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Effect of water activity on hydrolytic enzyme production by *Fusarium moniliforme* and *Fusarium proliferatum* during colonisation of maize

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Abstract

The effect of different water availabilities (water activity, a_w; 0.98–0.93) and time (up to 15 days) on the production of seven hydrolytic enzymes by strains of *F. moniliforme* and *F. proliferatum* during early colonisation of gamma-irradiated living maize grain were examined in this study. Both the total activity (μmol 4-nitrophenol min^{-1} g^{-1} maize) and specific activity (nmol 4-nitrophenol min^{-1} μg^{-1} protein) were quantified using chromogenic *p*-nitrophenyl substrates. The dominant three enzymes produced by the fungi on whole colonised maize kernels were α-D-galactosidase, β-D-glucosidase, and *N*-acetyl-β-D-glucosaminidase. The other four enzymes were all produced in much lower total amounts and in terms of specific activity (β-D-fucosidase, α-D-mannosidase, β-D-xylosidase and *N*-acetyl-α-D-glucosaminidase), similar to that in uncolonised control maize grain. There were significant increases in the total production of the three predominant enzymes between 3–15 days colonisation, and between 3–6 days in terms of specific activity when compared to untreated controls. The total and specific activity of the α-D-galactosidase, β-D-glucosidase and *N*-acetyl-β-D-glucosaminidase, were maximum at 0.98 a_w with significantly less being produced at 0.95 and 0.93 a_w, with the exception of the total activity of α-D-galactosidase which was similar at both 0.95 and 0.93 a_w. Single factors (time, a_w, and inoculation treatment), two- and three- way interactions were all statistically significant for the three dominant enzymes produced except for specific activity of β-D-glucosidase (two and three-way interactions) and for total activity of α-D-galactosidase in the time $\times a_w$ treatment. This study suggests that these hydrolytic enzymes may play an important role in enabling these important fumonisin-producing *Fusarium* spp. to rapidly infect living maize grain over a wide a_w range. © 1998 Elsevier Science B.V.

Keywords: Hydrolytic enzymes; Water activity; Maize grain; *Fusarium* spp.; Total activity; Specific activity; Infection

1. Introduction

Fusarium moniliforme and *Fusarium proliferatum* are commonly present on maize causing severe ear or kernel rot resulting in significant deterioration in

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quality and production of a number of mycotoxins including fusarins, moniliformin and fumonisins (Nelson, 1992; Nelson et al., 1993). Recent work has shown that *F. moniliforme* can infect maize systemically via seed, but that infection predominantly occurs during silking (Munkvold et al., 1997). It has also been shown that these *Fusarium* spp. are able to germinate and grow over a range of water availabilities (0.90–0.995 water activity, a_w) (Marín et al., 1995; 1996) which parallel moisture contents during silking and during post-harvest drying and storage. However, little information is available on the type of enzymes and the enzymatic capacity of these *Fusarium* spp. under environmental conditions conducive to infection.

Infection of maize grain by *Aspergillus flavus* has received more interest and pectinases, amylases and cutinases have been implicated in this process (Cotty et al., 1990; Woloshuk et al., 1996; Guo et al., 1996). The pericarp is the outermost layer of the maize kernel and provides effective protection from fungal invasion. It consists of several layers of cells which differ in their degree of degradation and cell wall thickness (Wolf et al., 1952). Thus the production of a range of enzymes may be advantageous for infection of maize kernels by *Fusarium* spp. pre- or post-harvest.

It has been suggested that the production of specific hydrolytic enzymes by spoilage fungi on temperate cereals can be a good early indicator of the initiation of moulding in grain post-harvest (Magan, 1993a,b). For example, both Stevens and Relton (1981) and Jain and Lacey (1991) using chromogenic 4-nitrophenyl substrates found that the largest quantities of enzymes produced by *Aspergillus, Penicillium* and *Eurotium* spp. were *N*-acetyl-β-D-glucosaminidase and α-D-galactosidase. Indeed, Jain and Lacey (1991) found that a range of xerophilic spoilage fungi (*Aspergillus, Eurotium* and *Penicillium* spp.) were able to produce β-glucosidase and *N*-acetyl-β-glucosaminidase, while only some of them showed α-galactosidase, β-galactosidase and α-glucosidase activity during colonisation of barley/wheat grain. However, practically none showed β-glucuronidase, α-mannosidase and α-fucosidase activity. Other semi-quantitative and agar-based enzymatic tests have suggested that *F. moniliforme* and *F. proliferatum* produce large amounts of the hydrolytic enzymes such as *N*-acetyl-β-D-glucosaminidase and β-glucosidase (Sala, 1993), although effects of environmental parameters were not investigated.

The objectives of this study were to quantify in detail the effect of a_w on the temporal production of seven glycosidase enzymes by strains of *F. moniliforme* and *F. proliferatum* and their potential role in infection of living gamma-irradiated maize grain.

2. Materials and methods

2.1. Fungal isolates

Two strains each of *Fusarium moniliforme* Sheldon (25N, 85N) and *Fusarium proliferatum* (Matsushima) Nirenberg (73N, 131N) were used in all experiments. All isolates were maintained on malt extract agar (MEA -20 g malt extract, 20 g glucose, 1 g peptone, 20 g agar, 1000 ml destilled water, pH = 5.5). All the strains are held in the Food Technology Dept. collection of the University of Lleida, Spain.

2.2. Grain

Spanish maize grain was irradiated with 12 kGrays of gamma irradiation and stored at 4°C. Following this treatment the grain contained no fungal infection but had retained germinative capacity (unpublished information). The initial water content and water activity (a_w) of the grain were 13.9% and 0.71, respectively.

2.3. Rehydration, inoculation and incubation of grain maize

Irradiated grain, 150 g, were placed in sterile flasks and rehydrated to the desired treatment a_w levels (0.93, 0.95 and 0.98) by addition of sterile destilled water. The amount of water added was calculated from a moisture adsorption curve for the grain. The grain treatments were allowed to equilibrate at 4°C for 48 h, with periodic shaking. Finally, the a_w values were confirmed by using a Novasina Thermoconstanter TH200 (Axaid Ltd., Pfäffikon, Switzerland).

Spore suspensions were obtained by harvesting spores from cultures of each isolate maintained on

MEA and suspending them in sterile distilled water with 0.002% Tween 80. The final concentration of the suspensions was assessed by using an haemocitometer and was adjusted to 3×10^7 spores ml^{-1}. A 500 μl of spore suspension was added to each treatment flask and shaken vigorously to obtain an even inoculation of the maize grain. This was taken into account in relation to the initial addition of sterile water to the grain. Uninoculated controls of the same a_w were also prepared. After equilibration, 10 g subsamples of inoculated grain were placed in 9 cm sterile Petri plates. Replicates of the same a_w treatment were placed in impermeable to water plastic containers together with two 100 ml beakers containing a glycerol–water solution (Dallyn, 1978) of the same equilibrium relative humidity value as the a_w of the maize grain substrate. These were incubated at 25°C for the duration of the experiment.

Samples were taken after 3, 6, 9, 12 and 15 days incubation and removed for analyses of enzymes. All enzymatic assays were carried out within 8 h. All treatments were repeated three times.

2.4. Extraction of enzymes from grain

Two different methods of sample extraction, similar to those described previously by Jain and Lacey (1991), were applied to all replicates and treatments.

(i) *Ground samples*: Enzymes were extracted by grinding 4 g of sample with 8 ml of 10 mM potassium phosphate extraction buffer (pH 7.2) using a Commercial Waring blender (Waring Ltd., UK). A 2 ml aliquot of this suspension was then centrifuged in eppendorf tubes in a benchtop microfuge (MSE Cenetaur 2, Norwich, UK) for 15 min at $450 \times g$ to obtain a clear supernatant.

(ii) *Unground samples*: Enzymes were extracted by weighing 2 g samples in glass Universal bottles containing 4 ml of 10 mM potassium phosphate extraction buffer (pH 7.2). These were shaken on a wrist action shaker (Ikalabortechnik KS250 basic, Janke and Kunkel GmbH and Co., Staufen, Germany) for 1 h at 4°C. The washings were then decanted into 1 ml plastic eppendorf tubes and centrifuged in a benchtop microfuge (MSE Cenetaur 2, Norwich, UK) for 15 min at $450 \times g$ and supernatant removed with care. Aliquots of all the extracts were frozen and stored at −80°C for subsequent protein analysis.

2.5. Total enzyme activity determination

The activity of seven glycosidases was assayed using the 4-nitrophenyl substrates (Sigma, England). The concentrations and buffers used for each enzyme assay are listed in Table 1. Enzyme activity was measured by the increase in optical density at 405 nm, caused by the liberation of 4-nitrophenol upon enzymatic hydrolysis of the substrate, three minutes after stopping the reaction with 1M Na_2CO_3. To allow rapid assay of multiple samples a Multiscan plate reader (Life Sciences International, Cambridge, UK) was used (Jain and Lacey, 1991).

For analyses the reaction mixture consisted of 40 μl of substrate solution in appropriate buffer, 40 μl of enzyme extract (previously diluted, if necessary) and 20 μl of the appropriate buffer. These were placed in the wells of the microtitre plate, along with appropriate controls and incubated for an hour at

Table 1
Summary of the hydrolytic enzymes assayed for in this study, their substrates, concentrations, buffer and pH used

Enzyme	Substrate	conc. (mM)	Buffer	pH
β-D-fucosidase	4-nitrophenyl-β-D-fucopyranoside	2.0	25 mM acetate	5.0
α-D-galactosidase	4-nitrophenyl-α-D-galactopyranoside	4.0	25 mM acetate	5.0
β-D-glucosidase	4-nitrophenyl-β-D-glucopyranoside	2.0	25 mM acetate	5.0
α-D-mannosidase	4-nitrophenyl-α-D-mannopyranoside	4.0	25 mM acetate	5.0
β-D-xylosidase	4-nitrophenyl-β-D-xylopyranoside	2.0	25 mM acetate	5.0
N-acetyl-α-D-glucosaminidase	*p*-nitrophenyl-*N*-acetyl-α-D-glucosaminide	2.0	25 mM acetate	4.2
N-acetyl-β-D-glucosaminidase	*p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide	2.0	25 mM acetate	4.2

37°C. The reaction was stopped by adding 5 μl Na_2CO_3. A calibration curve of 4-nitrophenol concentration vs. absorbency at 405 nm was previously constructed. Total enzyme activity was expressed as μmol 4-nitrophenol released min^{-1} g^{-1} dry maize.

2.6. Specific enzyme activity determination

Total protein in the extracellular enzyme extract was measured using a bicinchoninic acid protein assay (BCA) procedure. In order to carry out rapid analysis of multiple samples, a micro-assay method using a microtitre plate reader was used. A BCA protein assay reagent kit (Pierce, England) consisting of BCA reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.2N sodium hydroxide), BCA reagent B (solution containing 4% cupric sulphate) and Albumin standard (containing bovine serum albumin at a concentration of 2.0 mg ml^{-1} in a solution of 0.9% saline and 0.05% sodium azide) was used.

BCA reagent A, 50 parts, were mixed with 1 part of reagent B to obtain the working reagent, which is stable for at least 1 day when stored in a closed container at room temperature. A 10 μl volume of each standard or unknown sample were pipetted into the appropriate microtitre plate wells, while 10 μl of the diluent were used for the blank wells. A 200 μl volume of the working reagent were added to each well, and after shaking the plates were incubated at 37°C for 30 minutes. After cooling the plates to room temperature, the absorbance at 540 nm was measured in a plate reader. A calibration curve made with albumin standards was used in order to obtain protein concentrations in the enzyme extracts. These values were related to enzyme concentrations to calculate specific activity of the different enzymes tested in nmol 4-nitrophenol released min^{-1} μg^{-1} protein.

2.7. Statistical analyses of the results

Variance of both total and specific enzyme activity results were analysed and effects of the different factors involved in the experiments and their interactions were shown at the 99% level ($P < 0.01$). LSD tests were also made ($\alpha = 0.05$) for the significant factors found. All statistical analysis were made by using SAS 6.11 (SAS Institute Inc.).

3. Results

3.1. Comparison of the total enzyme activity and specific activity in irradiated unground and ground maize grain

Table 2 compares (i) the total activity and (ii) the specific activity of β-D-glucosidase and *N*-acetyl-β-D-glucosaminidase at 0.95 a_w after 15 days incubation for *F. moniliforme* (25N) and *F. proliferatum* (131N). This shows that although the total enzyme activity was higher in ground samples, the specific activity was often significantly higher in unground maize grain inoculated with strains of *F. moniliforme* and *F. proliferatum* when compared with untreated controls. Because of our interest in determining the role of enzymes and their activity in the infection of maize grain by these fungi all results are presented for total and specific activity in unground samples as

Table 2
Effect of the enzyme extraction method in both total activity (TA, μmol 4-nitrophenol min^{-1} g^{-1} dry maize) and specific activity (SA, nmol 4-nitrophenol min^{-1} μg^{-1} protein) of *F. moniliforme* (25N) and *F. proliferatum* (131N) found in samples at 0.95 a_w after 15 days

		β-D-glucosidase				*N*-acetyl-β-D-glucosaminidase			
		TA	LSD[a] groups	SA	LSD[a] groups	TA	LSD[a] groups	SA	LSD[a] groups
Ground samples	Uninoculated	1.748	A	0.905	A	10.535	A	5.533	A
	25N	108.819	C	11.532	C	45.358	B	5.371	A
	131N	95.862	B	9.897	B	48.992	C	5.154	A
Unground samples	Uninoculated	0.882	A	0.479	A	1.444	A	0.785	A
	25N	4.824	B	9.058	C	8.220	B	12.292	C
	131N	6.128	C	6.033	B	8.225	B	5.590	B

[a] groups with different letters are significantly different ($P < 0.05$).

we were specifically interested in their involvement in penetration of the grain surface by the mycelia of the *Fusarium* species. Furthermore enzymes extracted from the mycelia growing on the grain surface better represents their potential in the early stages of the infection.

By using both methods of extraction it was shown how total enzyme activity was markedly higher in extracts from ground samples in both inoculated and uninoculated maize due to the higher amount extracted from the maize grain itself. In general, specific activity (Table 2) in ground samples was also higher than that from unground whole kernels, although the differences were not as marked as in the measurement of the total enzyme activity.

The total enzyme activity expresses the overall increase or decrease in activity which can be due to both an increase in the enzyme amount or in the specific activity of the ones present. However, the specific activities obtained in relation the amount of protein present reflects the level of activity of each enzyme. Thus the specific activity measurement represents important complimentary information to the overall total enzymes quantified and reflects the possible enzyme dynamics and behaviour in infection of maize kernels. Because of this, all data are presented for both total and specific activity by the *Fusarium* spp. in unground maize samples.

3.2. Comparison of the total and specific activity of the seven glycosidase enzymes produced by F. moniliforme and F. proliferatum on whole maize grain at different water availabilities

In extracts from uninoculated control maize the enzyme β-D-glucosidase was found to be predominantly present with the most activity (1.94 μmol 4-nitrophenol min^{-1} g^{-1} dry grain; 1.05 nmol 4-nitrophenol min^{-1} μg^{-1} protein), followed by *N*-acetyl-β-D- glucosaminidase (1.62 μmol 4-nitrophenol min^{-1} g^{-1} dry grain; 0.88 nmol 4-nitrophenol min^{-1} μg^{-1} protein) and α-D-galactosidase (0.61 μmol 4-nitrophenol min^{-1} g^{-1} dry grain; 0.33 nmol 4-nitrophenol min^{-1} μg^{-1} protein).

Table 3 compares the total and specific activity of the seven enzymes produced by the *Fusarium* spp. on maize after 15 days at 0.95 a_w. The most important enzymes produced by strains of the two *Fusarium* spp. in terms of total and specific activity were α-D-galactosidase, β-D-glucosidase and *N*-acetyl-β-D-glucosaminidase. The other four enzymes examined were all present at levels similar to those of the uninoculated control grain, both in terms of concentration and specific activity.

3.3. Interactions between assayed factors

Table 4 shows the analysis of variance of the results and the effect of single, two and three-way factors on the total and specific activity of the three enzymes of interest. For α-D-galactosidase, β-D-glucosidase and *N*-acetyl-β-D-glucosaminidase, single factors (time -*t*-; water activity -a_w-, and inoculation treatment -*s*-), and most of two and three way interactions were significant ($P < 0.01$). All two- and three-way interactions were significant except for $a_w \times s$, and $t \times a_w \times s$ for production of α-D-glucosidase and for $t \times a_w$ for total activity of α-D-galactosidase. This wide significance of interactions means that single factors, beside being significant, have different trends depending on the levels of the

Table 3
Comparison of both total activity (TA, μmol 4-nitrophenol min^{-1} g^{-1} dry maize) and specific activity (SA, nmol 4-nitrophenol min^{-1} μg^{-1} protein) of *F. moniliforme* (25N) and *F. proliferatum* (131N) found in samples at 0.95 a_w after 15 days for the seven enzymes tested

Enzyme	*F. moniliforme* (25N)		*F. proliferatum* (131N)		Uninoculated	
	TA	SA	TA	SA	TA	SA
β-D-fucosidase	0.1326	0.4489	0.1256	0.2942	0.2087	0.1134
α-D-galactosidase	1.5607	5.0122	2.9311	6.8927	0.6085	0.3305
β-D-glucosidase	2.9108	9.4702	5.1953	12.8688	1.9413	1.0544
α-D-mannosidase	0.1508	0.4937	0.0865	0.2019	0.3296	0.2695
β-D-xylosidase	0.3179	1.0380	0.1322	0.3219	0.1857	0.1008
N-acetyl-α-D-glucosaminidase	0.1512	0.5099	0.1438	0.3513	0.2903	0.1577
N-acetyl-β-D-glucosaminidase	1.4549	4.6107	3.8795	8.9933	1.6176	0.8786

Table 4
Analysis of variance of *N*-acetyl-β-D-glucosaminidase, β-D-glucosidase and α-D-galactosidase total (TA, μmol 4-nitrophenol min^{-1} g^{-1} dry maize) and specific (SA, nmol 4-nitrophenol min^{-1} μg^{-1} protein) activity at different levels of incubation time (*t*), water activity (a_w), and inoculation treatments (*s*)

Enzyme	Factor	DF	TA MS	F	SA MS	F
α-D-galactosidase	t	4	49.16	23.55[a]	65.56	20.53[a]
	a_w	2	22.90	10.97[a]	51.16	16.02[a]
	t × a_w	8	3.17	1.52	14.96	4.68[a]
	s	4	88.79	42.53[a]	202.61	63.44[a]
	t × s	16	17.33	8.30[a]	18.95	5.93[a]
	a_w × s	8	8.60	4.12[a]	9.49	2.97[a]
	t × a_w × s	29	3.83	1.84[b]	5.79	1.82[b]
β-D-glucosidase	t	4	73.58	35.10[a]	92.80	15.50[a]
	a_w	2	112.35	53.59[a]	48.69	8.13[a]
	t × a_w	8	9.51	4.54[a]	16.69	2.79[a]
	s	4	139.61	66.59[a]	226.90	37.90[a]
	t × s	16	15.54	7.41[a]	24.94	4.17[a]
	a_w × s	8	26.07	12.44[a]	8.27	1.38
	t × a_w × s	29	7.49	3.57[a]	6.72	1.12
N-acetyl-β-D-glucosaminidase	t	4	130.62	19.66[a]	140.93	14.14[a]
	a_w	2	681.55	102.57[a]	171.68	17.22[a]
	t × a_w	8	42.49	6.40[a]	37.91	3.80[a]
	s	4	182.24	27.43[a]	336.98	33.80[a]
	t × s	16	32.65	4.91[a]	52.96	5.31[a]
	a_w × s	8	81.87	12.32[a]	28.57	2.87[a]
	t × a_w × s	29	24.78	3.73[a]	23.71	2.38[a]

[a] Significant $P < 0.01$.
[b] Significant $P < 0.05$.

other single factors, which makes the results more difficult to be interpreted.

3.4. Effect of water activity and time on the three predominant glycosidases produced by the F. moniliforme and F. proliferatum on maize grain

For the total enzyme activity, Fig. 1 compares the effect of a_w and time on the activity of the three main glycosidase enzymes produced by a strain of *F. moniliforme* (25N) and *F. proliferatum* (131N). In general, activity of α-D-galactosidase, β-D-glucosidase and *N*- acetyl-β-D-glucosaminidase, all increased with increasing a_w and time. However, a_w had an important influence on activity of these three dominant enzymes produced by both *Fusarium* spp. The α-D-galactosidase activity was least affected by a_w, while *N*-acetyl-β-D-glucosaminidase and β-D-glucosidase activity was higher at 0.98 a_w, but did not show much difference at 0.95 and 0.93 a_w (Table 5).

For the specific activity, a comparison of the three predominant enzymes is shown in Fig. 2. Considering the whole testing period, optimum activity was at 0.95 a_w for α-D-galactosidase and β-D-glucosidase, while activities were similar at 0.93 and 0.98 a_w. The exception was *N*-acetyl-β-D-glucosaminidase where specific activity was optimum at 0.98 a_w, and much lower and similar at both 0.95 and 0.93 a_w (Table 5). The maximum activity of this enzyme was found after 6 days and 9 days for *F. moniliforme* and *F. proliferatum*, respectively, at 0.98 a_w. However, at 0.95 and 0.93 a_w the enzyme was produced later.

3.5. Strain differences

Table 6 shows the statistical comparison of the total and specific activity of the three major hydrolytic enzymes assayed in this study. Significant differences in the specific activity of α-D-galactosidase, β-D-glucosidase and *N*-acetyl-β-D-glucosaminidase produced by the *F. proliferatum* strains on maize kernels were observed. Furthermore, *F. moniliforme* isolates had higher β-D-glucosidase

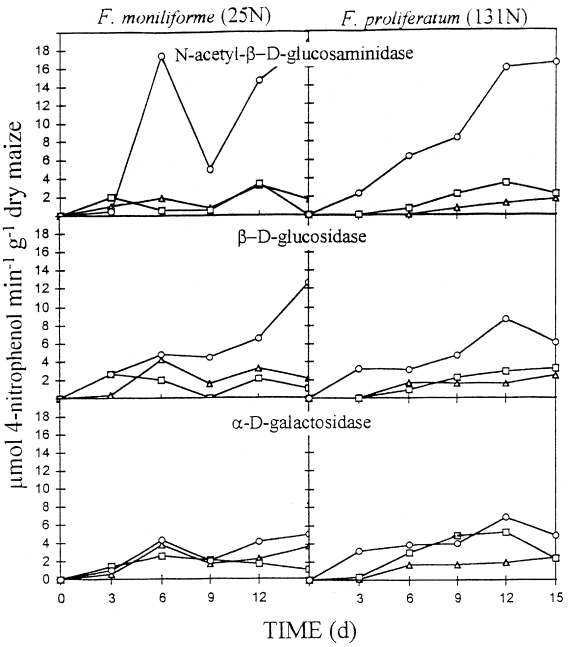

Fig. 1. Effect of time and water activity, 0.93 (△), 0.95 (□) and 0.98 (○), in total *N*-acetyl-β-D-glucosaminidase, β-D-glucosidase and α-D-galactosidase activity from *F. moniliforme* (25N) and *F. proliferatum* (131N) inoculated on maize grain. Blanks have been substracted from the results given.

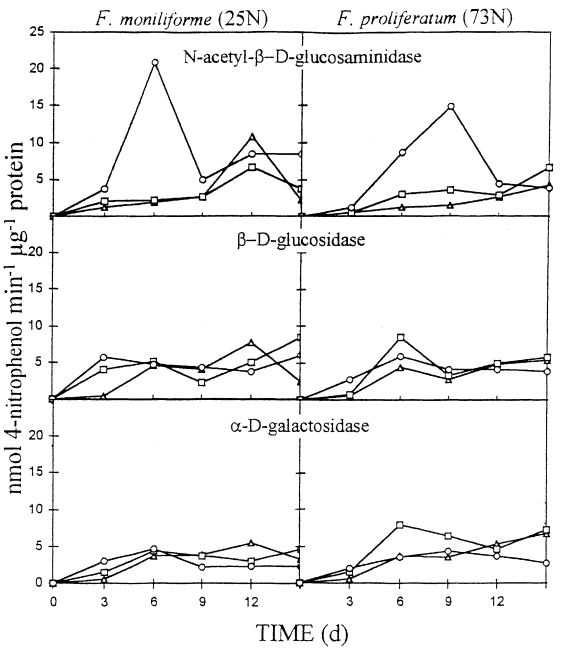

Fig. 2. Effect of time and water activity, 0.93 (△), 0.95 (□) and 0.98 (○), in specific *N*-acetyl-β-D-glucosaminidase, β-D-glucosidase and α-D-galactosidase activity from *F. moniliforme* (25N) and *F. proliferatum* (73N) inoculated on maize grain. Blanks have been substracted from the results given.

and *N*-acetyl-β-D-glucosaminidase total activity than *F. proliferatum.*

F. proliferatum isolates had significantly higher α-D-galactosidase specific activity than those of *F. moniliforme*, while for the other two enzymes there was significant difference between isolates of the same species.

4. Discussion

This study has shown that over a wide range of a_w levels *F. moniliforme* and *F. proliferatum* are able to colonise living maize kernels and produce a range of hydrolytic enzymes. The three enzymes with the highest total and specific activity produced during

Table 5
LSD test showing the effects of water activity in α-D-galactosidase, β-D-glucosidase, and *N*-acetyl-β-D-glucosaminidase total activity (TA, μmol 4-nitrophenol min^{-1} g^{-1} dry maize) and specific activity (SA, nmol 4-nitrophenol min^{-1} μg^{-1} protein)

	α-D-galactosidase			β-D-glucosidase			*N*-acetyl-β-D-glucosaminidase		
(i) TA	Level	LSD[a] Group	Mean	Level	LSD[a] Group	Mean	Level	LSD[a] Group	Mean
a_w	0.98	A	3.3387	0.98	A	5.0455	0.98	A	8.2281
	0.95	A	3.0319	0.95	B	3.5439	0.95	B	3.0213
	0.93	B	1.9040	0.93	C	1.9651	0.93	B	2.2458
(ii) SA	Level	LSD[a] Group	Mean	Level	LSD[a] Group	Mean	Level	LSD[a] Group	Mean
a_w	0.95	A	4.3913	0.95	A	5.2831	0.98	A	6.9092
	0.93	B	3.0693	0.98	B	3.9898	0.95	B	4.1744
	0.98	B	2.7670	0.93	B	3.3274	0.93	B	3.8511

[a] Groups with different letters are significantly different ($P < 0.05$) UNIN.: uninoculated.

Table 6
LSD test showing the differences between species and isolates in α-D- galactosidase, β-D-glucosidase, and *N*-acetyl-β-D-glucosaminidase total activity (TA, μmol 4-nitrophenol min^{-1} g^{-1} dry maize) and specific activity (SA, nmol 4-nitrophenol min^{-1} μg^{-1} protein)

	α-D-galactosidase			β-D-glucosidase			*N*-acetyl-β-D-glucosaminidase		
(i) TA	Level	LSD[a] Group	Mean	Level	LSD[a] Group	Mean	Level	LSD[a] Group	Mean
F.moniliforme	25N	B	2.8157	73N	C	3.3268	73N	B	3.6231
	85N	A	4.1883	85N	A	6.5070	85N	A	6.6864
F.proliferatum	73N	B	3.4306	131N	C	3.5626	131N	A	5.4397
	131N	B	3.4470	25N	B	4.2209	25N	A	6.1862
	UNIN.	C	0.3661	UNIN.	D	0.8821	UNIN.	C	1.4446
(ii) SA	Level	LSD[a] Group	Mean	Level	LSD[a] Group	Mean	Level	LSD[a] Group	Mean
F.moniliforme	25N	C	3.3931	85N	B	4.7373	85N	C	4.9856
	85N	C	2.9770	73N	B	4.6026	73N	C	4.7984
F.proliferatum	73N	B	4.4931	25N	B	5.0138	25N	B	6.3545
	131N	A	5.9679	131N	A	6.4496	131N	A	8.1938
	UNIN.	D	0.1964	UNIN.	C	0.4793	UNIN.	D	0.7857

[a] Groups with different letters are significantly different ($P < 0.05$). UNIN.: uninoculated.

the first 3–9 days were α-D-galactosidase, β-D-glucosidase and *N*-acetyl-β-D-glucosaminidase, when compared with uninoculated maize grain. Previous work has shown that both these fumonisin-producing fungi are able to rapidly germinate, and grow over a wide range of temperatures and water availabilities (Marín et al., 1995; 1996) enabling them to compete effectively in this niche.

This study has also shown that more useful information can be obtained by quantifying enzymes produced on the surface of the grain by these pathogens than by grinding samples. This may be particularly so where live irradiated grains are used as opposed to autoclaved dead grain. Irradiated grain may be more realistic as it has retained germinative capacity and some of the natural defences, such as phytoalexins, present in natural harvested grain (Cuero et al., 1986). The high specific activity of the predominant enzymes produced by both *Fusarium* spp., at 0.98–0.93 a_w is indicative of a potential role in the maize infection process.

Fungi have evolved different strategies to invade seeds and other plant tissue. Generally, fungi secrete a cocktail of hydrolytic enzymes including cutinases, cellulases, pectinases and proteases (Knogge, 1996). There are three major types of enzyme required for the hydrolysis of crystalline cellulose to glucose. These are endoglucanases, exoglucanases and β-glucosidases. Penetration of plants may be achieved quite simply by entry through the plant's natural openings or by utilising a route through non-cellulosic areas. It has been shown that *A. flavus* can produce cell-wall degrading pectinases, and may secrete extracellular cutinases to enable effective infection through the pericarp of maize kernels (Guo et al., 1996). There is now evidence that *F. moniliforme* colonises maize grain predominantly during silking but that it is also able to systemically infect seed and be transported within the plant to the next generation (Munkvold et al., 1997). Therefore the production of a range of enzymes is important for effective colonisation of ripening or harvested seeds.

Recently Sala (1993) in complementary work on maize mycoflora investigated *Fusarium* species in a similar way. She found that only three *F. proliferatum* isolates out of a total of 13 *F. moniliforme* and *F. proliferatum* strains had α-galactosidase activity, while all of them had β-glucosidase and *N*-acetyl-β-glucosaminidase activity. The β-glucosidase and *N*-acetyl-β-glucosaminidase activities were high in general, but there were no differences between *F. moniliforme* and *F. proliferatum* isolates. She found no β-galactosidase, β-glucuronidase, α-glucosidase, α-mannosidase and α-fucosidase in any of the isolates. However, in her studies no detailed quantification of the effects of a_w or time on total or specific activity were carried out. In our experiments all the *F. moniliforme* and *F. proliferatum* isolates used were able to produce α-D-mannosidase on moist maize (0.98 a_w) although in small amounts. In

contrast, α-galactosidase activities were higher than suggested by these semi-quantitative studies.

The present study suggests that α-D-galactosidase, β-D-glucosidase, *N*-acetyl-β-D-glucosaminidase, and, perhaps, β-D-xylosidase may be suitable indicators of *F. moniliforme* and *F. proliferatum* colonisation of maize. In general, expressing total activity in relation to maize weight may be a more useful and rapid method than measurements of specific activity on a protein basis because the former method is more sensitive in detecting fungal production of enzymes than the latter. Furthermore, the washing and extraction method was more useful for early detection because it enabled quantification of the increase in fungal activity after 3 days. Grinding of samples was less sensitive, but could be useful for examining fungal enzyme production during internal mycelial colonisation of the grain although interference from internal maize enzymes could make interpretation of the data more difficult.

It has been suggested that some of these hydrolytic enzymes may be good indicators of the early colonisation of cereal grain by spoilage fungi, particularly xerotolerant and xerophilic species (Jain and Lacey, 1991; Magan, 1993a, Magan, 1993b). Indeed, increases in some of these enzymes were observed prior to any visible moulding had occurred. In the present study on maize, enzyme activity by *Fusarium* spp. from washing extracts showed that *N*-acetyl-β-D-glucosaminidase, followed by α-D-galactosidase and β-D-glucosidase were important indicators of infection. In experiments with barley and wheat grain, *Penicillium* or *Aspergillus* spp. were found to produce little or no *N*-acetyl-α-D-glucosaminidase and α-D-glucosidase, while more α-D-mannosidase and β-D-xylosidase activity was generally found, but varied with cereal type. The activity of α-D-galactosidase, *N*-acetyl-β-D-glucosaminidase, and β-D-glucosidase was always much more greater than that in uninoculated control grain. The latter three enzymes were found to be produced in greatest amounts at 25% moisture content ($=0.98\ a_w$) after 30 days incubation. They suggested that *N*-acetyl-β-D-glucosaminidase, α-D-galactosidase, β-D-xylosidase/xylopiranosydase and perhaps β-D-glucosidase would be suitable candidates to detect activity of spoilage by xerophilic fungi, and pointed out that other enzymes (e.g. β-D-xylopiranoside) detected in greater amount in $>25\%$ moisture content, might be useful for detecting activity of more mesophilic fungi (Jain and Lacey, 1991). Flannigan and Bana (1980) reported how β-xylosidase and α-glucosidase activities increased with relative humidity (81–92%) of storage atmosphere in extracts from barley inoculated with *A. chevalieri* and stored for 5 months.

This study has shown that some of these hydrolytic enzymes may play an important role in enabling these fumonisin-producing *Fusarium* spp. to rapidly colonise maize grain over a range of water availability conditions. This together with the capability for rapid germination and growth (Marín et al., 1995, 1996) could partially explain their competitiveness in the maize grain niche and their ability for excluding other fungi once becoming established.

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