Selective effect of propionates and water activity on maize mycoflora and impact on fumonisin B_1 accumulation

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Abstract

The effect of a commercial mixture of propionates at two different doses (0.05% and 0.1%) on fungal spoilage of natural maize stored at 0.85, 0.90 and 0.95 water activity (a_w) was investigated. Parallel treatments with added inoculum of *Fusarium* Liseola section isolates (*Fusarium moniliforme* and *F. proliferatum*) were carried out in order to determine the effect of fungal interactions on the development of fumonisin-producers on maize in relation to preservative efficacy. Fungal colonisation of grain was measured as fungal counts (CFUs g^{-1} maize). In general, no differences were found between inoculated and uninoculated samples. Besides the selective effect of a_w on maize mycoflora, it was demonstrated that most genera which colonise maize remained unaffected by the preservative concentrations applied. However, *Penicillium* populations (CFUs g^{-1} maize) counts decreased significantly. As they represent a major component of the total fungal counts, an overall control of total mycoflora was observed. Furthermore, there was a significant statistical interaction between preservative and a_w levels, with the preservative activity enhanced at low a_w. The concentrations of fumonisin B_1 were unaffected by treatment with no significant differences in concentrations found. This suggests that the natural mycoflora of maize may act as an inhibitor of *Fusarium* development, and consequently of fumonisin biosynthesis. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Propionic acid and propionate formulations are highly effective mould inhibitors, commonly used in the food industry (cakes, bakery products, cheese) as calcium and sodium salts. Several reports exist on the efficacy of these fungistats as preservatives of stored high moisture cereals (Skrinjar et al., 1995). However, little information exists on whether such preservatives may be effective in controlling the growth of *Fusarium* spp or their mycotoxins, either pre-harvest or post-harvest.

For maize, environmental factors during ripening and storage can exist which favour rapid invasion and concomitant production of fumonisins by *Fusarium* Liseola species. The most important factors influencing growth and fumonisin production are water availability, and temperature (Marín et al., 1995a,b; 1996). Other related factors include mechanical and biological damage and microbial competition (Marín et al., 1998a,b). However, as environmental factors are quite difficult to control in the field, where conditions are exceptionally suitable for fumonisin production, effective post-harvest storage treatment would be needed to ensure that production is inhibited. It may be possible to use a combination of a slightly reduced water activity (a_w) combined with the addition of chemical preservatives for this purpose. However, few studies have investigated this possibility. Previous studies with these organic acids, particularly propionic acid, have shown that a 0.07% dose of propionate may be useful to avoid *Fusarium* Liseola section from growing in moist maize (0.93–0.98 a_w). However, a certain level of fumonisin B_1 was still detected (< 40 ppm). Most previous studies deal with *Aspergillus flavus* and other aflatoxigenic fungi, and aflatoxin production (Calori-Domingues et al., 1996). Propionic acid was shown to control growth of aflatoxigenic fungi and aflatoxin production in high moisture maize kernels (Smith and Moss, 1985; Rusul et al., 1987).

The objectives of the present work were to examine (a) the efficacy of a propionate-based preservative and interactions with a_w in preventing fungal development in naturally-contaminated maize, and (b) by the addition of *Fusarium moniliforme* and *Fusarium proliferatum* inoculum, to test the ability for control of both *Fusarium* populations and other species, and fumonisin B_1 production in relation to preservative concentration $\times a_w$ interaction.

2. Materials and methods

2.1. Culture material

Three isolates each of *F. moniliforme* Sheldon (25N, 85N, 123N) and *F. proliferatum* (Matsushima) Nirenberg (55N, 73N, 131N) were used in this study. These isolates have previously been found to be fumonisin producers (Sala, 1993), and are held in the Food Technology Department Collection of the University of Lleida (Spain).

2.2. Preservatives

A commercial antifungal preservative (Luctamold LS 1467-Z, LUCTA, S.A., Spain) based

on a mixture of propionates was purchased from a local supplier. This product consists of sodium propionate, ammonium propionate, propionic acid, 1,2-propanediol monoesters and fatty acids. Recommended dosages were 0.5 g kg^{-1} for sound, well-dried grain (14% m.c.), 1 g kg^{-1} for initially spoilt grain, with a m.c. of 14–16%, and finally 3 g kg^{-1} for very contaminated grain with a m.c. >16%. Suitable solutions were prepared from the mixture and filter-sterilised through a 0.22 μm filter, and stored at 7°C until used.

2.3. Rehydration and inoculation of maize in microporous bags

A factorial experiment was designed consisting of 3 factors, a_w (0.85, 0.90 and 0.95), preservative concentration (0, 0.5 and 1 g kg^{-1}), and either *Fusarium* inoculation or uninoculated treatments. Each treatment was repeated three times. The initial mean concentration of FB_1 in the maize grain used was 0.4 μg g^{-1}.

Natural maize subsamples of 150 g were weighed into sterile flasks and adjusted to 0.85, 0.90 and 0.95 a_w by addition of sterile distilled water and propionate solution, and flasks stored at 4°C for 48 h to modify the grain to the required a_w. They were regularly shaken to obtain a uniform treatment. For treatments which were spiked with inocula of the *Fusarium* spp 1 ml of a 3×10^7 spores ml^{-1} suspension containing a mixture of spores of the six *Fusarium* isolates mentioned above in equal parts was added. Additional volumes represented by both preservative and spore suspension had been subtracted from the initial amount of water added to the grain. After shaking flasks, grain was transferred to surface-sterilised microporous bags, and sealed. Bags were then enclosed in sealed environmental chambers containing beakers of glycerol–water solutions of the same a_w as the treatments, in order to maintain the correct equilibrium relative humidity. Chambers were incubated at 25°C for a 28-day period.

2.4. Assessment of fungal populations

Colonisation of grain was assessed as colony forming units (CFUs) g^{-1} after 7, 14 and 21 days incubation. 10 g subsamples were destructively analysed by serial dilution using malt extract agar (MEA). Plates bearing between 5 and 150 CFUs were enumerated for total *Aspergillus, Penicillium, Eurotium* and *Fusarium* colonies, as well as *A. flavus*.

2.5. Determination of fumonisin B_1 formation

The production of fumonisin B_1 in maize by natural and inoculated *Fusarium* species was determined after 28 days of incubation by HPLC. A modification of Shephard's method (Shephard et al., 1990) was followed. Briefly, 10 g subsamples were ground and extracted by blending in 20 ml methanol–water (3 + 1). Extracts were filtered and centrifuged. Filtrate (5 g) was loaded on a preconditioned SAX column and eluted with 0.5% acetic acid in methanol. The eluate was evaporated to dryness in a rotavapour, redissolved in methanol, and finally evaporated under a gentle stream of nitrogen and dissolved in methanol for HPLC. Fumonisin was coupled to OPA and assayed by HPLC, by comparison with external standards, using methanol: 0.1 M dihydrogen sodium phosphate (3 + 1) (pH 3.35) as mobile phase at a flow rate

of 0.8 ml min^{-1}. Reference standard of FB$_1$ was purchased from CSIR, Division of Food Science and Technology, Pretoria, South Africa.

2.6. Dry matter determination

The percentage of dry matter in each sample was determined by drying subsamples of approximately 10 g at 105°C for 17 h (ISTA, 1976). Thus, all results are presented on a dry weight basis.

2.7. Statistical treatment of data

Analysis of variance of the effect of a_w, doses of propionates, time and addition of *Fusarium* were performed for each separate genus (*Aspergillus, Eurotium, Penicillium, Fusarium*), for *A. flavus*, and for total counts. Fumonisin B$_1$ concentration in samples was analysed in a similar way. All statistical analyses were made using SAS version 6.12 (SAS Institute Inc.).

3. Results

3.1. Relative incidence of species

Aspergillus flavus was present at the lowest level in maize grain at 0.85–0.90 a_w, while *Eurotium* species were isolated in the least amounts at 0.95 a_w. *Penicillium* species were most abundant at 0.95 a_w, while at 0.85–0.90 a_w, *Aspergillus, Penicillium, Eurotium* and *Fusarium* species accounted for a major or minor part depending on propionate concentration. *Penicillium* species were the most abundant at 0.90–0.95 a_w in the absence of propionate. However, *Aspergillus* populations reached similar levels at both 0.5–1 g kg^{-1} propionate concentrations.

Statistically, there was no significant change in either *Aspergillus, Penicillium* or *Fusarium* counts when an additional inoculum of *Fusarium* was used (Table 1). However, the presence of populations of *Eurotium* species increased in *Fusarium*-inoculated maize grain samples.

3.2. Effects of water activity and time on fungal populations

Interestingly, total populations and single genera increased significantly with time, except for *Fusarium* populations, which remained unchanged throughout the experimental period.

Water activity was the most significant factor ($P < 0.01$) because of its influence on fungal populations. *A. flavus, Fusarium* and *Penicillium* species increased their populations with increasing a_w, although other *Aspergillus* species showed similar populations at 0.90 and 0.95 a_w, with lower levels at 0.85 a_w. In contrast, *Eurotium* species showed higher populations at 0.85–0.90 a_w and lower at 0.95 a_w (Fig. 1). Both time and a_w showed a significant interaction ($P < 0.01$). Thus, for example, *Eurotium* populations decreased significantly from 0.90 to 0.95 a_w, after 14 and 21 days storage.

Table 1
Analysis of variance of *A. flavus, Eurotium* sp., *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., and total counts ($\log(\text{CFU g}^{-1})$) on naturally contaminated maize. Significance of additional inoculation of *Fusarium* (I), time (t), preservative dose (P) and water activity (a_w), and their interactions

	DF	A. flavus		Eurotium		Aspergillus		Penicillium		Fusarium		Total	
		MS	F	MS	F	MS	F	MS	F	MS	F	MS	F
I	1	0.3	1.3	6.2	15.5**	0.6	1.9	0.0	0.3	0.9	1.8	0.1	0.8
t	2	3.0	15.5**	5.6	14.1**	13.0	40.2**	7.5	51.6**	0.2	0.4	6.3	74.4**
I × t	2	0.1	0.4	0.6	1.4	0.0	0.1	0.3	2.4	1.0	2.0	0.1	1.8
P	2	1.5	7.6**	2.4	6.0**	0.7	2.1	12.3	83.9**	0.7	1.5	0.8	9.5**
I × P	2	0.1	0.7	0.7	1.9	0.1	0.2	0.3	2.3	0.3	0.6	0.0	0.5
t × P	4	0.1	0.8	0.6	1.4	0.8	2.4	0.3	2.1	0.4	0.7	0.4	4.8**
I × t × P	4	0.0	0.2	0.4	1.0	0.0	0.1	0.4	2.6*	0.4	0.8	0.1	1.1
a_w	2	85.1	434.4**	7.7	19.3**	5.2	16.0**	76.4	522.1**	32.0	65.6**	22.8	269.7**
I × a_w	2	0.1	0.3	2.2	5.6**	0.7	2.3	0.3	2.3	0.9	1.8	0.1	0.9
t × a_w	4	2.8	14.5**	4.1	10.2**	2.4	7.3**	0.5	3.3*	0.7	1.4	0.4	5.0**
I × t × a_w	4	0.1	0.3	0.6	1.6	0.3	0.9	0.1	0.6	0.4	0.8	0.0	0.5
P × a_w	4	1.6	8.0**	3.3	8.2**	2.9	8.9**	1.0	7.0**	0.2	0.4	0.1	1.1
I × P × a_w	4	0.7	3.6**	1.2	2.9*	0.9	2.8*	0.3	1.8	0.0	0.1	0.1	1.3
t × P × a_w	8	0.1	0.6	0.6	1.6	0.5	1.7	0.8	5.6**	0.4	0.7	0.1	0.8
I × t × P × a_w	8	0.1	0.5	0.6	1.6	0.3	1.1	0.3	1.8	0.4	0.8	0.1	0.9

* Significant, $P < 0.05$.
** Significant, $P < 0.01$.

3.3. Effects of propionates on fungal populations

Total populations were equally inhibited by 0.5 and 1 g kg^{-1} propionate. However, detailed results showed that the *Penicillium* isolates were the only main group inhibited, with mean populations of 6.69, 6.23 and 5.87 (log CFU) for the control, 0.5 and 1 g kg^{-1} doses, respectively. The other genera had a low sensitivity to this preservative. As *Penicillium* species are often the major component of grain mycoflora, in terms of fungal counts (CFU), their sensitivity to propionates accounts for the major controlling effect in the grain. The efficacy of the propionate treatments against the *Penicillium* spp was significantly dependent on a_w ($P <$ 0.01), with better control at low a_w. However, for total fungal populations, the effect of propionates was closely related to the incubation time ($P < 0.01$), showing high inhibitory effects after 7 days, but lower efficacy after 14–21 days. On the other hand, populations of *Aspergillus* species seemed to be stimulated by preservative addition to the maize grain.

In general, the effect of propionates was closely linked to a_w for all of fungal groups. Thus, the statistical interaction a_w × preservative was significant in most cases ($P < 0.01$). Fig. 2 shows how, in general, at 0.85 a_w the effect of a 0.1% preservative is quite marked, while at 0.90–0.95 a_w there is no effect, except on the *Penicillium* species.
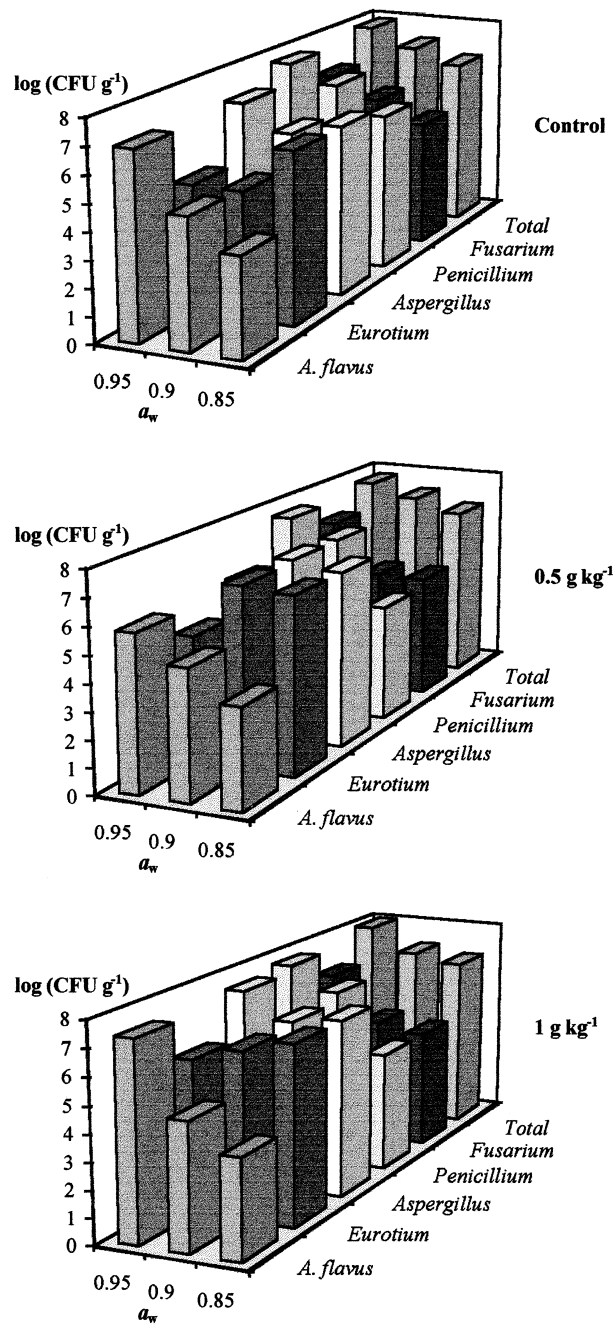
Fig. 1. Effect of a_w and concentration of propionates on maize mycoflora after a 21-day incubation period at 25°C.
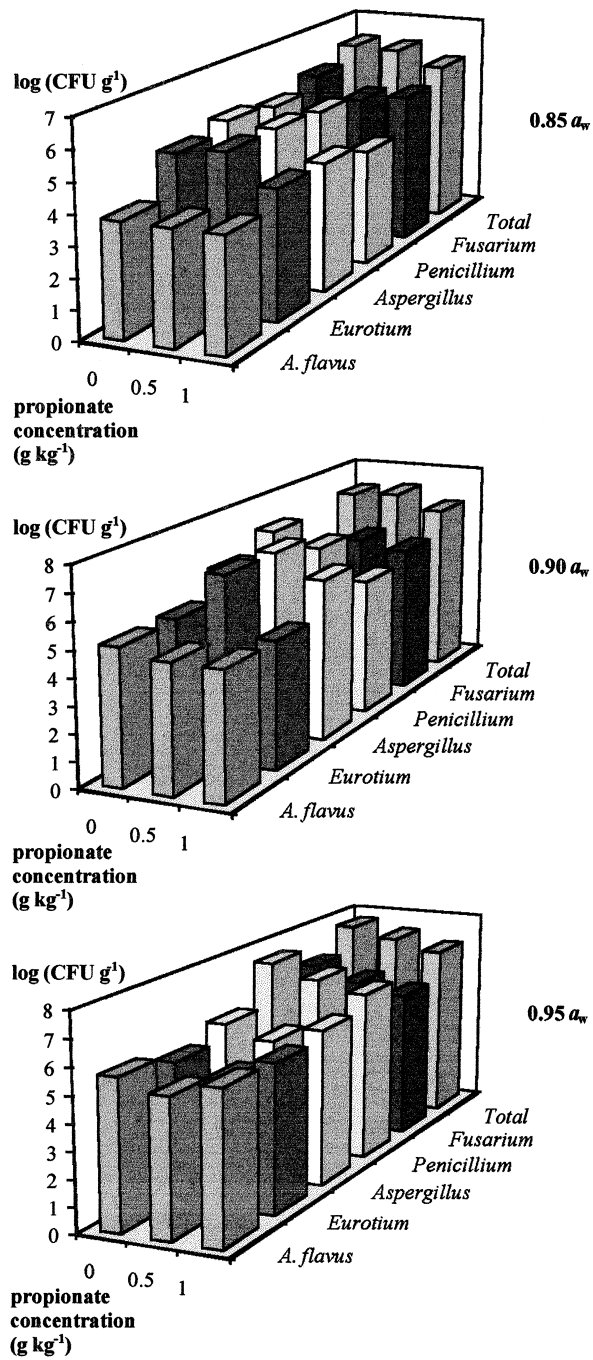
Fig. 2. Effect of propionates and a_w on maize mycoflora after a 7-day incubation period at 25°C.

Table 2
Fumonisin B_1 concentration ($\mu g\ g^{-1}$) in non-sterilised maize incubated at 25°C for 28 days

Water activity	Preservative concentration	Additional *Fusarium* inoculation	Non-additional *Fusarium* inoculation
0.85	0%	3.71	9.27
	0.05%	11.15	4.96
	0.1%	5.36	9.98
0.90	0%	4.78	5.65
	0.05%	5.72	7.34
	0.1%	3.38	8.04
0.95	0%	8.57	9.46
	0.05%	6.42	3.61
	0.1%	5.97	6.58

3.4. Effects of treatments on fumonisin B_1 production

No effect was exerted by either a_w or propionates on fumonisin concentration of samples (Tables 2 and 3). There was also no difference between fumonisin levels in maize grain inoculated with *Fusarium* species and uninoculated samples. The mean fumonisin level was 6.66 $\mu g\ g^{-1}$. The only interaction which proved significant was that of additional inoculation of *Fusarium* Liseola × propionate doses ($P < 0.05$).

4. Discussion

This study complements previous work in which the inhibitory effect of propionate on growth of *F. moniliforme* and *F. proliferatum* was demonstrated to be effective at low doses (0.07%), but only at high a_w levels (0.93–0.98 a_w) (Marín et al., 2000). However, while these conditions inhibited *Fusarium* growth, a low concentration of fumonisin B_1 was still detected (Marín et al., 2000). As discussed previously (Skrinjar et al., 1995), the effect of propionates

Table 3
Analysis of variance of fumonisin B_1 production by *Fusarium* sp. on non-sterilised maize. Significance of additional inoculation of *Fusarium* (I), preservative dose (P) and water activity (a_w), and their interactions

Factor	DF	MS	F
I	1	16.49	1.35
P	2	0.81	0.07
I × P	2	44.20	3.62[*]
a_w	2	11.48	0.94
a_w × I	2	9.51	0.78
a_w × P	4	15.52	1.27
a_w × I × P	4	16.58	1.36

[*] Significant, $P < 0.05$.

depends closely on the other environmental factors such as a_w and temperature. The present work focused on the biotic factors, i.e., the influence of natural microflora of maize on *Fusarium* development and its relationship with preservatives and a_w. The present study was carried out at 25°C with a_w levels of 0.85, 0.90 and 0.95 a regimen under which *Fusarium* spp should be inhibited by other fungal species which colonise maize grain (Marín et al., 1998d). However, at lower temperatures (e.g. 15°C) *Fusarium* has been demonstrated to grow better than *Aspergillus* species (Marín et al., 1998b,c), but not as well as *Penicillium* species (Marín et al., 1998a–c).

For the first time the selective effect of preservatives on maize mycoflora has been demonstrated. Furthermore, the tolerance of some fungal species to certain doses of propionates has been shown. For example, populations of *Aspergillus* species increased with concentration of preservative, while *Eurotium* species were unaffected by the preservative. Similarly, the incidence of all predominant storage fungi of stored rice, sorghum and groundnut under tropical conditions was found to decrease due to propionic acid treatment, except for *Eurotium* spp which increased (Patkar et al., 1995). With inshell groundnuts, 0.3 and 0.5% ammonium propionate was demonstrated to exert a selective effect on the natural mycoflora, with *Eurotium* spp representing more than 50% of the total fungal counts. This effect was evident with 0.3% propionic acid and controlled potentially aflatoxigenic fungi up to day 14 of incubation (Calori-Domingues et al., 1996).

It has been reported that subinhibitory doses together with inadequate distribution of chemicals could favour fungal growth on the treated material with an initial low level of contamination. A change in the dominance of genera and species can occur, enabling the growth of species tolerant to preservative treatment (Smith and Moss, 1985; Lacey, 1989). In vitro studies by Mutasa and Magan (1990a) with potassium sorbate (0.1–0.4%) on growth of tobacco spoilage fungi showed that at both 0.85 and 0.96 a_w and 25°C members of the *Eurotium* group were tolerant to sorbate, while *Aspergillus* and *Penicillium* species were less so, with some species able to metabolise the preservative rapidly (Mutasa et al., 1990b).

In fungi colonizing propionic-acid-treated hay, small additions of propionate to the medium tended to suppress conidial development and to enhance cleistothecium formation, but larger additions inhibited both. Other organisms isolated from hay, including *Fusarium culmorum*, failed to grow when more than 0.4% propionic acid was incorporated in the agar medium (Lord et al., 1981). The ability of certain fungi to metabolise propionic acid can be a problem. Thus *Paecilomyces variotii* and *Eurotium repens* grew well in 0.2 and 0.4% propionate, taking 1 and 2 weeks to metabolise the chemical completely in agar culture. However, no growth occurred in 0.8 and 1% propionate, even after 30 days, with propionic acid concentrations remaining unchanged. Members of the *Eurotium* group varied in their tolerance to propionate. The inhibitory action of propionic acid has been demonstrated in maize stored with high moisture content. A dose of 0.4% added directly to rehydrated maize controlled contamination for more than 35 weeks (Sauer and Burroughs, 1974). When high moisture maize was heavily inoculated with *Aspergillus flavus/parasiticus* and treated with 1% propionic acid, growth and formation of aflatoxins were inhibited for about 19 weeks (Vandergraft et al., 1975).

Similarly, production of aflatoxins in cultures by *A. flavus* and by isolates of the same fungus from moulded hay has been stimulated by up to 0.2% propionic acid (Al-Hilli and Smith, 1979; Clevstrom et al., 1981) and by 0.025% sorbic acid. The latter also stimulated production

of T-2 toxin by *Fusarium acuminatum* (Gareis et al., 1984) while higher concentrations were shown to inhibit mycotoxin production to varying degrees.

Efficacy of these preservatives is dependent on pH, propionic acid, like sorbic and benzoic acids, has optimal antimicrobial activity when 50% undissociated. The efficacy of these acids therefore depends on the dissociation constant, pK_a, which is 4.87 for propionic acid. Thus at pH 4.5, 5.0, 5.5, and 6.0, there is about 70, 43, 19, and 6.9% of undissociated acid, respectively (De Boer and Nielsen, 1995). The pH of treatments in this study varied from 4.7 to 5.7, with different patterns depending on a_w levels. Consequently, in most of the cases the percentage of undissociated acid was between 19 and 43%. This pH variation probably did not exert a great influence on preservative efficacy.

In the present study, propionate efficacy was improved at low a_w, and was most inhibitory during the first week of incubation. High treatment concentrations of propionic acid inhibit growth, but the organisms are not killed and growth may thus only be delayed (Lord et al., 1981). Consequently, in vitro screening for the efficacy of preservatives should take into account the combined effect of temperature and a_w on the growth of the microorganisms involved. Propionate concentrations of between 0.01–0.5% were assayed by Skrinjar et al. (1995), with up to 0.05% at 25°C markedly decreasing the growth and sporulation of *Penicillium verrucosum* (*Penicillium aurantiogriseum* var. *verrucosum*). Growth was completely inhibited at 0.1–0.5% at 30°C. Inhibition of ochratoxin (OA) production by propionate was also demonstrated. Complete inhibition of mycotoxin production was found using 0.1% propionic acid (25°C) or 0.05% (30°C), although some growth could be still observed in media with 0.1% propionic acid at 30°C. They concluded that rather than a single factor affecting OA production, multiple action of various inhibitors would be needed against fungal contaminants in food protection (Skrinjar et al., 1995).

Previously, potassium sorbate has been evaluated as a potential preservative for storage of whole kernel autoclaved yellow dent maize at 18, 24 and 30% moisture content. In general, mycotoxin and CO_2 production were reduced with increasing levels of sorbate. However, samples treated with 0.5% sorbate and inoculated with *A. parasiticus* contained about the same amount of aflatoxin as the control at the end of the incubation period. The sorbate was more effective on maize with lower moisture content and in sealed containers, where high concentration of CO_2 accumulated during incubation (Lee et al., 1986).

In this study a_w was shown to be the most significant factor in selecting maize mycoflora. Thus, *A. flavus* was the minor group at 0.85 and 0.90 a_w, while *Eurotium* populations were lowest at 0.95 a_w. The populations of *Penicillium* species increased at 0.90–0.95 a_w. The profiles of response of some of these species to a_w and temperature have been extensively reported elsewhere (Marín et al., 1998a).

It was interesting to note that there was no effect of preservative on fumonisin B_1 production. In our experiments only small amounts of fumonisins were found in the samples. This suggests that the competition between species can perhaps influence the synthesis of such mycotoxins. Probably, the low concentrations of fumonisin B_1 found in the samples were produced at the beginning of the incubation period. However as soon as the other species were able to colonise the grain, *Fusarium* isolates were unable to compete successfully and then neither growth nor fumonisin B_1 production occurred. This is supported by the fact that *Fusarium* counts were the only ones that did not change with time. The ability of some

Aspergillus and *Penicillium* to inhibit the growth of *Fusarium* species in maize grain at 25°C has been demonstrated previously (Marín et al., 1998b).

Herting and Drury (1974) studied the relative antifungal activities of volatile fatty acids on cereals and found propionic acid to be an effective antifungal agent at a level of 0.8% with cereals containing 20% moisture. Propionic acid was more effective than calcium propionate in controlling fungal population density in cereals where *Aspergillus* was dominant (Paster, 1979). De Boer (1988) suggests the application of propionic acid as a preservative in the food industry at levels of 0.1–0.3%. Propionic acid concentrations of 0.2–0.3% have been showed to be suitable for traditional storage of maize in India for periods up to 12 months, effectively inhibiting the growth of fungi (Kumar et al., 1993).

Acknowledgements

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