INTEGRATED STRATEGIES FOR THE CONTROL OF MOULDING IN GRAIN

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Abstract

Attempts to prevent moulding and mycotoxin production in stored grain have mostly emphasised control of one factor. Management strategies that integrate environmental, physical and chemical methods for preventing moulding might enable less extreme use of any one method. Such treatments may need to include measures for the control of moulding and mycotoxins even before the crop reaches store. In laboratory experiments with a range of seed types, combined irradiation and propionic acid treatments, with both 1 kGy + 0.1% propionic acid and 0.5 kGy + 0.3% propionic acid, controlled moulding better than any of the component treatments. Superficial seed lipids, that control aflatoxin production by *A. flavus*, were little affected by the irradiation. A collaborative study, funded by the European Economic Community, is seeking to develop systems for using combined treatments that can be applied on farms, especially in developing countries.

Introduction

About 5% of the world's grain harvest is lost during storage but in developing countries such losses may reach 30% or more. Fungi are important causes of loss, not only through loss of dry matter but also through loss in quality, by decreasing nutritional value and through their ability to produce mycotoxins. Studies of ways of preventing such losses have mostly emphasised the control of one environmental factor. Synergism between different factors controlling fungal growth have not been explored although it has been shown that suboptimal conditions of one factor decrease the ability of fungi to tolerate extreme conditions of another factor. Thus, suboptimal temperatures decrease the ability of fungi to tolerate low water activities and, similarly, water activity interacts with intergranular gas composition and propionic acid treatment in controlling fungal growth. Water activity and temperature also affect mycotoxin production. The use of management strategies in which environmental, physical and chemical methods are combined to control moulding could enable the use of less extreme levels of any one factor than if they were used alone.

Factors affecting moulding of stored grain

Colonization of stored grain starts before harvest but the species generally differ from those important in store. One exception is *Aspergillus flavus* in humid tropical areas which may colonize maize grain and even produce aflatoxins before harvest. However, contamination with inoculum of storage fungi usually occurs either from grain that has lodged and been colonized by *Penicillium* spp., from the harvester, from drying equipment or from the grain store itself. Conditions in stored grain are more stable than in the field because the insulating effect of the grain prevents marked diurnal temperature fluctuations. The humidity of the intergranular air is largely controlled by the water content of the grain and air movement is by convection or by artificial ventilation. Which species then develop depends on the water content of the grain, temperature, intergranular gas composition and any chemical or physical treatments that the grain may receive before or during storage. The roles of these factors in controlling moulding and their possible integration into a single management strategy are discussed below.

Water availability

The availability of water for microbial growth in grain is determined by the water content and is usually expressed as water activity (a_w) so that the effects of water availability on microbial growth in different types of seeds can be compared. The a_w/water content relationship differs between starchy and oil seeds and to a lesser extent between different types of cereal and between varieties. However, regardless of seed type, fungi cannot grow below 0.60-0.65 a_w and most species require a greater a_w. Thus, as a_w decreases, the number of species that are able to grow also decreases and their ability to germinate, grow and sporulate is retarded. Storage at high a_w may lead to spontaneous heating and, without ventilation, to the formation of hot spots and to moisture migration. Consequently, the degree of drying determines how long grain can be stored without significant colonization and deterioration. Usually, drying grain to 0.65 a_w is recommended for long term storage although a higher a_w, about 0.72 a_w may be acceptable for storage up to three months only, because slow mould growth still occurs (Snow *et al.*, 1944). These a_w are often below those normal in commercial trading and in grading standards. Only very small changes in a_w are necessary to have a large effect on the rate of moulding of stored grain (Christensen, 1963) so that a_w above these limits are not safe for storage. The mixing of wet and dry grain to achieve a water content satisfying a grade requirement is also not safe as the wetter grain may already have deteriorated and may render the whole more susceptible to subsequent deterioration. Grain harvested with many unripe grains may also be more susceptible to heating and moulding than uniformly mature samples with the same mean water content (Hill & Lacey, 1983). Prior invasion of part of the grain with storage fungi may render the whole more liable to deterioration as the fungi can continue to grow at a slower rate after mixing. Moulding may also alter the water content a_w relationship, making the grain more susceptible to further moulding than indicated by the water content, especially if the grain is not well mixed (Christensen, 1974).

Temperature

Fungi grow within temperature ranges characteristic of the species and growth rates increase with temperature to a clearly defined optimum followed by a rapid decline as the maximum is approached. Storage fungi differ greatly in their temperature requirements. Some *Penicillium* spp. grow between -4 and 35°C and *Aspergillus* spp. between 10 and 55°C while thermophilic fungi, e.g., *Humicola lanuginosa*, grow only between 30 and 60°C. Field fungi, as well as some storage fungi, can survive at low temperatures and may sometimes even grow actively, especially if a_w is high (Christensen, 1974). In particular, *Fusarium*, *Cladosporium* and *Penicillium* can grow well at -6 to -8°C and can sometimes sporulate and produce mycotoxins at these temperatures (Sinha & Wallace, 1965). Change in temperature of stored grain may result not only from changes in ambient temperature but also from spontaneous heating. If a_w is sufficiently high for microbial growth and the energy released by respiration cannot escape, heating of the grain occurs. The maximum temperature reached depends on the a_w of the grain with maxima of about 65°C at close to 1 a_w.

Interaction of a_w and temperature

Temperature and a_w interact in determining the rate at which fungi grow under different conditions (Magan & Lacey, 1984a). Studies of the growth rate of fungi at different combinations of temperature and a_w have resulted in the production of isopleth diagrams (Fig. 1). From Fig. 1 it can be seen that the minimum a_w allowing growth occurs at about the optimum temperature while the greatest temperature range supporting growth occurs when a_w is optimum. Any departure from the optimum conditions of one factor leads to a decrease in tolerance of unfavourable conditions of the other. Mycotoxin production also changes with a_w and temperature in a similar manner, although the relationship differs for mycotoxins produced by a single fungus and also between the same mycotoxin produced by different species of fungi.

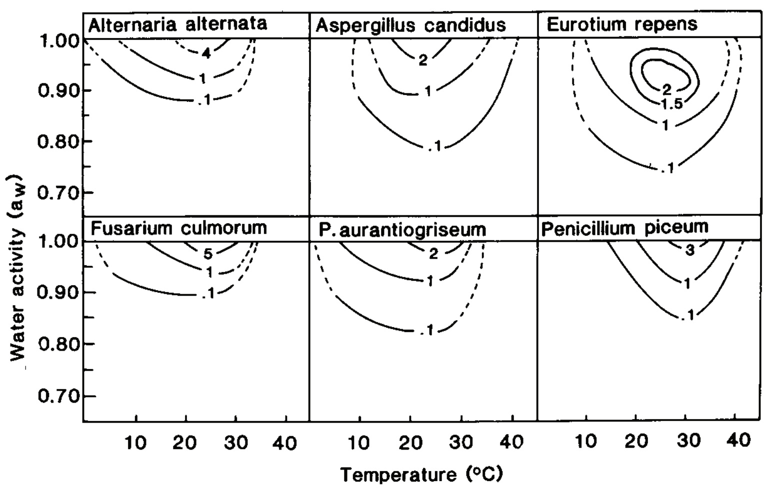

Fig. 1. A_w / temperature relationships of field and storage fungi

Intergranular gas composition

One of the products of respiration is CO_2, produced by the utilization of O_2. When grain is stored wetter than 0.65 a_w, microorganisms and grain are able to respire and CO_2 and O_2 accumulate within the bulk, sometimes in sufficient amount to inhibit fungal growth. Grain storage fungi are usually considered to be obligate aerobes but the partial pressure of O_2 necessary for growth is often overestimated. With many fungi, it is necessary to decrease the concentration of O_2 to <0.14% before linear growth is decreased by half. However, species differ much in their tolerance of both low O_2 and high CO_2 concentrations. Also, O_2 and

CO_2 concentrations interact in their effects on fungal growth so that a fungus may be more sensitive to high CO_2 when O_2 concentrations are low than when they are close to ambient levels. Fungal responses to gas composition are also affected by temperature and aw. Growth is sometimes stimulated by concentrations of 5-10% CO_2, especially at high a_w and often >15% CO_2 is required to halve growth rates. Indeed, *P. roquefortii* is reported to grow with up to 75% CO_2, providing O_2 is freely available, while it appears more sensitive to high CO_2 at 0.90 a_w than at 0.95-0.98 a_w (Golding, 1940; Magan & Lacey, 1984c). The sensitivity of fungi to high CO_2 and low O_2 concentrations is utilized in the storage of damp grain, especially barley and maize for animal feed, in sealed and unsealed silos and also in controlled-atmosphere storage of grain. Moist grain storage, whether in sealed or unsealed silos relies on the respiration of grain and its associated microflora to replace O_2 by CO_2, to give maximum CO_2 concentrations of up to 85% and minimum O_2 concentrations of <1% within the first 7 days of storage. By contrast, modified atmosphere storage relies on the addition of CO_2 or N_2 gases to grain in sealed bins, giving more rapid anaerobic conditions than would otherwise be possible with dry grain. However, the regimes proposed have usually been designed for insect control and are often insufficient to prevent fungal colonization. Control of moulding depends on the modified atmospheres being maintained throughout the storage period and with storage in N_2 atmospheres, depends on the total absence of O_2 from the atmosphere (Shejbal, 1980).

Ventilation

Ventilation removes respiratory heat, CO_2 and water vapour, preventing moisture migration, and contributing to the creation of uniform conditions throughout the grain bulk and to its cooling and drying. Low rate ventilation with ambient air is often used to cool the grain below 10°C so that mould development is slowed. This allows grain containing 18% water (about 0.87 a_w), cooled to 5-10°C, to be kept for up to 8 months in Britain although in warmer climates, drying to 12-13% (about 0.52-0.60 a_w) may be necessary before ventilation to prevent fungal growth. Care may also be necessary to prevent the addition of water to the grain during ventilation. However even in cool climates, musty odours may still occur during ventilation suggesting that some fungal growth has occurred, although not necessarily enough to become visible.

Chemicals

Chemicals have been used particularly for the prevention of moulding in animal feedstuffs. Volatile fatty acids have been widely used although many other chemicals have also been tested for this purpose. Few have shown satisfactory control of moulding while those that have also tend to inhibit germination. Mostly, the chemicals have been based on propionic acid, although formic and sorbic acids have also been used. These are all fungistatic rather than fungicidal and storage fungi differ in their susceptibility to inhibition by them. For instance, *Paecilomyces variotii* and *Eurotium* spp. can tolerate concentrations up to 1% in agar media while other species are inhibited by <0.2%, and can also degrade the propionate. Spore germination appears more susceptible to inhibition than mycelial growth. Consequently, application of the chemical must be uniform and adequate for the water content if control is to be maintained. Untreated or undertreated pockets can lead to the development first of tolerant species that metabolize the acid allowing subsequent development of less tolerant species and a succession characteristic of untreated grain at the same water content (Lord *et al.*, 1981). Sometimes *A. flavus* has been an important colonizer of grain treated inadequately with propionic acid and has formed aflatoxins (Hacking & Biggs, 1979), perhaps stimulated by the propionic acid (Al Hilli & Smith, 1979) or by other microorganisms colonizing the grain (Cuero *et al.*, 1987a, b).

Irradiation

Irradiation can be used to sterilize grain before storage but large doses are required. *Fusarium*, yeasts and *Bacillus* are particularly resistant to irradiation and require up to 12 kGy to ensure sterility. However, *Aspergillus* and *Penicillium* are killed by 1.2 kGy and Mucoraceae by 6 kGy (Cuero *et al*., 1986).

Other factors.

Broken grains and other foreign material enter the store with the grain and often segregate into discrete zones when the store is filled. They subsequently interfere with ventilation and may differ from the grain in a_w as well as provide a focus for fungal and insect infestation. Damaged grain is more susceptible to fungal colonization, especially by *Penicillium* spp. in barley at least, and may also show more visible mould subsequently.

Integrated control of moulding

Moulding of grain can be modified and controlled by altering the storage environment in different ways, although each attempts to maintain an unstable condition that could easily fail if environmental conditions change, management is deficient or if equipment fails. Almost always, control methods have relied on the control of one aspect of the grain ecosystem and have neglected the possibility of interactions and possible synergism between different factors. Thus, it has been shown that tolerance of unfavourable a_w and temperature is greatest when the other factor is optimal, that CO_2 tolerance is least when O_2 concentrations are decreased, that fungal interactions are affected by a_w, temperature, gas composition and chemicals (Magan & Lacey, 1984b, 1985) and that these may also affect the ability of particular species to produce mycotoxins (Cuero *et al*., 1987a, b). It has also been shown that different species differ in their abilities to tolerate low a_w, unfavourable gas compositions, chemicals and irradiation. Considering that these differences could result in synergistic effects where several factors were controlled, we planned a collaborative study using different seed types, grown in different regions and environments to see whether it was possible to develop integrated methods of control that would rely on the modification of several parameters to enable storage, particularly in difficult humid, tropical climates, without the use of extreme levels of any one factor. These could perhaps utilise biogenerators to produce CO_2 from local waste materials and, it was hoped, would obtain savings in costs without diminishing the efficiency of control. Possible combinations included the use of low levels of irradiation, small CO_2 concentrations and small applications of fungistatic chemicals on partially dried grain. The last part of this paper describes the first year's experiences.

Experimentation on integrated storage strategies

Methods

Preharvest colonization by *Aspergillus flavus*. Grain has been sampled from farms in two regions of Costa Rica (Brunca and Huetar), which are climatically similar but in one of which aflatoxin contamination of maize entering storage is a problem, to establish the extent of pre-harvest contamination and how much post-harvest handling contributes to the problem. The incidence of potentially toxigenic fungi on Indian grain was also assessed by plating grains at different times before harvest.

Development of integrated storage strategies. Barley, wheat, maize and sunflower seeds have been stored at different a_w after treatment with the treatments listed in Table I, alone or in combination, in one or more of the participating laboratories. Treatments were scored for visible moulding on an arbitrary scale during the course of incubation and grains were plated and assessed for respiratory activity at the end of each experiment. Seed surface lipids, which affect aflatoxin formation, were analysed to see whether they were affected by irradiation treatments.

Table I. Treatments applied during experiments.

Gamma-irradiation (kGy)	0.5, 1.0, 2.0, 4.0
Propionic acid (%)	0.1, 0.3, 0.5
Butylated hydroxyanisole (%)	0.03
Butylated hydroxytoluene (%)	0.03
Modified atmosphere CO_2/O_2 (%)	20/20
a_w	0.90, 0.80

Results

Pre-storage contamination of maize and rice with aflatoxin. Occurrence of aflatoxin in dry season maize crops in Costa Rica was erratic. However, mould invasion of shelled crops was greater than that of unshelled with, unexpectedly, *Fusarium* spp. the predominant colonizers, sometimes of >50% of the grains. On one farm in the Brunca zone, grain stored at 13 and 17% water content that had been shelled as a post-harvest treatment contained up to 1200 ng aflatoxin g^{-1} after 2 months storage. All grain that was not shelled until immediately before storage and most other grain shelled before the post-harvest period in Brunca zone contained <20 ng aflatoxin g^{-1} and in Huetar <65 ng g^{-1}. Incidence of *A. flavus* was similarly high in the grain shelled for the post-harvest treatment and much less in other treatments.

Preharvest fungi on rice paddy decreased in incidence as the crop dried but, unlike previous years, no *Aspergillus flavus, A. niger* or *A. fumigatus* was found.

Development of integrated strategies. Although many experiments are still incomplete, many treatments integrating physical and chemical control methods indicate better control of moulding than their component treatments. It remains to be determined whether there are any synergistic effects from the combined treatments and which treatments are likely to be optimum for field application.

Similar results were obtained in all the experiments with different seeds. For example with barley, visible moulding developed within one week both in untreated grain at 0.90 a_w and in that receiving 0.03% butylated hydroxyanisole (BHA) but was delayed for 3 weeks in irradiated grain and for 6 weeks in grain receiving 0.3% propionic acid (Table II). No combined treatment showed any moulding at 6 weeks nor was any moulding visible in any treatment of grain with 0.8 a_w throughout the 12 week incubation period. Most grains carried *Alternaria alternata* at the start of the experiment which could still be isolated from most directly plated grains at 0.80 a_w after 5 weeks but from only 62% at 0.90 a_w.

Table II. Development of visible moulding in barley grain at 25 °C and 0.90 a_w after treatment with propionic acid butylated hydroxyanisole (BHA; 0.03%) and gamma irradiation (4 kGy)

Treatment	Days to visible moulding
Untreated	7
Propionic acid (PA; 0.3%)	42
Butylated hydroxyanisole (BHA; 0.03%)	7
4 kGy gamma-irradiation	21
PA + BHA	>42
PA + 4 kGy	>42
BHA + 4 KgY	>42

No 0.80 a_w treatment showed visible moulding within a 12 week incubation period.

Eurotium amstelodami and *Aspergillus restrictus* were already common in the grain at the start of the experiment and increased further at 0.80 a_w, together with *E. rubrum* and *Penicillium aurantiogriseum*. At 0.90 a_w, *A. restrictus* had disappeared after only one week and already *E. ruber*, *P. aurantiogriseum* and *P. chrysogenum* colonized more than 72% of the grains, with *E. amstelodami* present in all. BHA caused little change in the amount of mould but treatments with propionic acid and irradiation caused a marked decrease. These changes were reflected in dilution plate counts where untreated grain at 0.90 a_w yielded 2.5 x 10^6, BHA treated 1.7 x 10^6, irradiated grain 4.0 x 10^5, propionic acid treated 1.2 x 10^5 and combined treatments 5 x 10^4 or fewer cfu g^{-1} after 3 weeks storage. The predominant species were similar to those found by direct plating but *Aspergillus candidus* appeared as one of the most numerous fungi in dilution plates of BHA-treated grain while *Penicillium* spp. were less numerous than in the untreated samples. Germination was poor in the untreated grain stored at 0.90 a_w, even at the start of the experiment, but deteriorated further during storage while it was immediately totally destroyed in treatments receiving propionic acid. Deterioration was less marked at 0.80 a_w except in treatments receiving propionic acid.

In the first experiment with maize, only treatments combined with 2 kGy gamma-irradiation showed no visible moulding. However, fungi grew from all untreated grains and from all those that received only propionic acid (0.1 - 0.5%) but from fewer grains that had been irradiated. Gamma-irradiation and propionic acid treatments were additive when used together but the only significant decrease in grain respiration at the end of the experiment was from 2 kGy + 0.5% propionic acid and 1 kGy + 0.5% propionic acid treatments. Grain treated with propionic acid and/or gamma-irradiation and then stored in modified atmospheres showed that 1.0 - 2.0 kGy gamma-irradiation was moderately effective in controlling fungi at 0.80 a_w while 0.1% propionic acid or modified atmosphere alone failed to prevent heavy moulding. Treatment with 2 kGy irradiation followed by propionic acid prevented moulding better than 0.5 and 1.0 kGy + propionic acid. Modified atmosphere combined with 1.0 or 2.0 kGy irradiation and/or 0.1% propionic acid, decreased fungal activity, as measured by respiration. With 0.90 a_w, only combined treatments prevented visible moulding and respiration although modified atmosphere alone was sufficient to prevent visible moulding throughout the incubation period. Propionic acid and gamma-irradiation treatments seemed much less effective in controlling fungi in sorghum than in rice.

As found by Cuero *et al.* (1986), irradiation favoured the development of *Aureobasidium pullulans* and yeasts rather than typical storage species. Fungi on seeds were not inhibited by up to 1 kGy gamma-irradiation fungi present while 4 kGy delayed fungal growth for 5 - 7 days before *Alternaria alternata* and other fungi developed grew from the seed. Lipids, mainly seed surface lipids (SSL), are important in inducing aflatoxin production in *Aspergillus parasiticus* and *A. flavus* (De Luca *et al.*, 1990). Gamma radiation doses of 0.5 - 4.0 kGy did not change the SSL composition of seeds but they slightly increased butylated hydroxytoluene (BHT) degradation, especially in sunflower seeds. However, this did not significantly affect aflatoxin production by a toxigenic isolate of *A. parasiticus* during 30 days incubation.

Volatile fatty acids were used as models of antifungal agents. Both propionic acid and gamma-irradiation seem more effective in controlling *A. parasiticus* in starchy seeds than in sunflower. Sunflower required 0.4% of propionic acid, twice as much as for wheat, for complete inhibition of the microflora. Propionic and formic acids were more effective than acetic acid in preventing moulding of rice and sorghum while sorbic acid appeared more effective against *A. flavus* on sorghum than on rice. However, propionic acid slightly stimulated aflatoxin production by *A. parasiticus* in some tests. The effect of BHT was greater when combined with propionic acid than alone and there was some evidence of synergistic interactions between the two chemicals. However, degradation of BHT must be limited by minimising seed water content. BHA and propionic acid appeared to differ in their effects on the grain microflora. BHA effectively prevented colonization by *Penicillium* spp. but only delayed colonization by *Eurotium* spp. A disadvantage of propionic acid is its deleterious effect on germination. As little 0.1% of propionic acid in wheat seeds greatly decreased germination and 0.2% caused complete inhibition within one week. Similarly, germination of barley was totally inhibited by 0.3% although inhibition was less at 0.80 a_w than at 0.90 a_w. With sunflower seeds, as with inhibition of moulding, larger amounts of propionic acid were required to inhibit germination. Germination was only totally inhibited by 0.4% of propionic acid while 90% of seeds still germinated with 0.2%. Whether this difference is due to the oil content of the sunflower, to differences in the metabolism of oily and starchy seeds or in the permeability of the seeds is uncertain since recovery of propionic acid from both seed types during experiments was similar. Species tolerant of propionic and sorbic acid were identified in rice and sorghum, including *Fusarium moniliforme*, *Aspergillus niger*, *Penicillium* spp. (PA), *Alternaria alternata* and *Phoma* spp. (sorbic acid).

Conclusion

Initial results from experiments support the hypothesis that combined treatments prevent moulding in stored grain more effectively than the component treatments alone. However, more work is required to make recommendations for integrated strategies for field trials. Alternative fungicides to volatile fatty acids are required that do not destroy the germination of the grain and which are safe to use on food grains. Use of ventilation in an integrated system has also to be tested.

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RESUME

Les essais destinés à empêcher l'apparition de moisissures sur les stocks de grains n'ont souvent mis en évidence que le rôle d'un seul facteur. Les stratégies de gestion intègrant les méthodes chimiques physiques et environnementales dans la prévention des moisissures peuvent permettre l'utilisation, à un degré moindre, de n'importe quel facteur pris séparément. Une étude faite en collaboration, demandée par la Communauté Economique Européenne, cherche à développer des modèles d'utilisation de traitements combinés qui peuvent être appliqués aux fermes, particulièrement dans les pays en voie de développement. De tels traitements pourraient comprendre des mesures d'élimination des moisissures et des mycotoxines, qui seraient prises avant l'entrée des récoltes en magasin, puisque, au Costa Rica, certaines récoltes de maïs sont colonisées par *Aspergillus flavus* avec développement d'aflatoxines, entre le moment de la récolte et le séchage. Des études sur le terrain, en Inde, ont montré les différentes fréquences d'apparition de plusieurs espèces de la mycoflore, peut-être en rapport avec l'activité de l'eau (a_w), les méthodes de stockage et, peut-être encore, la densité d'inoculum. Au cours d'expériences de laboratoire, *A. flavus* et *A. parasiticus* ont réagi différemment à la température et à l'(a_w), tandis que la densité d'inoculum affectait la colonisation de l'orge par *A. flavus* à 0,85 (a_w) mais pas à 0,97 (a_w). Deux traitements par irradiation, combinés à l'emploi d'acide propionique, comprenant 1 kGy + 0,1 % d'acide propionique pour le premier et 0,5 kGy + 0,3 % d'acide propionique pour le second, ont mieux empêché l'apparition des moisissures que n'importe quel autre composant pris séparément. Les lipides des couches périphériques du grain, qui affectent la production d'aflatoxine par *A. flavus*, ont été affectés par l'irradiation.