Effect of physical treatments on moulding and aflatoxin production in maize

H.S. Shetty*, P. Vijaya*, C.M. Usha*, K.L. Patkar* and J. Lacey†

Abstract

Maize (*Zea mays* L.) in India is generally grown in humid conditions and harvested with a fairly high water content. In these conditions, post-harvest losses caused by storage fungi can be large. Fungistatic agents and fumigants have been used to inhibit moulding and mycotoxin production but chemical treatments are hazardous and leave residues. Physical treatments can be used to complement and decrease dependence on chemical control methods. This paper describes the use of irradiation and modified atmosphere storage to prevent moulding and aflatoxin production in maize stored with different water contents. Treatment of maize with 4 kGy irradiation at both high and low water contents inhibited aflatoxin production but not moulding. Total inhibition of fungal colonisation could not even be achieved with as much as 14 kGy irradiation. Aflatoxin production by *Aspergillus flavus* isolates from irradiated maize was completely inhibited after 6 kGy irradiation. Modified atmospheres containing 60% CO_2/20% O_2 were more effective in controlling moulding and aflatoxin production than those containing less CO_2. Yeasts and yeast-like fungi became increasingly predominant as storage period increased. Integration of irradiation and modified atmosphere treatments showed synergism with the smaller doses of irradiation (4 kGy) and CO_2 (40% CO_2/20% O_2). Moulding and aflatoxin production were minimised compared to individual treatments.

Introduction

In India, maize is grown in humid conditions and is colonised by a range of fungi. Surface-sterilised maize kernels, from diverse geographical areas, predominantly yield species of *Aspergillus, Fusarium* and *Penicillium* (Lillehoj and Zuber 1988). These fungi decrease seed germination, vigour and nutritional quality as well as producing mycotoxins which present health hazards to humans and animals.

Traditionally, moulding and mycotoxin production in stored grain are controlled by drying, by using fungistatic agents, such as low molecular weight fatty acids, and by fumigation (Reichmuth 1991). In a humid climate, none of these methods is totally satisfactory. It is often difficult to dry to a safe water content. Low molecular weight fatty acids are volatile so that they may be lost during application, progressively escape from silos or be metabolised by fungi, leaving the grain unprotected (Multon 1989). Fumigation with highly diffusible gases is hazardous, none are fully effective against moulding and they may leave residues (Staufer 1991; Lacey 1993). Also, both acids and fumigants are corrosive.

* Department of Studies in Applied Botany, University of Mysore, Manasagangotri, Mysore 570 006, India.
† Institute of Arable Crops Research, Rothamsted Experimental Station, Harpenden, Herts AL5 2JQ, UK.

Interest is increasing in alternative methods of mould prevention, such as irradiation and modified atmosphere storage. Neither leave residues, and irradiation may uniformly disinfest stored seeds. However, doses of radiation may need to be large, e.g. 12 kGy (Cuero et al. 1986; Ramakrishna et al. 1991), to kill all microorganisms and reports of its effect on aflatoxin production appear contradictory. (Chang and Markakis 1982; Sharma et al. 1990). Inhibition of fungal growth requires greater than 60% CO_2 or less than 0.1% O_2 although there may be synergism when CO_2 concentrations are increased and O_2 concentrations are decreased simultaneously (Wilson et al. 1977; Lacey 1993). Low O_2 concentrations and high CO_2 can also inhibit mycotoxin production but at less extreme concentrations than are necessary to inhibit mould growth.

Different treatments applied together have been shown to act synergistically against moulds, especially when the levels of the different treatments are low (Paster et al. 1992). Thus, irradiation and modified atmospheres were shown to act synergistically in preventing moulding of maize and together to decrease the requirement for propionic acid. To seek to confirm and extend these findings in an Indian environment, we have tested the effects of water content and the combined effects of gamma irradiation and modified atmospheres on moulding and aflatoxin production in maize.

Materials and Methods

Preparation of seed samples

Freshly harvested maize seeds, cv. Mahyco, were collected from H.D. Kote taluk of Mysore District. Seed water content was determined by the high constant temperature oven method, following the recommendations of ISTA (1985). The water content of the seeds was modified by adding a calculated volume of distilled water and then equilibrating at 40•C for 4 days. The required volume was determined using the following formula:

$$W = \frac{A(b-a)}{100-b}$$

where W = volume of water required (mL); A = initial weight of sample (g); a = initial water content (%); b = required water content (%).

Irradiation treatment

Seeds adjusted to 12 or 18% water content were divided into 250 g lots and placed in polyethylene bags which were then sealed. They were then irradiated with 2, 4, 6, 8, 10, 12 or 14 kGy of gamma radiation from a ^{60}Co source at Kidwai Memorial Institute of Oncology, Bangalore. Two packets of seeds at each water content were irradiated with each dose. After treatment, one set of grain from each water content was stored under ambient conditions and the other was incubated in a desiccator adjusted to 90% r.h. using a saturated solution of barium chloride. Seed mycoflora, aflatoxin content and the

aflatoxin producing ability of *Aspergillus flavus* isolates were determined after 0, 15, 30 and 60 days storage.

Modified atmosphere treatment

Seeds containing 15 or 18% water were stored in atmospheres containing either 40% CO_2/20% O_2 or 60% CO_2/20% O_2. The modified atmosphere system was similar to that developed by Navarro and Donahaye (1972). The three atmospheric gases, N_2, O_2 and CO_2, were taken from gas cylinders through a series of pressure-reducing valves to give a pressure of about 12 mm Hg, measured using manometers. The gases were mixed in a chamber to give a flow of 100 mL/minute of the required mixture. Gas concentrations were confirmed using a CO_2 gas analyzer (Gow-Mac, Ireland) and an O_2 meter (Riken, Japan). The gas mixtures were humidified to 90% r.h., by passing through gas washing bottles containing saturated solutions of barium chloride, before being distributed to Erlenmeyer flasks containing maize seeds. Seed mycoflora and aflatoxin production were determined after 0, 15, 30 and 60 days storage.

Integrated treatments

Maize seeds, adjusted to 20% water content, was sterilised with 2 or 4 kGy gamma irradiation, then stored in a modified atmosphere of 40% CO_2/20% O_2. Grain was sampled after 0, 15, 30 and 45 days to determine mould and mycotoxin development.

Seed mycoflora

Fungal colonisation of seeds surface sterilised for 2 minutes in 2% sodium hypochlorite solution then rinsed and blotted dry was determined by plating on malt salt agar and incubating at 28±2°C for 7 days. Four replicates, each of 50 seeds, were plated from each treatment. Numbers of seeds infected were counted daily from the third day.

Aflatoxin B_1 assay

AFB_1 was extracted and assayed by indirect competitive ELISA as described by Ramakrishna et al. (1990). Aflatoxin B_1 aflatoxin B_1-BSA conjugate, goat antimouse IgG-HRP conjugate, 3,3',5,5'-tetramethyl benzidine (TMB) were purchased from Sigma Chemicals and the remaining chemicals were all analytical reagent grade. Monoclonal antibody (McAb) against aflatoxin B_1 was obtained from Rhône-Poulenc Diagnostic (Glasgow, U.K.).

Seed was extracted by blending 20 g with 100 mL of extraction solvent (acetonitrile + 0.5% KCl + 6% H_2SO_4, 89:10:1) for 2 min in a Waring blender. The extract was filtered through Whatman No. 1 filter paper and diluted to 1:10 with Tris-HCl buffer containing 9% acetonitrile before ELISA assay.

Aflatoxin production by *Aspergillus flavus* isolates

Aspergillus flavus in seed receiving different radiation doses were isolated and maintained on potato dextrose agar slants. Seven-day-old colonies then were used to inoculation yeast extract sucrose broth (YES). Culture filtrate (5 mL) was extracted with 25 mL extraction solvent and filtered through Whatman No. 1 paper before monoclonal antibody based indirect competitive ELISA as described above.

Results

Effects of irradiation on the seed microflora

Fungal colonisation of maize seeds decreased with radiation dose but fungi could still be isolated even after exposure to 14 kGy gamma radiation. Incidence of fungal infection increased with water content of the irradiated grain, from 24% in grain containing 12% water to as many as 40% in that containing 18% water. The percentage of seeds infected increased with storage period after all treatments so that nearly all seeds with 12% water content were infected after 30 days under ambient conditions (Table 1). Colonisation of untreated grain was slightly more rapid than that of irradiated and, except after 2 kGy irradiation, was more rapid with 18% water content that with 12%. Moulding of grain stored in 90% r.h. was more rapid than of that stored in ambient conditions and all seeds in all treatments were mouldy after 15 days.

The predominant fungi isolated from irradiated grain were *Aspergillus flavus, A. niger, A. versicolor* and *Penicillium* spp. *A. flavus* decreased in incidence with increasing radiation dose but, by the 15th day of incubation, it was isolated more frequently from grain irradiated with ≥8 kGy than from that receiving smaller doses. With incubation at 90% r.h., most *A. flavus* was found in untreated grain, followed by that receiving 2 kGy. Least occurred in grain irradiated with 4 kGy at 12% water content and with 6 kGy at 18% water content. With 90% r.h., *A. niger, A. versicolor* and *Penicillium* spp. were more common in grain with an initial water content of 18% than in that with 12% water content. *Penicillium* spp. were more common in grain incubated in 90% r.h. than in that incubated in ambient conditions.

Table 1. Effects of irradiation of maize seeds containing 12 or 18% water on fungal colonisation during storage under ambient conditions (26-30°C)

Storage period (days)	Radiation dose (kGy)							
	0	2	4	6	8	10	12	14
	12 % initial water content							
0	74.0	58.0	52.0	40.0	38.0	38.0	28.0	24.0
15	99.0	78.0	64.0	77.5	86.0	89.5	99.5	87.0
30	99.0	98.5	94.5	97.5	99.0	99.0	98.5	100
60	99.5	99.0	98.0	99.0	100	100	100	100
	18% initial water content							
0	76.0	78.0	56.0	40.0	52.0	54.0	44.0	40.0
15	100	51.0	97.0	97.5	100	84.0	94.0	99.5
30	100	89.5	97.5	86.5	93.5	93.0	81.0	100
60	100	89.5	98.5	92.0	93.5	93.5	97.0	100

Aflatoxin was already present in the maize at the start of the experiment although the amount recovered decreased with increasing radiation dose (Table 2). Subsequently, it occurred only in seeds containing 12% water irradiated with 2 or 4 kGy and in those with 18% water content treated with 2 kGy. Results were similar after incubation in 90% r.h. although 2.0 ng aflatoxin/g was also found in grain irradiated with 6 kGy after 15 days.

Effect of irradiation on aflatoxin production by *A. flavus*

Aspergillus flavus isolated from unirradiated seeds produced more AFB_1 than that isolated from irradiated seeds and no AFB_1 was produced by isolates from grain irradiated with 8 kGy or more at 12% water content or 4 kGy or more with 18% water content (Table 3). Production decreased with dose, and isolates from the drier grain tended to produce more AFB_1 than those from the wetter.

Modified atmosphere storage

Aspergillus flavus already colonised 26% of maize grains at the start of the experiment and *Penicillium* spp. 45% (Table 4). *A. niger* and yeast developed subsequently. During storage, *A. flavus* was slightly more common in grain with 18% water content stored in modified atmospheres than with 15% and was more common in atmospheres containing 40% CO_2 than in those containing 60%.

Generally, incidence of *A. flavus* decreased during storage in modified atmospheres although less so in the lower CO_2 concentration at the higher water content. After 60 days storage, no *A. flavus* could be isolated from treatments stored in 60% CO_2/20% O_2 while numbers in 40% CO_2/20% O_2 were slightly greater than those in grain stored in ambient conditions.

By contrast with seeds stored in ambient conditions, *Penicillium* spp. decreased initially in both modified atmosphere treatments. However, except at 15% water content, incidence later increased, specially with 40% CO_2. No yeasts were isolated at the start of the experiment but numbers increased through storage of all treatments, especially in modified atmospheres. *A. niger* was more common in seeds stored in ambient conditions than in those stored in modified atmospheres.

At the start of the experiment, the maize contained 52 ng AFB_1/g (Table 4). However, AFB_1 concentrations decreased during storage, especially at 18% water content.

Integrated treatment

The incidence of different fungi in maize grain irradiated with 2 or 4 kGy and stored in different atmospheres is shown in Table 5. *A. flavus* infected 10 and 8%, respectively, of grains receiving 2 and 4 kGy irradiation but numbers decreased in both modified atmospheres and increased in ambient air. *Penicillium* spp. and yeasts increased in all treatments. The increase in incidence of *Penicillium* spp. was greater after 4 kGy irradiation than after 2 kGy and slightly less after modified atmosphere storage. Yeasts increased more rapidly in air than in modified atmospheres, in contrast with the earlier experiment.

Initially the maize contained 26–30 ng aflatoxin/g (Table 5). AFB_1 concentration decreased slightly in seeds stored in ambient air while none could be detected in seeds stored 15 days or more in modified atmospheres.

Discussion

Losses of grain during storage occur worldwide but are especially large in developing countries where facilities are often lacking for the safe, technically sound storage of food grains and seeds. A range of different methods has been used to prevent deterioration of stored seeds and to maintain their quality. The present study has examined the potential of gamma irradiation and modified atmosphere storage, both alone and in combination, for preventing mould and mycotoxin development in stored maize seeds.

Different treatments interacted in their effects on the grain microflora. Low water contents were best for irradiation treatment and the subsequent storage of irradiated seeds.

Table 2. Aflatoxin B_1 concentrations (ng/g) in irradiation treated grain during storage under ambient conditions (26–30°C).

Storage period (days)	Radiation dose (kGy) 0	2	4	6	8	10	12	14
				12% initial water content				
0	10.0	9.7	9.6	8.6	4.3	4.0	3.2	3.0
15	8.0	5.6	3.2	0.0	0.0	0.0	0.0	0.0
30	6.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
60	5.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
				18% initial water content				
0	10.0	9.8	9.6	9.0	4.0	3.6	3.0	3.0
15	8.6	2.4	0.0	0.0	0.0	0.0	0.0	0.0
30	6.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
60	4.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 3. Production of aflatoxin B_1 (ng/mL medium) by isolates of *Aspergillus flavus* from gamma irradiated maize seeds

Seed water content (%)	Radiation dose (kGy) 0	2	4	6	8	10	12	14
12	10.8	9.9	9.3	4.4	0.0	0.0	0.0	0.0
18	9.3	8.8	0.0	0.0	0.0	0.0	0.0	0.0

Fewest *Aspergillus flavus* and *Penicillium* spp. were isolated from 12% water content grain after 4 kGy irradiation while their numbers were least in 18% water content grain only after 10 kGy irradiation. However, at neither water content were all fungi killed, even by 14 kGy irradiation. This is despite 12 kGy having previously been found sufficient to sterilise maize and barley seeds and 1.2 kGy to kill *Aspergillus* spp. (Cuero et al. 1986; Ramakrishna et al. 1991).

The concentration of aflatoxin B_1 present in the maize at the start of the experiment declined with increasing radiation dose and with increasing period of storage at both 12 and 18% water content. Although *A. flavus* could still be isolated from irradiated grain, its ability to produce AFB_1 appeared to be reduced by irradiation, especially at 18% water content, and isolates from irradiated grain were also less able to produce aflatoxin than those from unirradiated. Sharma et al. (1990) also found decreasing aflatoxin producing ability in *A. parasiticus* after increasing radiation doses. By contrast, Chang and Markakis (1982) found more aflatoxin in seeds with a high water content than in drier seeds and increasing concentrations with period of storage after irradiation.

Table 4. Fungal colonisation and aflatoxin production in maize seed with different water contents stored in modified atmospheres.

Fungi (% seeds infected)	Storage period (days)	Water content					
		15%			18%		
		Ambient air	MA[a]	MA[b]	Ambient air	MA[a]	MA[b]
A. flavus	0	26	26	26	26	26	26
	15	32	12	10	27	12	11
	30	13	11	2	14	22	2
	60	8	9	0	4	11	0
Penicillium spp.	0	45	45	45	45	45	45
	15	54	10	1 2	43	12	10
	30	50	48	14	82	61	38
	60	56	5 1	17	80	60	44
Yeast	0	0	0	0	0	0	0
	15	10	5	8	16	10	13
	30	12	18	20	20	30	26
	60	1 4	20	22	22	31	26
Aflatoxin (ng/g)	0	52	52	52	52	52	52
	15	36	30	32	42	24	26
	30	46	12	13	51	3	5
	60	28	10	14	20	5	6

[a]MA, modified atmosphere consisting of 40% CO_2 and 20% O_2
bMA, modified atmosphere consisting of 60% CO_2 and 20% O_2

Table 5. Fungal colonisation and aflatoxin production in maize seed during storage in a modified atmosphere after irradiation treatment.

Fungi (% seeds infected)	Storage period (days)	2 kGy		4 kGy	
		Ambient air	MA[a]	Ambient air	MA[a]
A. flavus	0	10	10	8	8
	15	8	8	10	4
	30	16	5	12	2
	60	29	4	10	1
Penicillium spp.	0	30	30	24	24
	15	38	32	56	29
	30	44	40	72	32
	60	62	53	86	76
Yeast	0	0	0	0	0
	15	20	2	35	3
	30	60	12	62	11
	60	100	19	100	17
Aflatoxin (ng/g)	0	30	30	26	26
	15	26	0	12	0
	30	28	0	24	0
	60	24	0	21	0

[a]MA, modified atmosphere consisting of 40% CO_2 and 20% O_2

During modified atmosphere storage, concentrations of aflatoxin B_1 and the incidence of *A. flavus* decreased, while incidence of *Penicillium* spp. and yeasts increased. Decreased aflatoxin B_1 production by *A. flavus* in modified atmospheres has previously been reported (Landers et al. 1967; Diener and Davis 1972), Large concentrations of CO_2 were more effective than small concentrations of O_2 in inhibiting aflatoxin production in groundnut and zearalenone in maize (Paster et al. 1991). Aflatoxin production was never completely inhibited in the modified atmospheres tested although a combination of high CO_2 and low O_2 might have been more effective (Lacey 1993).

Fungal colonisation was less in maize seeds which were first irradiated and then stored in a modified atmosphere than in any of the individual treatments, and no aflatoxin B_1 could be detected during storage of the combined treatments. The effect on aflatoxin production could have resulted both from the direct effects of the treatments on *A. flavus* and also from their effects on other components of the microflora. For instance, *Penicillium* spp. were much more tolerant of the treatments than were *A. flavus* and yeasts. This study has thus confirmed the findings of Paster et al. (1992) that a combination of different treatments can have synergistic effects on both mould development and mycotoxin production in stored grain. These can be utilised to prolong safe storage without deterioration.

Acknowledgment

This project was part funded by the Commission of the European Community under Contract No. TS2.0164.M(H).

References

Chang, H.G. and Markakis, P. 1982. Effect of gamma-irradiation on aflatoxin production in barley. Journal of the Science Food Agriculture, 33, 559–564.

Cuero, R.G., Smith, J.E. and Lacey, J. 1986. The influence of gamma irradiation and sodium hypochlorite sterilisation on maize seed microflora and germination. Food Microbiology, 107–113.

Diener, U.L. and Davis, N.D. 1972. Atmospheric gases and aflatoxin production in peanuts. Highlights of Agricultural Research, 19, 3.

ISTA (International Seed Testing Association) 1985. Moisture content determination. Seed Science and Technology, 13, 338–341.

Lacey, J. (1993). Effects of intergranular gas composition and fumigants on mould growth and mycotoxin production. In: Navarro, S. and Donahaye, E., ed., Proceedings of an International Conference on Controlled Atmosphere and Fumigation in Grain Storages, Winnipeg, Canada, June 1992. Jerusalem, Caspit Press, 131–149.

Landers, K.E., Davis, N.D. and Diener, U.L. 1967. Influence of atmospheric gases on aflatoxin production by *Aspergillus flavus* in peanuts. Phytopathology, 57, 1006–1090.

Lillehoj, E.B. and Zuber M.S. 1988. Distribution of toxin producing fungi in mature maize kernels from diverse environments. Tropical Science, 28, 19–24.

Multon, M. 1989. Control of post harvest loss in cereal. In: Multon, M. ed., Pre- and post-harvest spoilage of cereal grains. Boca Raton, CRC Press, 178.

Navarro, S. and Donahaye, E. 1972. An apparatus for studying then effect of controlled low pressure and composition of atmospheric gases on insects. Journal of Stored Products Research, 8, 223–226.

Paster, N., Blumenthal-Yonassi, J., Barkai-Golan, R. and Menasherov, M. 1991. Production of zearalenone *in vitro* and in corn grains stored under modified atmospheres. International Journal of Food Microbiology, 12, 157–160.

Paster, N., Menasherov, M., Lacey, J. and Fanelli, C. 1992. Synergism between methods for inhibiting the spoilage of damp maize during storage. Postharvest Biology and Technology, 2, 166–170

Ramakrishna, N., Lacey, J., Candlish, A.A.G., Smith, J.E. and Goodbrand, I.A. 1990. Monoclonal antibody-based enzyme linked immunosorbent assay of aflatoxin B_1, T-2 toxin and ochratoxin A in barley. Journal of the Association of Official Analytical Chemists, 73, 71–76.

Ramakrishna, N., Lacey, J. and Smith, J.E. 1991. Effect of surface sterilization, fumigation and gamma irradiation on the microflora and germination of barley seeds. International Journal of Food Microbiology, 13, 47–54.

Reichmuth, C. 1991. New techniques in fumigation research today. In: Fleurat-Lessard, F. and Ducom, P., ed., Proceedings of the Fifth International Working Conference on Stored-product Protection, Bordeaux, France, September 1990, 709–724.

Sharma, A., Padwal Desai, S.R. and Nair, P.M. 1990. Aflatoxin producing ability of spores of *Aspergillus parasiticus* exposed to gamma radiation. Journal of Food Science, 55, 275–276.

Staufer, J.E. 1991. Control without liquid fumigant. Cereal Food World, 31, 807.

Wilson, D.M., Jay, E., Male, D.M. and Huang, L.M. 1977. Controlled atmosphere storage of high-moisture corn and peanuts to control aflatoxin production. Annales de Technologie Agricole, 27, 339–342.