88: 177-184.

Dawar, S. and A. Ghaffar. 1991a. Detection of aflatoxin in sunflower seed. *Pak. J. Bot.*, 23: 123-126.

Dawar, S. and A. Ghaffar. 1991b. Detection of the seedborne mycoflora of sunflower. *Pak. J. Bot.*, 23: 173-178.

Goldblatt. L.A. 1969. *Aflatoxin Scientific Background Control and implications*, Academic Press, N. York pp. 472.

Gomez, K.A. and A.A. Gomez. 1984. *Statistical procedure for agricultural research*. J. Wiley, New York, 680 pp.

Lewis, J.A. 1976. Production of volatiles from decomposing plant tissues and effect of these volatiles on *Rhizoctonia solani* in culture. *Can. J. Microbiol.*, 22: 1300-1306.

Lutey, R.W. and C.M. Christensen. 1963. Influene of moisture content, temperature and length of storage upon survival of fungi in barley Kernels. *Phytopath.*, 53: 713-717.

Mahalingam, R.J. and S. Govindan. 1990. A study on aflatoxin detoxification by the aqua-ammonia method in poultry feed. *Indian. Vet. J.*, 67: 149-151.

Mercado, C.J., M.P.N. Real and R.R. Dez Rosario. 1991. Chemical detoxification of aflatoxin containing copra. *J. Food Sci.*, 56: 733-735.

Napaporn O-ariyakul, Songpan Wangjaisuk and Nantarit Choketha worn. 1991. Chemcial detoxification of Aflatoxin B1. pp. 252-253. In: *Proc. Fungi and mycotoxins in stored product* (Eds.) B.R. Champ, E. Highley, A.D. Hocking and O.I. Ittsangkok, Thailand.

Pongsawart Niyomca and Natenapit Chinanomwate. 1991. Potential of Ammonium benzoate for aflatoxin control. p.25. In: *Proc. Fungi and mycotoxin in storage products* (Eds.) B.R. Champ, E. Highley, A.D. Hocking and J.I. Pitt, Bangkok, Thailand.

Sastri, B.N. 1959. *The wealth of India*. A dictionary of Raw materials and Inustrial products. Vol.V. Council of Sciencetific & Industrial Research, New Delhi. pp. 332.

Young, J.C. 1986. Reduction in levels of deoxynivalenol in contaminated corn by chemical and physical treatment. *Journal of Agriculture and Food chemistry*, 324: 659-664.

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