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Effects of Calcium Propionate and Water Activity on Growth and Aflatoxins Production by *Aspergillus parasiticus*

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Abstract.- Efficacy of calcium propionate at two different doses (0.5% and 1%) against growth and aflatoxins production by *Aspergillus parasiticus* (PRR-2747) was investigated *in vitro* on Czapek yeast extract agar at different levels of water activity (a_w) in the range of 0.996-0.94a_w. *A. parasiticus* spores germinated on all calcium propionate and a_w treatments, however, 1% calcium propionate at 0.94 a_w delayed the germination process up to 9 days. Mycelial growth rate was also found slower (0.57mm day^{-1}) at 1% calcium propionate and 0.94a_w. Aflatoxins (B_1, B_2, G_1 and G_2) were also produced minimally (44.45, 2.72, 21.39 and 2.06 ng g^{-1} of media, respectively) at the aforementioned dosage of calcium propionate and water activity. It was concluded from the results that addition of calcium propionate and a_w amelioration can prove effective tools for suppressing the germination, growth rate and aflatoxins production by *A. parasiticus* in different substrates.

Key words: *Aflatoxins,* calcium propionate and fungal spore germination, water activity and fungal spore germination.

INTRODUCTION

Aflatoxins are a family of highly toxic and carcinogenic fungal metabolites produced by several species of *Aspergillus* such as *A. flavus* Link ex Fries and *A. parasiticus* Speare which frequently contaminate agricultural products all over the world (Kurtzman *et al.,* 1987; Beardall *et al.,* 1994). These fungi germinate, grow and produce aflatoxins over a wider range of environmental conditions. Optimum conditions for aflatoxin production and growth by *A. flavus* Link ex Fries and *A. parasiticus* Speare are 33ºC and 0.99a_w and 35ºC and 0.95a_w, respectively (Sanchis and Magan, 2000). Pitt and Miscamble (1995) showed that the impact of environmental factors on growth of *A. flavus, A. parasiticus* and *A. oryzae* was similar with minima of 0.82a_w at 25ºC and 0.81a_w at 30 and 37ºC.

There are as many as 20 fractions of aflatoxins but the important ones are aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) (Jay, 1996). A variety of chronic effects have been reported for these aflatoxins when ingested by human beings, livestock and poultry (IARC, 1987; Oguz *et al*., 2003). They are designated so because they fluorescence either blue or green in ultraviolet light. Amongst all fractions, AFB1 is normally predominant in amount in cultures as well as in food and feed products. Not all *A. flavus* isolates produce aflatoxin and those that do usually produce only B aflatoxins, whereas almost all *A. parasiticus* isolates produce aflatoxins and produce both B and G toxins (Klich and Pitt, 1988; Klich, 2007).

Strains of *A. flavus* and *A. parasiticus* that produce aflatoxins are ubiquitous in nature. Aflatoxins have been frequently detected in grains, oil seeds, tree nuts, fermented beverages, milk, edible animal tissues and many other agricultural commodities (Abdulkadar *et al.,* 2000; Fernandez *et al.,* 2006; Jaime-Garcia and Cotty, 2006; Giray *et al.,* 2007). Qazi and Fayyaz (2006) reviewed that aflatoxins may be present as a result of fungal action before, during harvest and during storage. Conditions favorable for natural aflatoxin contamination of food occur at latitudes between 40°N and 40°S of the equator. Contamination levels of various foods have been suggested to be associated with occurrence of liver cancer in Karachi.

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A. parasiticus can grow in the presence of sublethal concentrations of numerous food additives (Gunterus *et al.,* 2007; Klich, 2007). Therefore, there is dire need to search a better chemical preservative that can effectively control this species. This study was conducted to determine the effects of calcium propionate preservative and water activity (a_w) on growth and aflatoxin production by *A. parasiticus* (PRR-2747).

MATERIALS AND METHODS

Media and cultural conditions

A representative strain of *A. parasiticus* (PRR-2747) was isolated from poultry feed samples collected from local markets of Peshawar, North West Frontier Province (NWFP) of Pakistan. Initial studies on the sclerotial production pattern and subsequent comparison with known strains of *A. parasiticus* (PRR-2747), previously held in the Applied Mycology Dept. cultural collection of Cranfield University, UK suggested that it was an appropriate representative and aflatoxigenic in nature. The experiment reported in this communication was conducted in the Mycology laboratory of Applied Mycology department, Cranfield University, UK during 2007. The effect of calcium propionate preservative and a_w was studied for the first time on this strain.

Calcium propionate, 0.5 and 1%, was incorporated into molten cooled Czapek yeast extract agar (CYA) media modified to 0.996, 0.96 and 0.94 a_w with glycerol (Magan and Lacey, 1986). Spores suspensions were prepared from colonies of *A. parasiticus* (PRR-2747) grown on malt extract agar (ME, 20g, peptone 1g, agar 20g, distilled water 1000ml, pH 5.5) in 90mm Petri plates for 7 to 10 days at 25°C. Spores were harvested by using a sterile glycerol-water solution containing 0.1% Tween 80 with the a_w adjusted to match that of the growth medium (Magan and Lacey, 1986). Spore suspension was adjusted with the same solution to give a final spore concentration of 10^6 spores ml^{-1} and was utilized the same day.

Solidified control as well as the preservative containing CYA media in 90mm Petri plates were inoculated centrally with a needlepoint of the spore suspension. Plates of the same a_w were stacked and sealed in clean polythene bags. Treatments were replicated three times and cultures were incubated at 25ºC for up to 14 days. The time, in days, for spores germination was assessed daily using a dissecting microscope. Growth was measured along the two diameters at right angles to each other on daily basis. Radial growth rates were calculated and compared with control plates of the same a_w but without the preservative.

Extraction and analysis of aflatoxins

The presence of aflatoxins in all cultural media was tested on the 14th day of incubation. The colonies and the surrounding area of agar were placed in plastic bags (Seward, UK). Extraction was carried out using 20ml of chloroform (twice with 10 ml each), and homogenization for 5 min in a Stomacher® (IUL, USA)**.** The chloroform phase was filtered through Whatman No.3 filter paper and concentrated to dryness under a gentle flow of nitrogen. The residue was redissolved in 200 µl mobile phase of water-acetonitrile-methanol (64+13+23) (Rojas-Duran *et al.,* 2007). An aliquot of 50 µl of extract was injected into the HPLC system after derivatization with Trifluoroacetic acid (TFA). An HPLC system (Waters 600E System Controller) with a fluorescence detector (Waters 470) and an auto-sampler (Waters 712 WISP) was employed for aflatoxins quantification. The column was a superspher 100 RP-18 and the flow rate was 1 ml min^{-1} (Giorni *et al.,* 2007). Aflatoxins production was measured in ng g^{-1} of culture medium.

A commercial mixture of aflatoxin containing AFB1 = 1.6 x 10^{-6}M, AFG1 = 1.5 x 10^{-6}M, AFB2 = 4.7 x 10^{-7}M, AFG2 = 4.5 x 10^{-7}M (Sigma-Aldrich, USA) was used as standard.

Statistical analysis

Analysis of variance was made for all parameters by using SAS program version 6.12 (SAS Institute, Inc.). Means were compared by Least Significant Difference (LSD) test at $p \leq 0.05$.

RESULTS

Spore germination

The germination of spores of *A. parasiticus* (PRR-2747) on CYA significantly delayed with

increasing concentrations of calcium propionate in the media and decreasing levels of a_w (Table V). At 1% level of calcium propionate, *A. parasiticus* (PRR-2747) spores germinated after 5, 8 and 9 days of inoculation at 0.996, 0.96 and 0.94a_w, respectively. While on control media (without calcium propionate) it took only 0.75, 1 and 1.5 days for germination on the abovementioned respective a_w levels (Fig. 1). However, none of the treatments completely stopped the germination process.

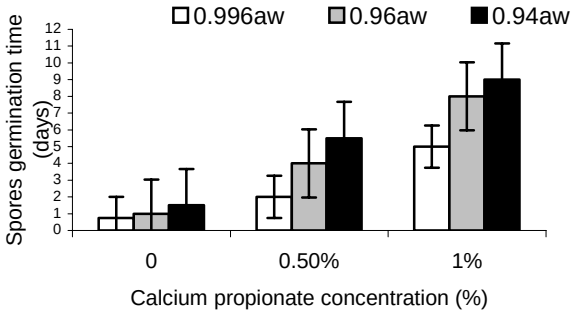

Fig. 1. Effect of calcium propionate on spores germination time (days) of *A. parasiticus* (PRR-2747) at 0.996, 0.96 and 0.94a_w. Bars represent S.E.M.

Radial growth rate

A. parasiticus (PRR-2747) grew faster on CYA with no calcium propionate preservative and at high a_w level (Fig. 2). The growth rate significantly decreased with increasing preservative concentration and decreasing a_w (Table V). Maximum growth rate (3.71mm day^{-1}) was recorded on control media at 0.996a_w while minimum (0.57mm day^{-1}) on 1% calcium propionate added media at 0.94a_w. The growth rate of *A. parasiticus* was sufficiently repressed by calcium propionate and modified a_w levels, however, none of them completely inhibited the growth.

Aflatoxins production

Preservative concentrations and a_w significantly affected aflatoxins production by *A. parasiticus* (PRR-2747) on CYA. Minimum AFB1 content (44.45 ng g^{-1}) was found in 1% calcium propionate added media at 0.94a_w while maximum amount (226.27 ng g^{-1}) was examined in control media at 0.996a_w (Table I). Similar trends were observed for AFB2 (Table II), AFG1 (Table III) and AFG2 (Table IV). It was concluded that amelioration of a_w and calcium propionate preservative can be considered for developing effective tools for controlling aflatoxins production by *A. parasiticus*.

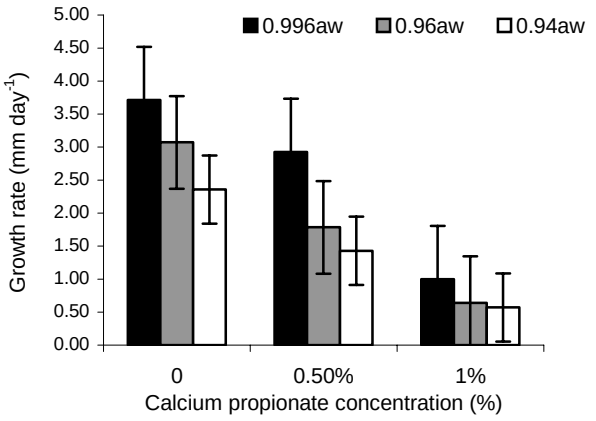

Fig. 2. Growth rate (mm day^{-1}) of *A. parasiticus* (PRR-2747) at different a_w levels (0.996-0.94a_w) in control, 0.5 and 1% calcium propionate added CYA media. Bars represent S.E.M.

Table I. Effect of calcium propionate on the production of AFB1 (ng g^{-1}) by *A. parasiticus* in CYA at different a*w* levels.

Preservative concentration	water activity (a*w*)			Mean ± S.E.M.
	0.996a_w	**0.96a_w**	**0.94a_w**	
0	226.27	191.22	138.11	185.20± 25.36a
0.50%	149.46	131.43	126.67	135.85± 6.84b
1%	118.42	51.32	44.45	71.39± 23.35c
Mean	164.71a	124.65b	103.08c	

*LSD value for preservative concentration (C) = 2.646
LSD value for a_w = 2.646
LSD value for C x a_w = 4.583
* Least Significant Difference

DISCUSSION

It was observed in the present study that the spores germination and growth rate of *A. parasiticus* (PRR-2747) were greatly influenced by calcium

Table II. Effect of calcium propionate on the production of AFB2 content (ng g^{-1}) by *A. parasiticus* in CYA at different a*w* levels.

Preservative concentration	water activity (a*w*)			Mean ± S.E.M.
	0.996a_w	0.96a_w	0.94a_w	
0	77.55	63.33	8.14	49.67± 20.95a
0.50%	31.46	51.53	4.47	29.15± 13.49b
1%	21.68	29.42	2.72	17.94± 7.85c
Mean	43.57a	48.09b	5.11c	

LSD value for preservative concentration (C) = 2.330
LSD value for a_w = 2.330
LSD value for C x a_w = 4.036
*Least significant difference.
Means in row and column followed by different letters are significantly different at p≤0.05.

propionate preservative and water availability in the media. Spores of *A. parasiticus* took longer time for germination on media which had higher concentration of calcium propionate and lower the water activity. After germination of spores, the radial colony growth rate was found much slower in presence of 1% calcium propionate than 0.5 and 0% calcium propionate at all a_ws tested. However, calcium propionate and a_w alone or interactively could stop neither the germination of fungal spores nor the colony radial growth rate completely at any of the level tested. By contrast, Gowda *et al.* (2004) found that propionic acid (0.1–0.5%), ammonia (0.5%) and benzoic acid (0.1–0.5%) completely inhibited *A. parasiticus* growth on potato dextrose agar. The reason is that salts of propionic acid are less fungistatic and show lower short-term efficacy (Marin *et al.,* 1999). In addition, the change in pH and composition of media also influences antifungal effects of these preservatives (Liewen and Marth, 1984; Mutasa *et al.*, 1990; Gqaleni *et al.,* 1996; Marin *et al.,* 2000). The media with propionic acid have lower pH than calcium propionate added media and hence strongly inhibit growth of *A. parasiticus*. However, it is known that the aliphatic acids alone are more volatile while their salts have good long-term efficacy.

A number of chemical compounds are known to affect *Aspergillus* growth and subsequent production of aflatoxins. Chourasia (1993) tested several food preservatives for their effect on growth and toxin production by *A. parasiticus*. He found that propionic acid stopped fungal growth at 0.1% concentration, while citric acid at 0.5% and 1.0%. Sodium metabisulfite inhibited fungal growth in liquid culture at all three concentrations, but not in solid medium where it still produced aflatoxins. Cultures with benzoic acid, sodium acetate and sodium chloride continued to grow but generally produced less amounts of aflatoxins.

Table III.- Effect of calcium propionate on the production of AFG1 content (ng g^{-1}) by *A. parasiticus* in CYA at different a*w* levels.

Preservative concentration	water activity (a*w*)			Mean ± S.E.M.
	0.996a_w	0.96a_w	0.94a_w	
0	248.75	210.52	99.41	186.23± 44.33a
0.50%	159.26	171.27	39.53	123.35± 41.62b
1%	114.17	61.31	21.39	65.62± 26.59c
Mean	174.06a	147.70b	53.44c	

LSD value for preservative concentration (C) = 2.308
LSD value for a_w = 2.308
LSD value for C x a_w = 3.998
Means in row and column followed by different letters are significantly different at p≤0.05.

Table IV.- Effect of calcium propionate on the production of AFG2 content (ng g^{-1}) by *A. parasiticus* in CYA at different a*w* levels.

Preservative concentration	water activity (a*w*)			Mean ± S.E.M.
	0.996a_w	0.96a_w	0.94a_w	
0	82.42	9.67	7.97	33.35± 24.29a
0.50%	7.69	4.23	3.31	5.08± 1.320b
1%	5.26	3.16	2.06	3.49± 0.929c
Mean	31.79a	5.69b	4.45c	

LSD value for preservative concentration (C) = 1.239
LSD value for a_w = 1.239
LSD value for C x a_w = 2.145
Means in row and column followed by different letters are significantly different at p≤0.05.

Table V.- Sum of Squares for spores germination (SG), radial growth rate (RGR) and aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2).

SoV	d.f.	Sum of squares					
		SG	RGR	AFB1	AFB2	AFG1	AFG2
CP	2	176.63**	24.16**	58626.32**	4661.32**	65491.73**	5080.70**
a_w	2	34.88**	5.55**	17608.86**	10040.60**	72387.92**	4291.41**
CP x a_w	4	10.50**	1.19**	5099.12**	2503.86**	8556.39**	6594.21**
Error	18	0.19	0.03	128.46	99.67	97.80	28.15
Total	26	222.19	30.93	81462.76	17305.44	146533.85	15994.47

CP = Calcium propionate; d.f. = Degree of freedom
** Significant at P≤0.01

Quantitative studies of aflatoxins in the media showed that the production of all the four types of aflatoxins *i.e.* AFB1, AFB2, AFG1 and AFG2 were affected by calcium propionate concentrations and a_w levels. Highest quantities of the toxins were produced in the media with no calcium propionate and uppermost a_w tested. Although aflatoxins production by *A. parasiticus* was not completely stopped by calcium propionate and modified a_w levels, however, a significant reduction was noted. Previous studies on the effect of calcium propionate preservative and a_w on aflatoxins have shown that calcium propionate is less effective than propionic acid in controlling the fungal population density and subsequent production of aflatoxins (Paster, 1979). In the present study propionate efficacy enhanced at low a_w level. High treatment concentration of propionate at low a_w levels, inhibited the growth, but the organisms were not killed. Similar results have been reported by Lord *et al.* (1981) and Marin *et al.* (2000).

Propionic acid has been recommended in several studies as effective feed preservative for preventing growth of aflatoxigenic fungi (Rusul *et al.,* 1987; Gowda *et al.,* 2004). However, it is known that propionic acid alone is more volatile than its salts with good short-term efficacy. Therefore, under longer incubation periods the salts such as calcium propionate or sodium propionate may be more effective (Marin *et al.,* 1999). Different concentrations of the preservative at varying levels of a_w are to be worked out for different substrates to generate valid data for application in food preservation.

In perspective of toxicity of aflatoxins, it is alarming for the public health authorities of Pakistan to design strategies to make the public aware with seriousness of the aflatoxin issue. Due to improper storage and post-harvest processes, grains and other commodities are highly prone to be contaminated with the toxigenic strains of *Aspergillus* sp. This situation coupled with poorly practiced food regulations and increasing incidence of the hepatitis epidemics in this country are suffice to open the eyes. Strong regulations and surveillance program and facilities for testing the food and feeds for the aflatoxin contamination are highly imperative for improving the increasingly decreasing health status of the public. The present study indicates that incorporation of proper amount of calcium propionate and water activity amelioration in different feeds are likely to reduce aflatoxins contaminated food consumption's associated health problems in Pakistan. In context of the aforementioned facts it is urgently required to try different physical, chemical and biological methods for controlling aflatoxins in food chains both at national and international levels.

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