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Effect of water activity and temperature on growth and ochratoxin production by three strains of *Aspergillus ochraceus* on a barley extract medium and on barley grains

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Abstract

Aspergillus ochraceus Wilhelm is a widespread storage fungus that has been isolated from grains such as barley. The objective of this study was to determine the effects of water activity (a_w), temperature, time and their interactions on (a) growth on a barley extract agar medium, (b) growth on barley grains and (c) ochratoxin production on barley grains by three strains of *A. ochraceus*. For the three *A. ochraceus* isolates examined (NRRL 3174, 3.113 and 3.38), optimal a_w levels for growth on agar media were in the range 0.98–0.96, with temperature optima of 30°C for two of the isolates and 25–30°C for the other isolate. Growth was observed at 10 and 37°C, but only at higher a_w levels assayed. Two dimensional profiles were constructed for the range of a_w and temperature conditions that allow growth of the three isolates. Maximum growth on barley grains was reached at 30°C, at both a_w levels assayed (0.96 and 0.98), with fungal growth rates in the 4–5 mm day^{-1} range. Maximum amounts of ochratoxins were produced at the highest a_w treatment (0.98 a_w) and after a three-week incubation time at 25–30°C. The range of ochratoxin concentrations varied considerably, from 1.7 to 12,949 ppm, depending on the a_w and temperature interactions.

Keywords: *Aspergillus ochraceus*; Fungal growth; Ochratoxins; Water activity; Barley

1. Introduction

Fungal contamination is one of the main sources of deterioration of stored grains. Fungi are able to change the quality of grains, decreasing the germinability and significantly affecting their nutritional value (Airede and Esuruoso, 1987; Lacey et al., 1991). *Aspergillus ochraceus* Wilhelm is a widespread storage fungus that has been frequently isolated from grains, oilseeds and different vegetables (Gourama and Bullerman, 1988; Manabe and Tsuruta, 1991; Sanchis et al., 1988; Trojanowska, 1991). The natural incidence of *A. ochraceus* on Spanish barley has been reported (Sala, 1993).

Ochratoxins were originally discovered in cultures of *A. ochraceus* (Van der Merwe et al., 1965), although now it is known that *Penicillium ver-*

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rucosum and several *Aspergillus* species are able to synthesize these compounds on different grains, including barley (Kuiper-Goodman and Scott, 1989), and that they can be present in other foods and feeds (Krogh, 1987; Marquardt and Frohlich, 1992). Ochratoxin contamination of beer has been detected (Kuiper-Goodman and Scott, 1989) and *A. ochraceus* contamination of barley and conditions of mycotoxin production are of great interest to the brewing industry. Several studies have demonstrated that these mycotoxins are nephrotoxic, hepatotoxic, teratogenic, immunosuppressive and carcinogenic in different species, including farm livestock (Manning and Wyatt, 1984; Pohland et al., 1992).

Environmental conditions such as moisture, temperature, incubation time and substrate type (Häggblom, 1982; Lillehoj and Elling, 1983), as well as other factors such as the presence of competitive flora and the integrity of the seed (Marquardt and Frohlich, 1992) play an important role in the colonisation by *A. ochraceus* and the amount of ochratoxin produced. The most important factors influencing fungal development in stored grain ecosystems are the water availability (water activity, a_w), storage temperature and the intergranular gas composition (Magan and Lacey, 1988; Sinha, 1973, 1995).

Several studies have been carried out to determine *A. ochraceus* development on culture media (Ciegler, 1972; Garza et al., 1993; Gourama and Bullerman, 1988; Lisker et al., 1983; Madhyastha et al., 1993a; Paster and Chet, 1980; Paster et al., 1983, 1985) and on different grains, such as barley (Chelack et al., 1991; Damoglou et al., 1984; Häggblom, 1982), wheat (Madhyastha et al., 1990, 1993a,b), maize (Madhyastha et al., 1990) and oilseeds (Madhyastha et al., 1990). However, with the exception of a few reports (Damoglou et al., 1984; Häggblom, 1982; Northolt et al., 1979), little attention has been paid to the influence of incubation temperature and changes in a_w, either in media or in grains, on growth and ochratoxin production. Fungal growth has previously been mainly correlated with fungal counts (Chelack et al., 1991; Damoglou et al., 1984) or glucosamine concentration (Häggblom, 1982; Madhyastha et al., 1990, 1993a,b) on grains. Very few detailed comparisons have been made of the capacity for growth and ochratoxin production by isolates of *A. ochraceus* in vitro or in situ in barley grain.

The objective of this study was to determine the effects of a_w, temperature, time and their interactions on (a) the growth on a barley extract agar medium, (b) growth on barley grains and (c) ochratoxin production on barley grains by three strains of *A. ochraceus*.

2. Materials and methods

2.1. Organisms

One culture collection isolate (*A. ochraceus* NRRL 3174), and two isolates from Spanish grains (isolates 3.113 and 3.38) were used in this study. The isolates are deposited in the Food Technology Department Collection of the University of Lleida, Spain.

2.2. Media

Spanish barley (1995 harvest) with an initial moisture content of 11.22% (0.54 a_w) was used in this study. The basic medium used was a 3% (w/v) barley meal extract agar (BMEA) that was made by boiling 30 g of dry ground barley/1 l of water for 30 min. The resulting mixture was filtered through a double layer of muslin and the volume was made up to 1 l. The water activity (a_w) of this basal medium was 0.99. To determine the influence of a_w on mycelial growth on this medium, experiments were carried out on the basal medium (0.99 a_w), and with media to which glycerol had been added to 0.77, 0.81, 0.83, 0.90, 0.92, 0.94, 0.96, 0.98 a_w. Autoclaved media (20 ml) were poured into 9 cm diameter sterile plastic Petri dishes.

To determine growth patterns on barley grains, two different amounts of water (29.6 and 48.0 ml), calculated from a moisture absorption curve for the barley, were added to sub-samples of 100 g of grain in flasks. The barley was allowed to equilibrate at 4°C for 48 h, with periodic shaking. Once sealed, flasks were autoclaved for 20 min at 121°C and then single layers of grain were carefully placed in 9 cm sterile plastic Petri dishes in a flow bench. The final a_w levels of each treatment were 0.96 and 0.98, respectively.

Ochratoxin production on barley grain was investi-

gated by using sterile barley grain prepared as indicated above with a_w levels of 0.96 and 0.98.

All of the a_w determinations were carried out with a Novasina Humidat IC I Thermoconstanter (Novasina, Zurich, Switzerland).

2.3. Inoculation, incubation and measurement of growth

Actively growing 10–14 day-old colonies of the strains grown on potato dextrose agar (PDA) were used for the experiment of growth on BMEA. Agar plugs (5 mm diameter) taken from the growing margin of the colonies were aseptically placed in the centre of each treatment Petri plate. Petri plates of the same a_w were sealed in polyethylene bags. The incubation conditions were at the eight a_w levels detailed previously and at eight different temperatures (5, 10, 15, 20, 25, 30, 37 and 42°C). The Petri plates were examined daily or as necessary, and the diameter of the growing colonies was measured in two directions at right angles to each other. The increase in radial growth was determined and used to calculate the growth rate (mm day^{-1}) under each set of treatment conditions for each strain. All treatments were repeated three times.

The plates with a single layer of barley grains (ca. 25 g plate) were inoculated and measured in the same way as BMEA plates. The incubation conditions for growth and ochratoxin production on barley grains were two a_w levels (0.96 and 0.98) and three temperatures (15, 25 and 30°C). Petri dishes of the same treatment were stacked in sealed containers together with two beakers containing 100 ml glycerol/water solutions of the same a_w to maintain the equilibrium relative humidity in containers (Dallyn, 1978). All treatments were made in triplicate.

After one, two and three weeks, grains for ochratoxin determination were transferred to plastic bags and frozen (−20°C) until mycotoxin analyses were carried out.

2.4. Ochratoxin analyses

Total ochratoxin concentration in 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 α (<0.1%). Extraction of the samples was carried out as described in the kit.

2.5. Statistical treatment of results

In all cases, the linear regression of increase in radius against time was used to obtain growth rates on both BMEA and barley grains under each set of treatment conditions. Analysis of variance for the different sets of results were carried out using the SAS package (version 6.11, SAS Institute Inc.). Ridasoft version 2.0 (R-Biopharm GmbH) was used to calculate the ochratoxin concentration in the samples.

3. Results

Fig. 1 gives a diagrammatic representation of the interaction of a_w and temperature on the growth rate (mm day^{-1}) of assayed *A. ochraceus* strains on BMEA medium (see also Fig. 2). *A. ochraceus* NRRL 3174 had an a_w optimal for growth at 0.96 at 30°C, whereas the optimal for the other two isolates was at 0.98 at 30°C (3.113 strain) and 25°C (3.38 strain). These data were used to construct profiles of growth for the three isolates of *A. ochraceus* at different $a_w \times$ temperature levels. Water activity and temperature significantly influenced fungal growth of all three isolates, while there was no significant difference between the growth rates of isolates.

Fig. 3 shows the interaction between a_w and temperature on the growth rate of *A. ochraceus* on barley grains. It can be seen that maximum growth on barley grains was reached at 30°C, at both a_w levels assayed, with fungal growth rates in the 4–5 mm day^{-1} range. Growth rates of 5 mm day^{-1}, as obtained with NRRL 3174 and 3.113 strains growing on BMEA, were never reached, which could confirm a substrate influence on fungal growth. The analysis of variance of the effect of single (isolate, a_w and temperature), two- and three-way interactions were all statistically significant except for isolate $\times a_w$ effects.

Table 1 shows the mean ochratoxin concentrations obtained on barley grains inoculated with the three *A. ochraceus* isolates in relation to a_w, temperature and incubation time. The highest amounts of

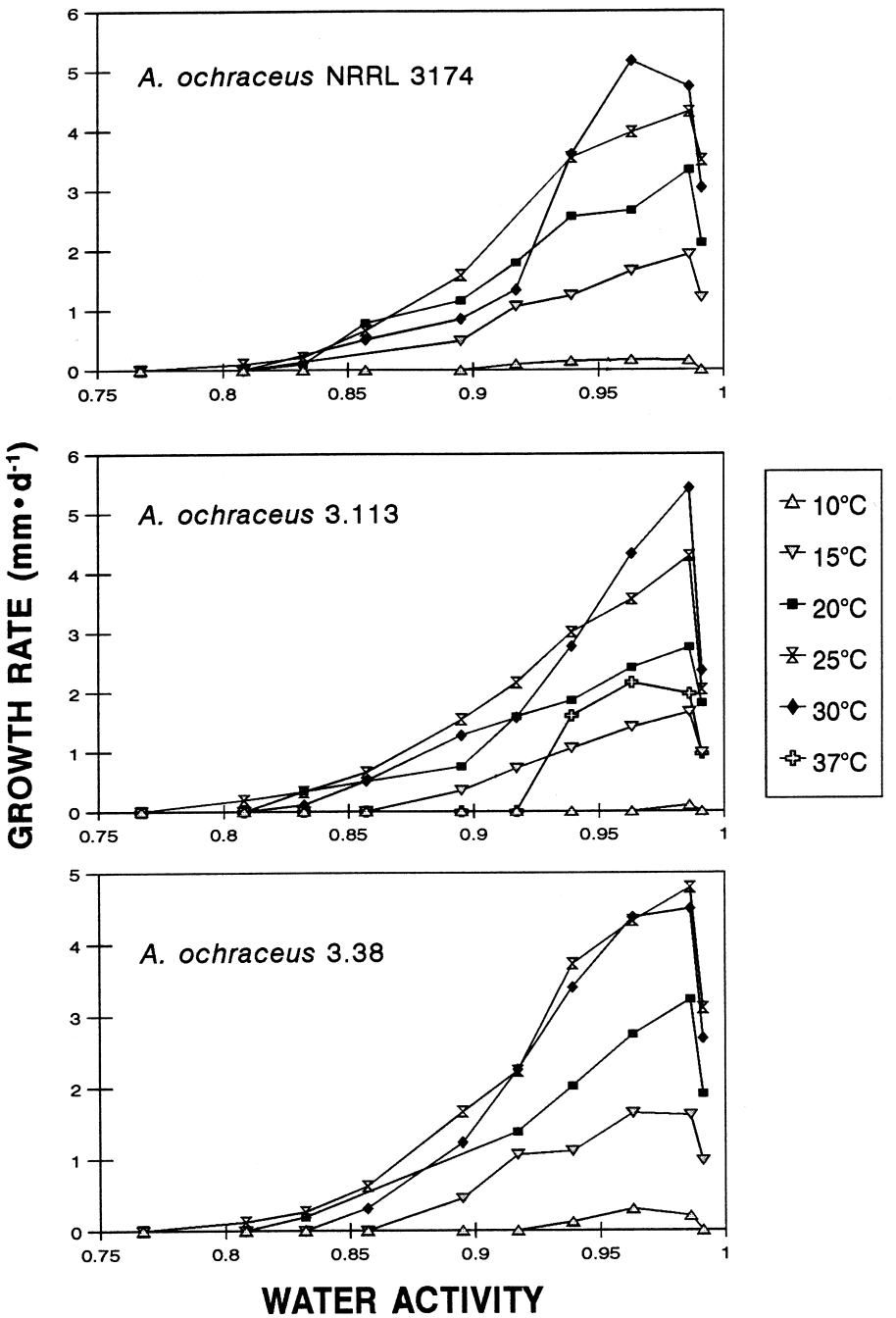

Fig. 1. Diagrammatic representation of interaction of water activity (a_w) and temperature on growth rate (mm day^{-1}) of assayed *A. ochraceus* strains on BMEA medium.

ochratoxins were always obtained at 0.98 a_w, regardless of the temperature considered, with maximum ochratoxin production at 25°C for the isolates 3.113 and 3.38 (12,949 and 840.5 ppm, respectively) and at 30°C for NRRL 3174 strain (8450 ppm). The only statistically significant factor was the isolate factor,

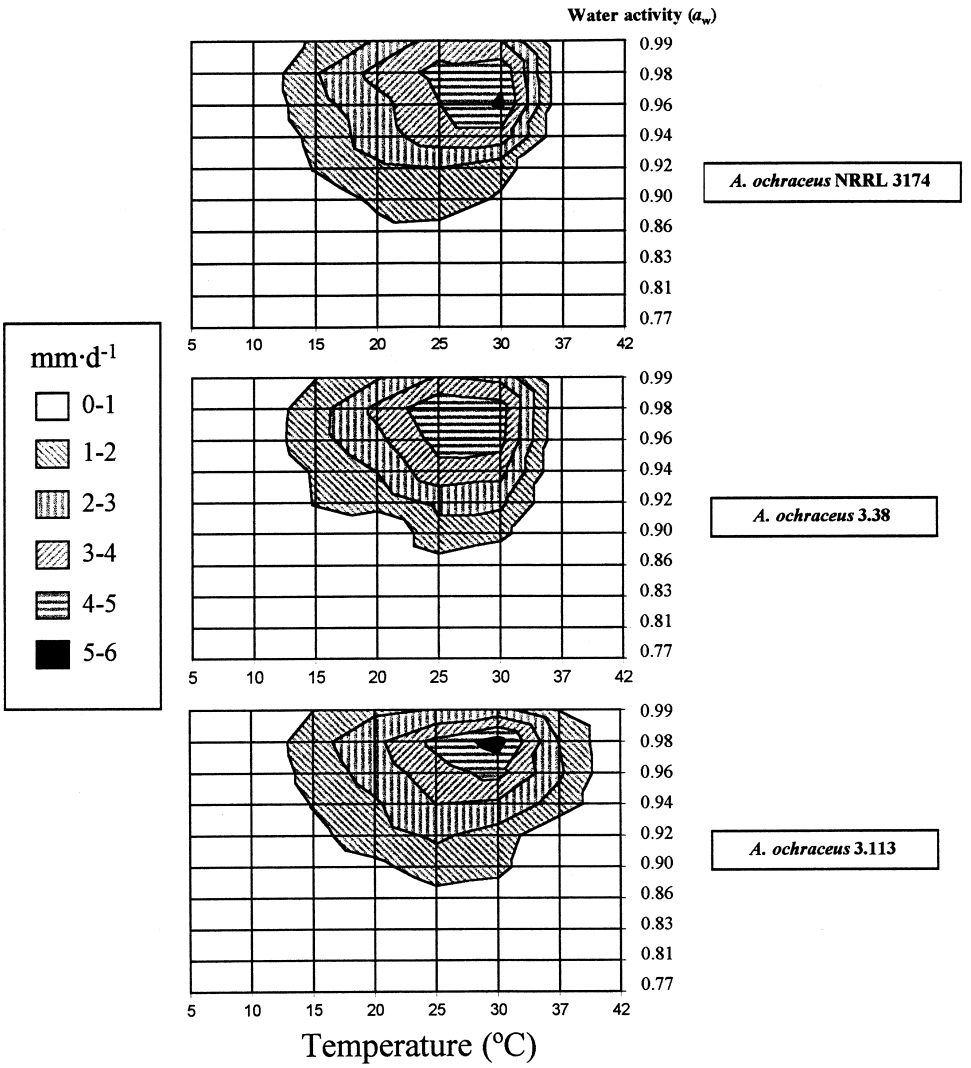

Fig. 2. Effect of water activity and temperature on growth rate (mm day^{-1}) of *A. ochraceus* isolates on barley meal extract agar (BMEA).

which influenced ochratoxin production on barley grains.

4. Discussion

This study has shown that, for the three *A. ochraceus* isolates examined in vitro on agar based media, optimal a_w levels for growth were in the range 0.98–0.96 with temperature optima of 30°C for two of the isolates (NRRL 3174, 3.113) and 25–30°C for the other isolate. No growth was observed at 5 and 42°C, regardless of the a_w level, but growth was observed at 10°C at the higher a_w levels assayed. Only the 3.113 isolate was able to growth at 37°C, with an optimum at 0.96 a_w. The a_w minima in the present study were 0.81 at 25°C, with none being able to grow at 0.77 a_w during the experimental period of our studies. Previous studies suggest that a_w minima for *A. ochraceus* vary from 0.76 to 0.88 depending on substrate (Christensen, 1962; Lopez and Christensen, 1967). Previous studies with *A. ochraceus* (NRRL 3174) have shown optimum temperatures for growth at 28–32°C on a defined medium (Paster and Chet, 1980). Unfortunately, this was only carried out in media with freely available

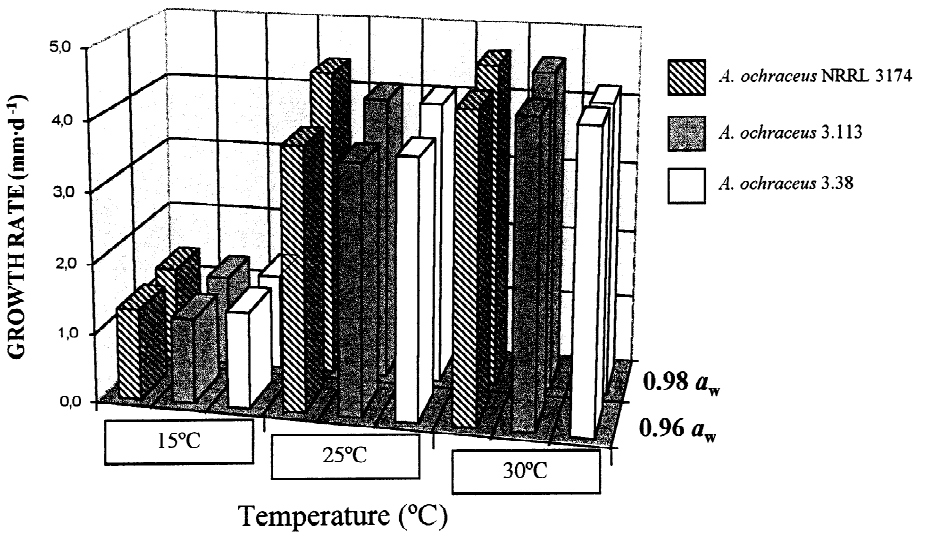

Fig. 3. Interaction of water activity (a_w) and temperature on growth rate (mm day^{-1}) of assayed *A. ochraceus* strains on barley grains.

Table 1
Mean ochratoxin concentrations (μg ochratoxin/g barley) on barley grains at two water activities (a_w) inoculated with three different strains of *A. ochraceus* at 15, 25 and 30°C after one, two and three-week incubation periods

A. ochraceus strain	Temperature (°C)	Ochratoxin (μg ochratoxin/g barley)					
		0.96 a_w			0.98 a_w		
		1-week	2-week	3-week	1-week	2-week	3-week
NRRL 3174	15	959.0	1419.0	1162.0	3072.0	8393.0	4268.0
	25	5907.0	5313.0	7360.0	5044.5	5889.0	1603.3
	30	947.0	2547.0	2295.3	6039.0	8365.0	8450.0
3.113	15	1100.5	1769.0	N.D.	1260.5	1361.0	740.0
	25	1987.5	889.5	1453.0	1427.5	920.0	12949.0
	30	2572.6	1697.5	1884.0	1501.5	1031.5	1171.7
3.38	15	9.4	3.00	2.7	1.7	2.7	2.5
	25	4.3	2.9	1.9	1.7	4.2	840.5
	30	2.5	4.9	12.3	2.2	2.7	798.5

N.D., not determined.

water (0.99 a_w), which makes direct comparisons difficult. Other studies have examined *A. ochraceus* growth but often in liquid media only, which are less relevant to potential activity on cereal-based substrates (Gourama and Bullerman, 1988).

It has been suggested that the nutrient source can affect the minimal a_w for growth (Wearing and Burgess, 1979), and that studies on artificial substrates may not accurately represent the real capacity to grow on a natural substrate. For this reason, a study was carried out on whole barley grain kernels. It was noticeable that, unlike growth on BMEA, statistically significant effects on growth were observed not only for a_w and temperature, but also between isolates. However, general growth patterns obtained on BMEA were similar to those obtained on barley grains, although growth rates on grains were always slightly lower.

Previous studies with *A. ochraceus* have also demonstrated the effect of either temperature or a_w on growth or biomass production, but interactions between these parameters were not investigated

(Damoglou et al., 1984; Häggblom, 1982; Häggblom and Ghosh, 1985; Madhyastha et al., 1990, 1993a,b). This makes direct comparisons with our work more difficult. In laboratory studies on ochratoxin production by *A. ochraceus*, Northolt et al. (1979) found that a minimum a_w of 0.83–0.87 was required for ochratoxin A production, with maximum toxin yields at 0.99 a_w. At optimum a_w, the temperature range for ochratoxin A production by *A. ochraceus* was 12 to 37°C. This study was carried out on malt or Czapek maize extract media modified with the solutes sucrose or glycerol to the desired a_w levels.

In the present study, the NRRL 3174 and 3.113 isolates produced significantly higher concentrations of total ochratoxin than the 3.38 strain. Maximum amounts were produced at the highest a_w treatment (0.98 a_w) and after a three-week incubation time at 25–30°C. These conditions were not optimal for growth of all three isolates. The range of ochratoxin concentrations varied considerably, from 1.7 to 12,949 ppm, depending on treatment a_w and temperature interactions.

Studies by Madhyastha et al. (1993a) found no direct relationship between fungal growth and ochratoxin production when *A. ochraceus* NRRL 3174 was grown on autoclaved barley with 24–25% water (ca. 0.94 a_w). They found ochratoxin A in the barley at a level of about 30 μg/g after 15 days at 28°C, which is lower than those obtained in the present study after 14 days at 25–30°C (5313–2547 μg ochratoxins/g) with a grain water content of 29% (ca. 0.96 a_w).

According to Damoglou et al. (1984), *A. ochraceus* IMI 132429 grew very slowly at 10°C on autoclaved barley at 0.85 a_w (20% moisture content) and did not produce ochratoxins. However, at 20°C, a maximum of 0.28 μg ochratoxin A/g barley was produced. Häggblom (1982) obtained higher levels of ochratoxin A using *A. ochraceus* 589.68 (17–48 μg ochratoxin A/g grain) and SLV (6–8 μg/g) strains grown at 25°C on autoclaved barley with a water concentration of 22% (ca. 0.91 a_w), although no ochratoxin production was detected at 10°C. Temperature was again a critical factor influencing ochratoxin production and had a pronounced influence on intra-strain production.

The influence of a_w, temperature and incubation time on growth and ochratoxin production of *A. ochraceus* on barley grains has been demostrated in this study, but more research is needed on the interaction of these factors with other ecophysiological parameters that could determine the colonisation patterns of this fungus in a specific niche. Recent work by Magan et al. (1997) demonstrated that complex interactions and niche overlap between *Fusarium* spp. and other spoilage fungi can occur, depending on the prevailing environmental factors. Special attention is needed on such interactions between *A. ochraceus* and other *Aspergillus* species, as has been described by Paster et al. (1992). The impact that such interactions may have on ochratoxin production should be investigated.

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