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International Journal of Food Microbiology 61 (2000) 11–16

INTERNATIONAL JOURNAL OF
Food Microbiology

www.elsevier.nl/locate/ijfoodmicro

Impact of environment and interspecific interactions between spoilage fungi and *Aspergillus ochraceus* on growth and ochratoxin production in maize grain

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Received 21 June 1999; received in revised form 10 January 2000; accepted 20 March 2000

Abstract

Using layers of irradiated but still fertile maize grain, the effects of water activity (0.995, 0.95 a_w) and temperature (18, 30°C) on interspecific interactions between *Aspergillus ochraceus* and five other spoilage fungi were examined. *Asp. ochraceus* was not competitive against *Asp. flavus*, *Asp. niger*, or *Alternaria alternata* at 18°C when water was freely available (0.995 a_w), while at 0.95 a_w it was dominant against *Asp. candidus*, *Asp. flavus* and *Alt. alternata*. At 30°C and 0.995 a_w, *Asp. ochraceus* was dominated by other fungi, except *Alt. alternata*, and was mutually antagonistic to *Asp. candidus* and *Eurotium amstelodami*. However, at 30°C and 0.95 a_w it was competitive against *Asp. candidus* and *Alt. alternata*, but not against the other species examined. The overall Index of Dominance showed that *Asp. ochraceus* was not competitive under the conditions examined here. At 18°C ochratoxin production by *Asp. ochraceus* was inhibited significantly by *Asp. candidus* (0.995 and 0.95 a_w) and *Asp. niger* (0.995 a_w). When grown on maize grain at 30°C, ochratoxin production by *Asp. ochraceus* was significantly inhibited by other spoilage fungi when both were grown on maize grain, especially by *Asp. niger* and *E. amstelodami* (0.995 a_w) and *Asp. flavus* at 0.95 a_w. These results suggest that, to a large extent, *A. ochraceus* is not as competitive as some other spoilage fungi in primary resource capture on maize grain at a_w of 0.95 or above, although it may modify resource quality and influence secondary colonisation by other species under the conditions tested.

Keywords: Ochratoxin; *Aspergillus ochraceus*; Water activity; Temperature; Interactions; Spoilage fungi; Interspecific interactions; Primary resource capture

1. Introduction

Aspergillus ochraceus is a xerophilic spoilage fungus which contaminates a range of temperate and tropical cereals, often producing ochratoxins (Pohland et al., 1992). It is an important coloniser of maize and inevitably interacts and competes with other contaminant *Fusarium*, *Aspergillus* and *Penicillium* species for the maize grain niche. We recently demonstrated that niche overlap and domi-

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0168-1605/00/$ – see front matter
PII: S0168-1605(00)00385-8

nance by *Asp. ochraceus* is influenced by water availability and temperature, and that in vitro interactions and competition markedly influenced the production of ochratoxin by this species (Lee and Magan, 1999a,b). Previous work by Ramakrishna et al. (1993, 1996a,b) and Marin et al. (1998b) demonstrated that interactions between mycotoxigenic species and other spoilage fungi on barley or maize grain significantly influenced mycotoxin production. Toxin production was stimulated under some environmental conditions. Substrate colonisation by interacting species on cereal straw was also found to be influenced by resource quality, although in vitro and in situ results were found to be very similar (Robinson et al., 1993).

The objective of the present work was to examine the effect of a range of common maize colonists on the capacity of *Asp. ochraceus* for primary resource capture on irradiated maize grain under different a_w and temperature conditions. Examination of hyphal interactions, relative growth rates during interaction, and the effect on ochratoxin production in the maize grain niche was carried out.

2. Materials and methods

2.1. Fungal species

Single isolates of the following species were used in this study. *Aspergillus ochraceus* (NRRL 3174), *Alternaria alternata* (HBL100), *Aspergillus candidus* (HBL101), *A. flavus* (HBL103), *A. niger* (HBL104), and *Eurotium amstelodami* (HBL105). Stock cultures are all held in the Cranfield Biotechnology Centre, Cranfield University. All fungi were isolated from maize grain.

2.2. Grain preparation and a_w modification

Spanish dent maize grain (initial moisture content 13.9%, 0.71 a_w) was irradiated at 12 kGy of gamma irradiation and stored aseptically at 4°C. The grain contained no fungal infection or contamination but had retained fertility. Irradiation has been demonstrated to result in less chemical and physical change than autoclaving (Frankland et al., 1990).

For all experiments, irradiated maize grain was weighed into sterile flasks and rehydrated to the desired a_w levels (0.995 and 0.95) by addition of sterile distilled water. The amount of water added was calculated from an 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 levels were confirmed by using a Humidat IC II Thermoconstanter (Novasina, Switzerland).

2.3. Inoculation, incubation and growth assessment

Rehydrated maize was placed in sterile 9 cm Petri dishes (ca. 20 g plate^{-1}) forming single layers of grain (Marin et al., 1995). A 4 mm agar disc was taken from sporing cultures of each species after approx. 48 h growth and either inoculated centrally (control treatments) or in dual cultures, 4.5 cm apart, on the surface of the single layers of maize. Treatments containing grain of the same a_w were placed in sealed closed containers containing beakers of glycerol/water mixtures of the same a_w as the plates to maintain the equilibrium relative humidity. Containers were incubated at 18 and 30°C. All treatments were repeated three times.

Every day during the incubation period of 14 days, growing colonies were measured with the aid of a binocular magnifier. Two diameters at right angles were obtained for each colony. Growth rates, expressed as mm day^{-1}, were calculated by linear regression of colony radius against time for each strain at each set of conditions tested (Marin et al., 1995). Interacting species were also observed macroscopically and the type of interactions occurring were assessed using the categories given by Magan and Lacey (1984). Subsequently, scores were given to each type of interaction and were added to obtain a so-called Index of Dominance (I_D). The scores were based on mutual intermingling (1/1), mutual inhibition on contact (2/2), mutual inhibition at a distance (3/3), dominance on contact (4/0), and dominance at a distance (5/0). In the last two cases the former score is for the dominating species, and the second for the dominated. These scores were then added for each species individually to obtain a total I_D under different steady state environmental conditions.

2.4. Ochratoxin extraction and analyses

Total ochratoxin concentration in 14 d samples was determined in duplicate using a commercial

ELISA kit (Ridascreen Ochratoxin A; R-Biopharm GmbH, Darmstadt, Germany). The kit has the following cross-reactivity with different ochratoxins: A (100%), B (44%), C (14%) and $\propto$($<0.1\%$). Extraction of samples was carried out as described in the kit. Samples were diluted where necessary to keep within the range of detection and quantification.

3. Results

3.1. Effect of interactions between Asp. ochraceus and other species on indices of dominance

Table 1 shows the effect of changes in both a_w and temperature on the interactions between *Asp. ochraceus* and other competing species on irradiated maize grain. Interaction types were affected by both physical factors. For example, at 0.95 a_w *Asp. flavus* was dominated by *Asp. ochraceus* at 18°C, but at 30°C *Asp. flavus* was dominant. *E. amstelodami* was dominant at both 18 and 30°C at this a_w level.

With freely available water (0.995 a_w) *Asp. ochraceus* was dominant against only *Asp. candidus* and *E. amstelodami* at 18°C, and at 30°C against *Alt. alternata*. Mutual inhibition occurred between *Asp. ochraceus* and *Asp. candidus*, and with *E. amstelodami* at 30°C at this a_w level.

Table 1
Effect of water activity (a_w) and temperature on numerical interaction scores and Index of Dominance (I_D) for *Aspergillus ochraceus* (Ao) when grown with other species on irradiated maize grain

a_w		18°C	30°C	I_D[a]
0.950	*Alternaria alternata*	4/0	4/0	
	Aspergillus candidus	4/0	4/0	
	Asp. flavus	4/0	0/4	
	Asp. niger	0/4	0/4	
	Eurotium amstelodami	0/4	0/4	
	I_D	12/8	8/12	20/20
0.995	*Alternaria alternata*	0/4	4/0	
	Aspergillus candidus	4/0	2/2	
	Asp. flavus	2/2	0/4	
	Asp. niger	0/4	0/4	
	E. amstelodami	4/0	2/2	
	I_D	10/10	8/12	18/22

[a] *Aspergillus ochraceus* score/other species score.

3.2. Effects of fungal interactions on growth of Asp. ochraceus

Fig. 1 shows that interactions with other species led only to non-significant changes in growth of *A. ochraceus* at 18°C, at both a_w levels. However, at 30°C the growth of *Asp. ochraceus* was significantly reduced by all fungi tested, particularly by *E. amstelodami*, *Asp. flavus* and *Asp. niger* at 0.95 a_w and by the latter two species at 0.995 a_w as well.

3.3. Effect of fungal interactions on ochratoxin production

The impact that competition for the maize grain substrate had on ochratoxin production at different a_w and temperature levels is shown in Fig. 2. In the absence of competition at 30°C, *Asp. ochraceus* produced significantly ($P = 0.05$) more ochratoxin at 0.95 than at 0.995 a_w. However, ochratoxin levels were significantly reduced by interactions with other species. Significant reductions in ochratoxin occurred with all species, but particularly when interacting with *Asp. candidus* at 18°C, at both a_w levels. At 30°C, *Asp. niger* and *E. amstelodami* reduced ochratoxin production by *Asp. ochraceus* at both a_w levels, and also by *Asp. flavus* at 0.95 a_w.

4. Discussion

This study is the first detailed examination of the type of interspecific interactions which can occur between *Asp. ochraceus* and other spoilage fungi in situ on maize grain. This has shown that some fungi significantly inhibit growth of *Asp. ochraceus* at optimum and high temperatures and a_w levels. These interactions often resulted in a significant reduction in ochratoxin content. This differed from results in in vitro studies on an agar medium based on maize meal, where *Alt. alternata* (at 18°C and 0.995 a_w), *Eurotium* species (at 0.95 and 0.90 a_w, and 25 or 30°C) and *Asp. candidus* (at 0.90 a_w and 30°C) significantly stimulated ochratoxin production (Lee and Magan, 1999b).

Two aspects of competition must be taken into account: primary resource capture and combat. Prolific production of spores, rapid germination, possession of appropriate extracellular hydrolytic enzymes and high growth rates are all prerequisites

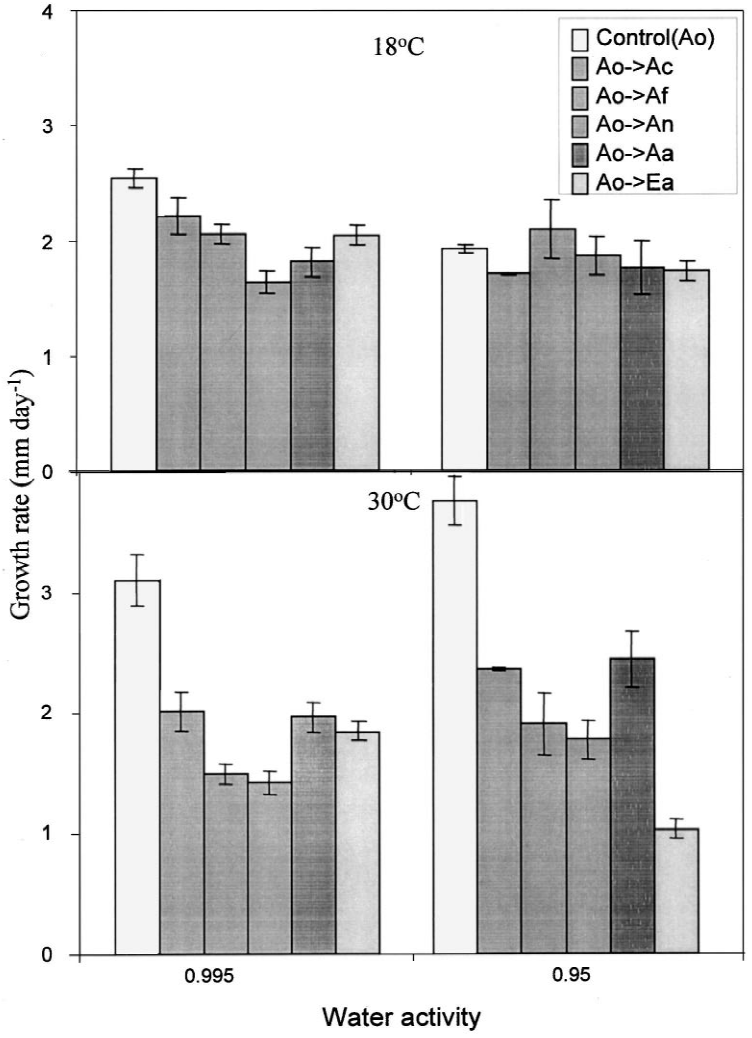

Fig. 1. Influence of five spoilage fungi on the growth rate (mm day^{-1}) of *A. ochraceus* at 0.995 and 0.95 a_w and 18 and 30°C on layers of irradiated maize grain. Bars indicate standard errors of the mean growth rates. Key to fungi: Ao, *Aspergillus ochraceus*; Aa, *Alternaria alternata*; Ac, *Aspergillus candidus*; Af, *Aspergillus flavus*; An, *Aspergillus niger*; Ea, *Eurotium amstelodami*.

for effective primary resource capture. I_D probably reflects more the potential for combat and dominance. However, in naturally contaminated grain the relative proportions of different species is variable and the outcome of interactions in such mixed populations are more complex, although previous studies suggest that the dominance may be in a state of flux, and particularly dependent on the prevailing environmental parameters (Marin et al., 1998a). Changes in resource quality over time affect interactions and dominance of the domain. Types of in situ interaction observed were similar to those obtained in vitro on an agar medium based on maize meal. The influence of a_w and temperature on dominance of different species in the maize grain niche correlates with the in vitro studies with these same species (Lee and Magan, 1999b). In recent studies, no correlations were found between the niche overlap index (NOI) and growth (Lee and Magan, 1999a). Previous studies by Marin et al. (1998b) showed some significant correlations between *Fusarium moniliforme*, *F. proliferatum* and *Asp. ochraceus* in

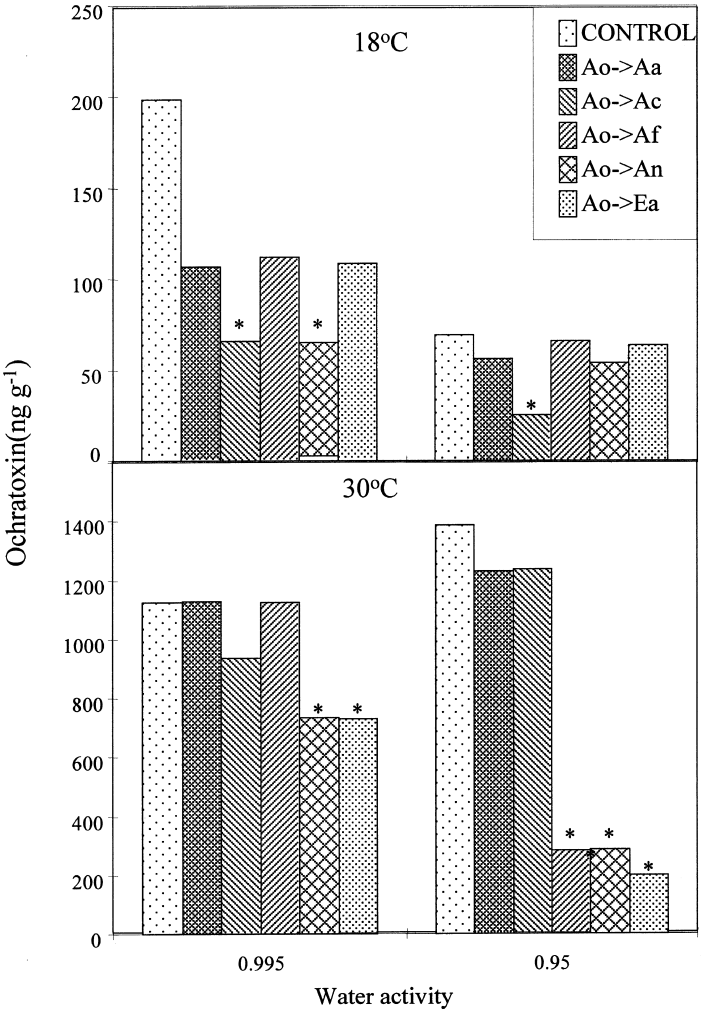

Fig. 2. Influence of five spoilage fungi on ochratoxin (ng g^{-1}grain) production by *Aspergillus ochraceus* at 0.995 and 0.95 a_w and 18 and 30°C on layers of irradiated maize grain. Asterisks indicate statistically significant differences ($P = 0.05$) from controls. Key to fungi: Ao, *Aspergillus ochraceus*; Aa, *Alternaria alternata*; Ac, *Aspergillus candidus*; Af, *Aspergillus flavus*; Ag, *Aspergillus niger*; Ea, *Eurotium amstelodami*.

relation to NOI, I_D and growth, and for counts of *F. proliferatum* and *A. ochraceus* against production of fumonisins.

This difference in in vitro and in situ results in relation to mycotoxin production suggests that *Asp. ochraceus* may not be as competitive on moist maize grain as *Fusarium* species (Marin et al., 1998a) or *Asp. flavus* (Wicklow et al., 1988). This could partially be due to differences in exploitative rather than exploratory mycelial growth. Marin et al. (1998c) previously demonstrated that a range of hydrolytic enzymes were rapidly released by *Fusarium* species colonising fresh maize grain, indicative of rapid exploitative growth and competitiveness. This is indicative of rapid primary resource capture of the available resource, perhaps preventing other species, such as *Asp. ochraceus*, from competing effectively, and then perhaps only in secondary resource capture (Cooke and Rayner, 1984). Marin et al. (1998b) demonstrated that, in the presence of certain species, e.g. *Asp. niger*, *F. moniliforme* and *F. proliferatum* were stimulated to produce a signifi-

cant increase in fumonisins on irradiated maize. It was suggested that increased production could play a role in niche exclusion of competing fungi and maintaining the occupation of colonised substrate by the fungus. Previously, Ramakrishana et al. (1996a,b) reported that, on barley grain, competition by *Asp. flavus*, *Penicillium verrucosum*, or *Hyphopichia burtonii* inhibited *F. sporotrichoides* and T-2 toxin production. Studies with *P. verrucosum* suggested that fungal counts or percentages of seed infection gave little indication of ochratoxin production in barley grain in dual cultures (Ramakrishana et al., 1996a,b).

A negative relationship between the presence of high concentrations of fumonisins and low amounts of aflatoxins in naturally contaminated maize grain in Thailand (Yoshizawa et al., 1996) suggested some interaction between these two species. However, as the history of these naturally contaminated samples was not known, comparisons cannot be made with the present work.

Acknowledgements

Dr. H.B. Lee is grateful to the Korea Research Foundation for a fellowship held in the Applied Mycology Group, Cranfield Biotechnology Centre, during which this work was carried out.

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