CONTROL OF MOULDING AND MYCOTOXIN PRODUCTION IN STORED SORGHUM (SORGHUM BICOLOR (L.) MOENCH) AND RICE (ORYZA SATIVA L.) USING ORGANIC ACIDS AND ANTIOXIDANTS

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Abstract

Propionic, acetic, and formic acids, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were applied singly or in combination to sorghum and rice seeds at 0.90 a_w to assess their effectiveness in preventing moulding. Moulding, as assessed by direct plating, was decreased more by treatment with 0.15 % (v/w) organic acids than by 0.015 % (w/w) BHA or BHT there was more moulding in rice than in sorghum. *Aspergillus flavus* increased on sorghum after treatment with BHA or BHT alone or in combination with propionic acid. Combined organic acid treatments controlled *A. flavus* , *A. niger* and *Eurotium sp.* better storage than the individual acids alone. Formic and propionic acids controlled *Eurotium sp.* better than acetic acid. Formic acid failed to control *Paecilomyces varottii* in rice. More aflatoxin B_1 was formed in sorghum treated with single acids, with most in formic acid treatments , than with combinations of acids.

Introduction

Microbial deterioration of stored rice (*Oryza sativa* L.) and sorghum (*Sorghum bicolor* (L.) Moench) is an important limitation on their use for food purposes (Castor & Frederiksen, 1980). Deterioration of rice (with husk) is particularly important in India as it is widely grown and stored under humid conditions. This leads to deterioration by storage fungi, decreased grain viability and, occasionally, mycotoxin contamination. Short chain organic acids, especially propionic acid because of their solubility, taste and low toxicity are frequently used to control moulding in insufficiently dried grain (Ray & Bullerman, 1982). The undissociated molecule of the acid provides the antifungal activity.

Other preservatives that could be used in cereals and grain products are the phenolic antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). BHA (0.025 - 0.1 %) added to spores or mycelium of *A. parasiticus in vitro* inhibits growth and aflatoxin production but lower concentrations (0.001%) stimulated aflatoxin production (Chang & Branen, 1975). The activity of BHA and BHT *in vivo* prevents moulding and mycotoxin production by decreasing peroxidation and production of organic free radicals from seed lipids. Combinations of formic, acetic, propionic, butyric and isobutyric acids may show synergistic activity against moulds in different cereals (maize, sorghum, wheat, oats, barley and soyabeans) (Herting & Drury, 1974) but generally synergism between chemicals and other factors controlling moulding has not been tested. This paper reports on results from the use of chemicals, alone or in combination, for control of moulding in sorghum and rice.

Materials and methods

Aspergillus flavus (IMI 102556) was maintained on 2 % malt extract slopes at 25 °C. Spores for inoculation of grain were produced on sorghum or rice grains at 25 % water content. Inoculated grain was incubated in a closed desiccator at 0.97 a_w and 25 °C for 20 days.

For experiments, 50 g grain was inoculated with 1 ml of a suspension containing 10^6 spores ml^{-1} and incubated in a desiccator with humidity maintained at 90 % with saturated KNO_3. Propionic, formic, and acetic acids were applied singly or in combination at 0.15 % v/w in the water required to hydrate the grain to the desired water content. Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) were applied in 0.5 ml methanol and mixed with the grain before adding the water. Fungal colonisation of incubated grain was assessed visually using an arbitary 0 - 5 scale and by direct and dilution plating.

Aflatoxin B_1 (AFB_1) was assayed by direct competitive ELISA (Ramakrishna, Lacey, Candlish, Smith, & Goodbrand, 1990). Sorghum and rice extracts in acetonitrile, diluted 1:8 with buffer, still caused significant interference in AFB_1 ELISA which was overcome by diluting 1:10. Standards were prepared in grain extract diluted to 18 % in acetonitrile.

Germination was assessed by placing replicate samples of 50 seeds on moistened filter papers in Petri dishes.

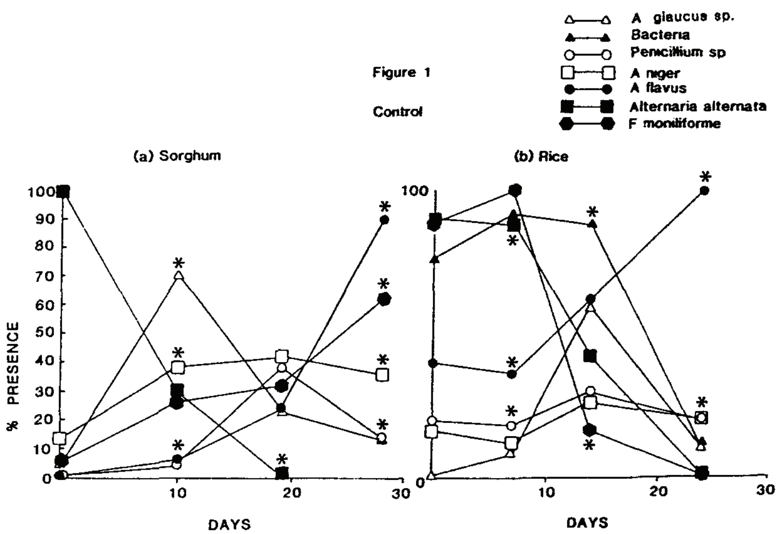
Results

(i) Moulding of sorghum and rice stored at 0.90 a_w and 25 °C

Changes in the microflora of sorghum and rice samples at 22.50 % water content (0.90 aw) over 28 and 24 days period respectively are shown in Figure 1a & b. A greater range of species of fungi was found on moulding rice (figure 1b) than that on sorghum (figure 1a). *Aspergillus flavus* increased from < 5 % to 90 % in sorghum and from 40 % to 100 % in rice during incubation. Significantly more of *Eurotium sp*. occurred on sorghum than on rice and of bacteria on rice than on sorghum, but bacteria decreased in incidence from approximately 90 % to 10 % during the 24 day incubation. Sorghum and rice were similiarly contaminated with *Penicillium sp*. and *Aspergillus niger*

Figure 1

Control



* denotes points significantly different from each other at p = 0.05

(ii) Moulding of sorghum and rice treated with preservatives

(a) Organic acids: Rice and sorghum treated with propionic, acetic or formic acids, either singly or in combination, were significantly more contaminated with Eurotium rubrum than untreated controls, with approximately 90 % of rice grains contaminated after 7 days when treated with 0.15 % acetic and 0.15 % propionic acid (Figure 2). Application of acids in combination was more effective in controlling Eurotium sp. than any single acid. Combinations containing formic acid were more effective in controlling Eurotium sp. in rice while those containing propionic acid were more effective in sorghum. A. flavus in rice and sorghum was decreased by organic acids to approximately 10 % but Aspergillus candidus increased significantly to 90 % after 19 days in sorghum receiving either 0.15 % propionic or 0.15 % acetic acids. However, when organic acids were applied in combination or formic acid was applied alone, only 0 - 5 % of the grain yielded A. candidus after 28 days incubation (Figure 3a).

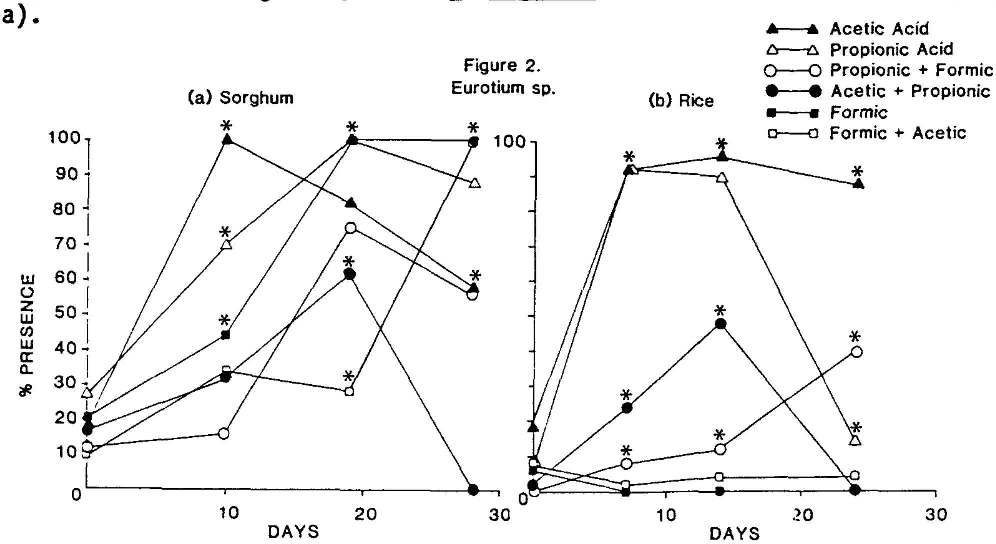


Figure 2.
Eurotium sp.

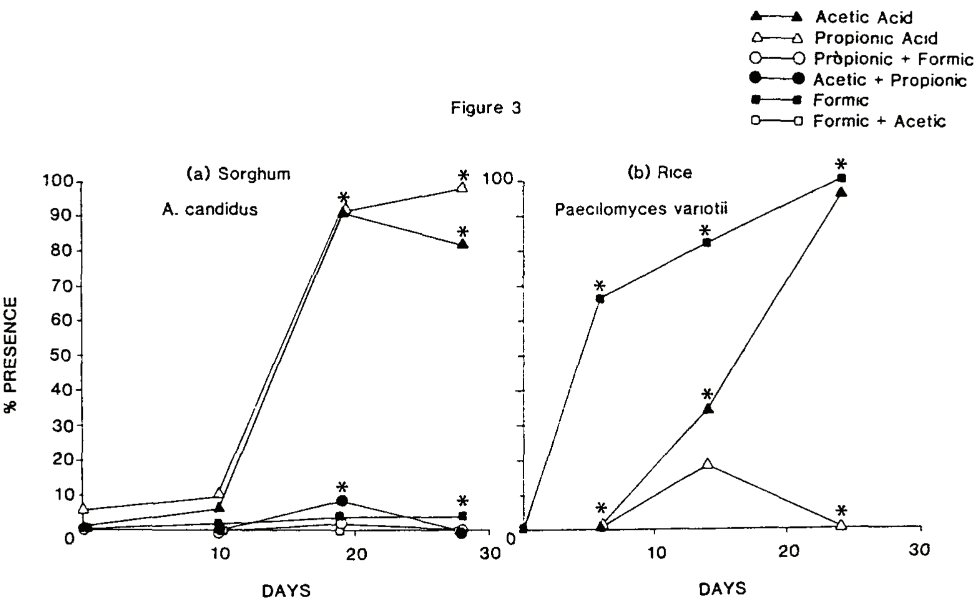


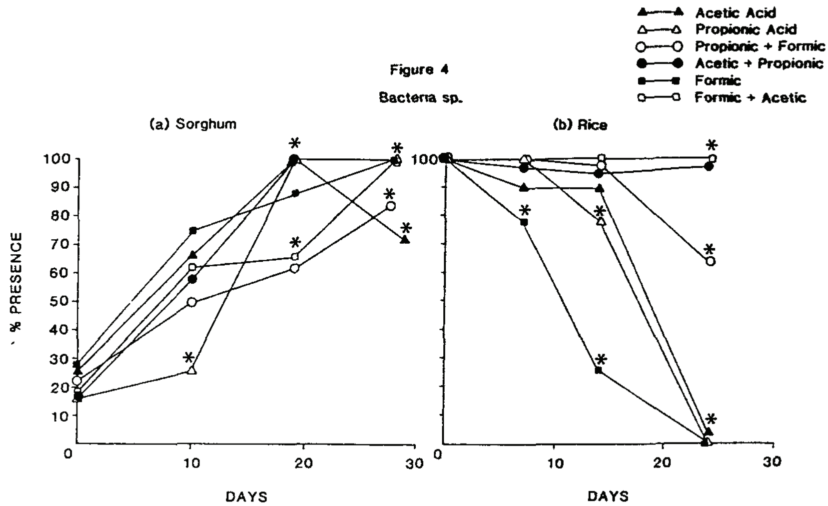
Figure 3

denotes points significantly differently from each other p = 0.05

Paecilomyces variotii contaminated all rice grain treated with formic or acetic acids alone after 24 days incubation (Figure 3b). However, only 18 % were contaminated 14 days after treatment with propionic acid and none after combined treatments (Figure 3b). Bacteria increased in chemically treated sorghum during incubation (Figure 4a). All rice grain treated with organic acids yielded bacteria (Figure 4b) but that receiving combined acid treatments yielded significantly more than that receiving single treatments.

Figure 4

Bacteria sp.



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(b) Antioxidants: Treatment with BHA or BHT at 0.015 % w/w singly or in combination did not decrease A. flavus significantly on sorghum and rice. BHA or BHT applied in combination with propionic acid (0.15 % v/w) did not significantly affect the incidence of A. flavus from that observed in untreated controls. The effects of BHA and BHT on rice and sorghum were similiar (Figure 5a) except that BHA applied in combination with propionic acid and BHT applied alone stimulated the growth of A. flavus on rice (Figure 5a). The occurrence of Penicillium sp. on rice was significantly decreased in treatments receiving combinations of either BHA or BHT with propionic acid or BHA alone to < 20 % (Figure 6a). BHT applied alone or in combination with BHA did not control the incidence of Penicillium sp.. The incidence of Aspergillus nidulans was significantly increased by BHA and BHT applied in combination with propionic acid (Figure 6b). Treatment of sorghum and rice with BHA or BHT in combination with propionic acid decreased incidence of F. moniliforme more than treatment with BHA or BHT alone or in combination (Figure 7).

(iii) Effect of organic acids on colonisation by A. flavus

(a) Growth of A. flavus on sorghum: Treatment of sorghum grain with acetic or formic acid (0.15 %) prior to inoculation with A. flavus did not significantly decrease colonisation compared to untreated controls over the 28 days incubation (Figure 9). Propionic acid alone significantly decreased growth of A. flavus up to 21 days but after 28 days incubation there was no significant difference in the growth on propionic acid treated and untreated sorghum. All combinations of organic acids applied to sorghum prior to inoculation with A. flavus significantly decreased its growth and colonisation, compared both to untreated grain and that receiving organic acids singly.

Figure 5

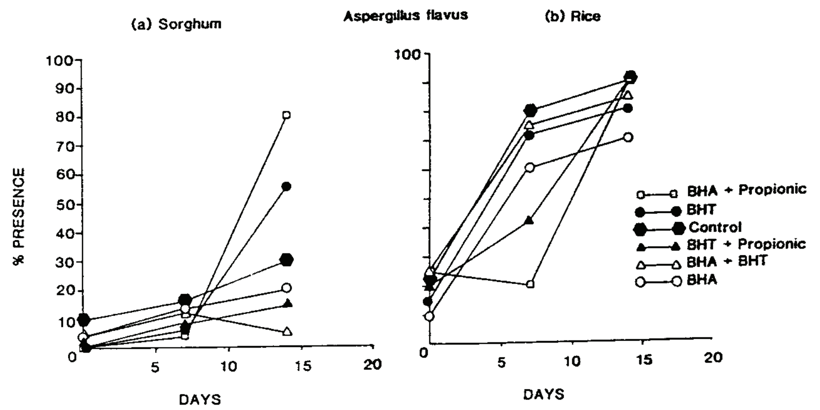


Figure 6

RICE

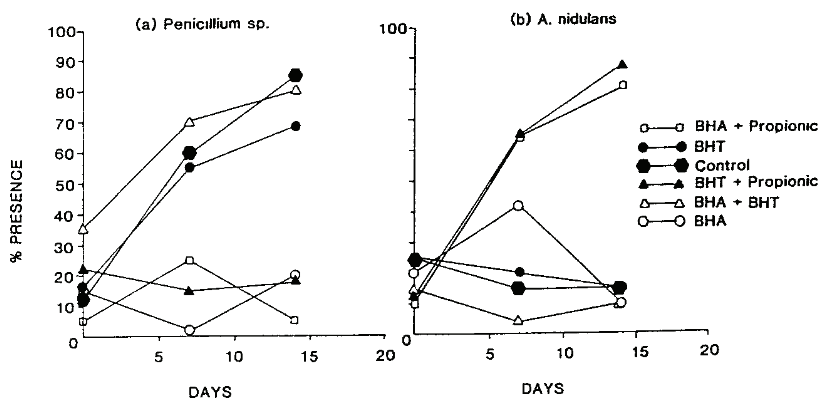
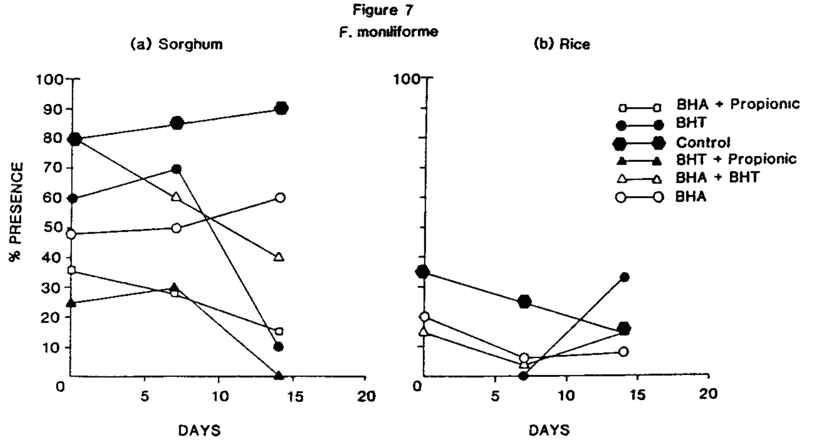


Figure 7



(b) Visible moulding of sorghum: Visible mould scores for sorghum and rice inoculated with A. flavus during incubation at 0.90 a_w and 25 °C are shown in Table 1.

Table 1. Visible moulding of sorghum inoculated with A. flavus and treated with organic acids singly or in combination.

Treatment	Days after inoculation			
	7	14	21	28
Control	3.0	3.0	4.0	4.0
Acetic	1.1	3.0	4.0	4.0
Formic	2.7	3.3	4.0	4.0
Propionic	0.0	0.7	1.0	3.0
Formic + Propionic	0.0	0.0	0.0	0.0
Acetic + Propionic	0.0	0.0	0.0	0.0
Formic + Acetic	0.0	0.0	0.0	0.0

Untreated sorghum inoculated with A. flavus showed visible moulding after 7 days. Acetic and formic acid treatments moulded less than untreated grain after 7 days but there was no difference after 14 days while propionic acid delayed visible signs of moulding until 14 days. Combined acid treatments allowed no visible moulding throughout the 28 day incubation period.

(c) Aflatoxin B_1 accumulation in sorghum: Aflatoxin B_1 production in untreated sorghum inoculated with A. flavus increased from 131.1 ng g^{-1} after 7 days to 611.1 ng g^{-1} after 21 days (Table 2). Formic acid (0.15 % v/w) significantly stimulated aflatoxin B_1 production throughout the 21 day incubation period.

Table 2. Aflatoxin B_1 production (ng g^{-1}) in sorghum inoculated with A. flavus and treated with organic acids singly or in combination.

Treatment	Days after inoculation		
	7	14	21
Control	131.18±23.13	342.18±109.14	611.11±38.47
Acetic	>0.50±0.21	90.08±19.12	598.37±60.94
Formic	578.06±101.67	630.00±39.19	793.37±130.0
Propionic	>0.50±0.21	47.37±10.20	646.01±77.21
Acetic + Formic	115.50±10.53	146.35±65.13	134.43±14.00
Acetic + Propionic	57.73±9.89	95.57±27.78	89.96±11.91
Propionic + Formic	206.68±29.31	186.62±36.56	137.70±38.19

Propionic acid inhibited aflatoxin B1 production after 7 and 14 days but toxin production was not significantly different from that in untreated grain after 21 days. Combined acid treatments decreased aflatoxin accumulation. The combinations of propionic and acetic acid was the most effective. Large concentrations of aflatoxin occurred in grain receiving formic acid alone.

(iv) Effect of organic acid treatment on germination of sorghum and rice: Germination of untreated control samples declined from approximately 90 - 100 % to 10 % in sorghum and to 70 % in rice at 25 C and 0.90 aw (Figure 8a & b). Seeds of sorghum and rice treated with formic acid, alone or in combination had very poor germination. Acetic acid had no significant effect on germination of sorghum but decreased germination of rice to 15 % after 7 days. Sorghum and rice incubated for 7 days after treatment with propionic acid failed

Figure 8
Germination

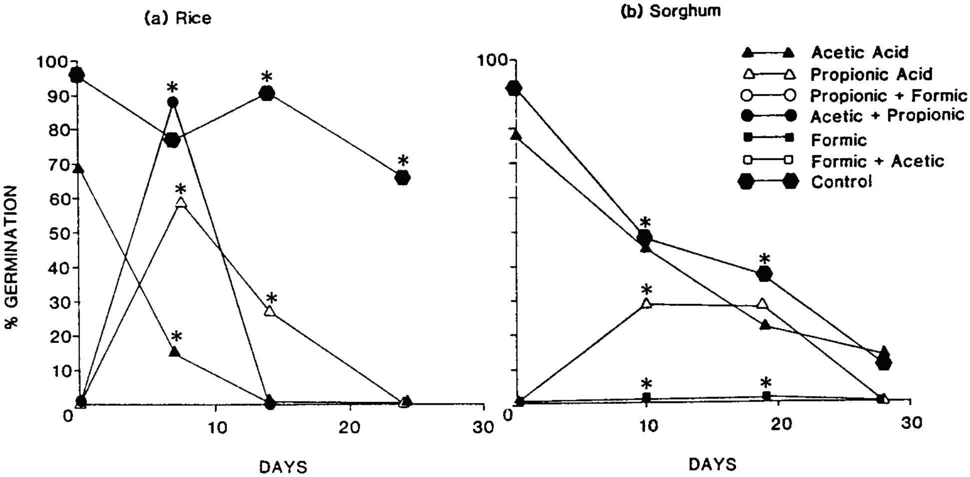
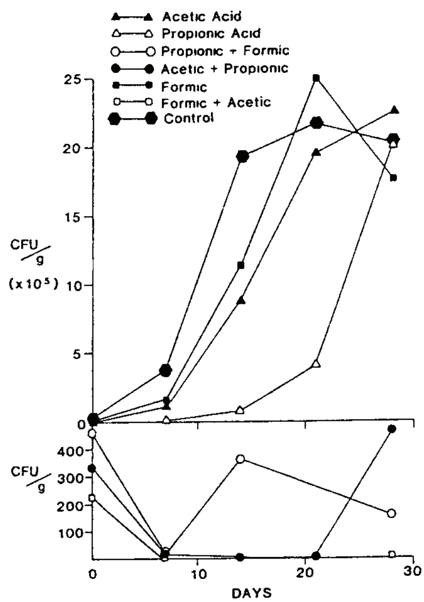


Figure 9
A. flavus
Sorghum



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to germinate but after 7 - 10 days 60 and 30 % germinated, respectively although no germination was again found at the end of the incubation period. Combined acid treatments generally prevented germination completely.

Discussion

Short chain organic acids have been frequently used to prevent mould growth in grain including rice and sorghum but germination and seed quality factors were often greatly affected (Herting & Drury, 1974) Combined chemical treatments offer the potential for control of moulding in sorghum and rice with less chemical and less damage to germination and seed quality. Untreated sorghum and rice in the present study was colonised by storage moulds including Eurotium sp., A. flavus and Penicillium sp. as reported by other workers (Christensen, 1969, 1970). Treatment with propionic, formic and acetic acids significantly affected the microflora. The most commonly isolated organisms from both sorghum and rice treated with organic acids were Eurotium sp. (usually E. rubrum). Other commonly occurring species included A. candidus on sorghum treated with propionic and acetic acids and P. variotii present on rice treated with formic, acetic or propionic acids.

Tolerance of Eurotium sp. and P. variotii to propionic acid has been reported previously (Lord et al.,1981, Clevström et al., 1989). Propionic, acetic acid and isomers of butyric (C_4) and valeric acids (C_4) are also degraded by P. variotii and Eurotium sp. (Lord et al.,1981).

Combined acid treatments decreased the amount and speed of colonisation by Eurotium sp. possibly because these treatments had larger amounts of undissociated molecules which penetrate cells more readily than corresponding ions (Byrde, 1969) and so are more fungicidal (Herting & Drury, 1974). This may explain, at least in part, the slow increase in tolerant species. Thus, presence of different organic acids on grain could inhibit mould development while small differences in the ability of fungi to metabolise different organic acids could alter their ability to colonise grain treated with the combined acid treatments. Metabolism of organic acids enables more sensitive fungi to subsequently colonise the substrate (Lord, Cayley & Lacey, 1981).

In our study, combined organic acid treatments controlled colonisation of sorghum and rice by storage moulds. Untreated sorghum was progressively colonised by A. flavus while colonisation of grain treated with organic acids was negligible. A. flavus maintained its ability to grow in grain treated with 0.15 % of each organic acid alone. Visible moulding was present in single acid treatments of sorghum but not in combined acid treatments which contained only few colony forming units throughout the incubation period (single acid treatments yielded 10^5 CFU g^{-1} grain). There was very little difference in aflatoxin production between treatments at first but over the whole incubation period less was produced in combined acid treatments than in single acid treatments or in untreated grain. Aflatoxin B_1 production was stimulated by formic acid treatment as found previously (Clevström et al., 1989).

Treatment with BHA or BHT alone or in combination had little effect on colonisation of sorghum and rice but their combination with propionic acid significantly decreased initial colonisation of rice by A. flavus. This decrease was probably due to the propionic acid. In sorghum, BHA with propionic acid and BHT alone enhanced colonisation by A. flavus perhaps because there are more superficial lipids in the hulls of these seeds. The small effect of BHA and BHT in our experiments may have resulted from use of too little chemical, although Passi et al, (1986) used similiar concentrations to control growth and aflatoxin production by A. parasiticus on sunflower seeds, or from the insolubility of antioxidants making uniform coverage of seeds difficult.

Penicillium sp. in rice were inhibited by the combination of propionic acid with BHA but not BHT as in barley (Lacey, Paster, et al., 1990). There was little inhibition of A. nidulans by BHA or BHT and growth was stimulated by combination of propionic acid and antioxidants, suggesting possible tolerance. Both BHA and BHT alone or in combination decreased the incidence of F. moniliforme but less than treatments including propionic acid.

While combined organic acid treatments inhibit moulding and, generally, mycotoxin production, those containing formic acid stimulated aflatoxin B_1 production and are thus unsuitable where A. flavus is present. Further work will study the effects of organic acids alone and in combination on colonisation and mycotoxin production by species other than A. flavus in different grain types. Control of aflatoxin B_1 by BHA and BHT also requires further investigation in rice and sorghum.

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INHIBITION DE LA CROISSANCE DES MOISISSURES ET DE LA FORMATION DES MYCOTOXINES DANS LES STOCKS DE SORGHO (*SORGHUM BICOLOR* L. Moench) ET DE RIZ (*ORYZA SATIVA* L.) PAR L'UTILISATION D'ACIDES ORGANIQUES ET D'ANTIOXYDANTS

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RESUME

Les acides propionique, acétique, formique, l'hydroxyanisole butylé (BHA) et l'hydroxytoluène (BHT) ont été appliqués seuls ou combinés, à des grains de sorgho et de riz, à une a_w de 0,90 afin de mesurer leur efficacité dans la prévention de la formation de moisissures. Une réduction de la formation de moisissures, mesurée par la méthode directe de chromatographie sur couches minces a été meilleure après traitement à 0,15 % (w/v) qu'après traitement à 0,015 % (w/v) de BTA ou de BHT. La présence d'*Aspergillus flavus* augmente sur le sorgho lorsqu'il est stocké pendant 14 jours après traitement au BTA ou au BHT seuls ou combinés à l'acide propionique. Les traitements par des mélanges se sont avérés plus efficaces dans l'élimination de la colonisation de *A. flavus*, *A. niger* et *Eurotium spp.*, pendant 30 jours de stockage, que les composés acides appliqués seuls. *Eurotium spp.* a été éliminé plus efficacement par les acides formique et propionique que par l'acide acétique. La présence de *A. flavus*, *A. niger*, *A. versicolor* et *Eurotium spp.* a été plus élevée pour le riz que pour le sorgho et leur élimination par les acides organiques a été beaucoup moins efficace. Les grains traités aux antioxydants différaient peu des grains non traités tandis que le développement de moisissures était inhibé plus efficacement pour le riz par l'acide formique que par les acides propionique et acétique.