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Influence of physiological factors on growth, sporulation and ochratoxin A/B production of the new *Aspergillus ochraceus* grouping

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Abstract

Recently, new species within the *Aspergillus* section *Circumdati* group responsible for ochratoxin production were reported. This study has examined the impact of interactions between water activity (a_w,0.99-0.90), temperature (20-35°C) on growth, asexual spore production and ochratoxin A & B (OTA and OTB) on strains of each of the three species from this new grouping (*A. ochraceus*, *A. steynii*, and *A. westerdijkiae*) for the first time. The maximum growth occurred at 0.95 a_w and 30°C for both *A. ochraceus* and *A. westerdijkiae*, while it was at 0.99 a_w and 30°C for *A. steynii*. No conidial spore production occurred at 0.99 a_w in cultures of *A. ochraceus* and *A. steynii* but large numbers of spores (2.3×10^7 cm^2) were produced by *A. westerdijkiae.* Optimum temperature for spore production was 0.95 a_w and 30°C for *A. westerdijkiae* and *A. ochraceus*, and 0.95 a_w and 35°C for *A. steynii*. Quantification of OTA showed that optimum was produced at 0.99 a_w, by *A. steynii* at 30°C, for *A. westerdijkiae* at 25°C and for *A. ochraceus* at 20°C. As water stress was imposed (0.95 a_w), the temperature for maximum OTA production changed. For example, for *A. steynii* and *A. westerdijkiae* this was at 35°C, for *A. ochraceus*, 25°C. Much less OTB was produced relative to OTA, but the production followed the same pattern at all a_w levels and temperatures. This is the first detailed study to examine the similarities and differences in ecology of these related species in this important mycotoxigenic group.

1. Introduction

Frisvad *et al.* (2004) recently examined the *Aspergillus* section *Circumdati* and separated two new ochratoxigenic species, *A. westerdijkiae* and *A. steynii* from the more commonly reported *A. ochraceus* species responsible for contamination of a wide range of foodstuffs with ochratoxins. Indeed, since this separation was demonstrated there have been a number of reports of *A. westerdijkiae*, predominantly responsible for OTA contamination being isolated from a number of raw materials, especially coffee (Leong *et al.*, 2007, Morello *et al.*, 2007).

There have been many previous studies on the physiology of the species *A. ochraceus*. Ramos *et al.* (1998) examined effect of water activity (a_w) and temperature on two dimensional profiles for growth and OTA production on barley-based media and barley grain. The impact of environmental factors and presence of other fungal competitors on OTA production was shown by Lee and Magan (2000). More recently, Pardo *et al.* (2006) demonstrated the non-nutritional specificity of *A. ochraceus* strains isolated from coffee. It has now become apparent because of the study by Frisvad *et al.* (2004) that some of these strains examined may well have been *A. westerdijkiae* or *A. steynii*.

It has previously been shown that a_w and temperature are important criteria for understanding the ecology of spoilage fungi, especially mycotoxigenic species. A significant amount of information is now available on the growth and mycotoxin profiles for many of these species including ochratoxigenic ones (Sanchis and Magan, 2004). However, practically no information is available on the similarities and differences between the species in this group which can produce ochratoxins. Furthermore, sporulation profiles of these species has not

been studies although this could help in determining dominant contaminant species in food commodities. Sporulation has also been linked to biosynthetic pathways for mycotoxin production (Brodhagen & Keller, 2006). Thus, the objectives of this study were to examine representative strains of the three species, *A. westerdijkiae*, *A. steynii* and *A. ochraceus* and examine the effect of (a) interactions of water activity x temperature on growth, (b) on spore production and (c) quantify ochratoxin A and B production.

2. Material and Methods

Fungal strains

Strains from the new grouping of *A. ochraceus* (*A. ochraceus* IBT 11952, *A. steynii* IBT 22339, IBT 23096, and *A. westerdijkiae* IBT 10738, IBT 23971, IBT 21991) were used in these studies. For the latter two species representative strain data is presented. The strains were kindly supplied by Prof. J. Frisvad (Tech. Univ. of Denmark, Lynby, Denmark).

Media, inoculation, incubation and assessment of growth rate

Fungal strains were subcultured before examination on Malt Extract Agar [20.0 g Malt extract (Difco), 2.0 g Peptone (Difco), 15.0 g Agar (Sigma)] at 25 °C in the dark. The spores were gently dislodged from the colony surface into suspensions of 10 ml sterile distilled water containing 0.05 % Tween-20 in 25 ml Universal bottles. Fungal spore concentration was determined using a haemocytometer and adjusted to 10^6 spores ml^{-1}. This was used for inoculation.

All the strains were inoculated on Yeast Extract Sucrose (YES) medium (20.0 g l^{-1} Yeast extract, 150.0 g l^{-1} Sucrose, 15 g l^{-1} Agar) after adjusting a_w levels using glycerol to 0.99, 0.95 and 0.90 a_w. Glycerol/water solutions at these a_w levels were prepared and added to the

medium. Media were sterilized by autoclaving at 121 °C, for 20 min. Molten cooled media were poured into 9 cm Petri dishes. The same a_w treatments were kept in separate polyethylene bags and checked using a AQUALAB ® 3TE, USA for confirmation of actual levels.

The treatment Petri plates were inoculated with 5 µl of the spore suspension and incubated at 20, 25, 30, and 35°C. The experiments were carried out with at least three replicates pre treatment and some treatments repeated. Colony diameters of replicate plates were measured in two directions at right angles to each other. Measurements were recorded daily during growth until the Petri plates were completed colonised (Aldred *et al.*, 1999) and the growth rate was calculated by plotting the radial mycelium growth against time and the slope of the linear growth phase was used to obtain the radial growth rates (mm day^{-1}, Patriarca *et al*., 2001) .

Quantification of conidial production

The strains were inoculated on YES medium overlaid with cellophane (8.5 cm, P400, Cannings Ltd, Bristol, U.K.); this enabled the entire mycelial colony to be removed. The colony was suspended in 10 ml of sterile water containing a wetting agent (Tween 80, 0.1%) to wet the spores (Ramos *et al*., 1999). Spores were collected by filtering through sterile glass wool, and the filtrate was centrifuged to obtain a spore pellet. The number of spores was determined per cm of colony at the end of incubation period using a haemocytometer and a binocular microscope connected to a Video imaging unit (Parra *et al.*, 2004).

Determination of ochratoxin A and B

The treatments were harvested after 8 days. Plugs (3 mm diameter) were taken across the diameter of the colony using a sterile cork borer. The three plugs were placed into 2 ml

Eppendorf tubes and weighed. Ochratoxins were extracted by adding 1 ml HPLC grade methanol and shaken for 1 hour. The mycelial residue was discarded by centrifugation. The extracts were filtered directly into amber HPLC vials.

Samples were analyzed by HPLC using a 470 fluorescence detector (Millipore Waters, Corporation Masssachusetts USA) (λ_{exc} 333 nm, λ_{em} 460 nm) and a C_{18} column (Luna Spherisorb ODS2 150 x 4.6 mm, 5 µm) all under the control of Waters Millenium ^{32} software. The analysis was performed at a flow rate 1 ml min^{-1} with a mobile phase of 41 % Water: 57 % Acetonitrile: 2 % Acetic acid and a run time of 15 minutes. A stock solution of ochratoxin A and ochratoxin B standard (Sigma Aldrich, Supelco) were evaporated from benzene: acetic acid (9:1) and diluted with methanol to analyse the OTA and OTAB using standards (50, 100, 200, 400 and 800 ng; 10, 20, 40, 80 and 160 ng respectively. The linear regressions for OTA concentrations against peak height were plotted (OTA, R^2=0.985; OTB, R^2=0.993). The limit of detection for OTA was 5 ng $ml^{-1}$and for OTB was 0.6 ng ml^{-1}. The data is presented as log_{10} OTA and OTB in the results.

Statistical analysis

All experiments were carried out with 3-4 replicates and repeated. Statistical tests were performed using Statistica version 8 (StatSoft, Inc, 1984-2007) for three-way ANOVA and LSD Fisher was determined at the 95% confidence limits.

3. Results

Effects of a_w and temperature on growth.

Figure 1 compares the growth of all three strains of *A. ochraceus* group at different a_w levels and temperatures. This shows that at 0.95 a_w, maximum growth occurred at all temperatures

except at 20ºC for all strains except for *A. steynii* which was at 30ºC and 0.99 a_w. Overall, *A. ochraceus* 11952 grew faster than the other two species followed by *A. westerdijkiae* 10738 and *A. steynii* 22339 at all the temperatures and 0.95 and 0.90 a_w levels examined. Statistical analyses of the data sets showed that single and interacting factors for a_w, temperature treatments and their interactions were all significant (Table 1).

Effects of a_w and temperature on asexual sporulation of *A. ochraceus* strains.

Figure 2 shows the effect of the two abiotic factors on asexual sporulation by *A. ochraceus* group species. Overall, *A. westerdijkiae* 10738 produced higher amounts of spores than the other two species at all a_w levels and temperatures. In the absence of water stress, no conidial spore production occurred (0.99 a_w) at all temperatures by *A. ochraceus* 11952 and *A. steynii* 22339, while *A. westerdijkiae* 10738 produced spores at all temperatures except at 20 °C, with highest amount at 30 °C. However, as water stress was imposed there was significantly higher spore production especially at 0.95 a_w levels which was optimum for spore production for *A. westerdijkiae* and *A. ochraceus*, and at 0.90 a_w for *A. steynii*. *A. westerdijkiae* 10738 had markedly different sporulation capacities when compared to the other two strains examined. Table 2 shows the effect of single, two and three way interactions. Statistically significant differences were due to the single factors of temperature, a_w and their interactions with species.

Effects of a_w and temperature on ochratoxin A and B production of *A. ochraceus* group species

Figure 3 compares OTA production of *A. ochraceus* strains under different a_w and temperature regimes on YES medium. Overall, *A. steynii* 22339 produced higher amounts of OTA followed by *A. westerdijkiae* 10738 and *A. ochraceus* 11952 at all temperature and a_w

levels examined. At 0.99 a_w, *A. steynii* 22339 produced most OTA at 30°C, *A. westerdijkiae* 10738 at 25°C and *A. ochraceus* 11952 at 20°C. As water stress was imposed (0.95 a_w), maxima for OTA production were observed at different temperatures. For example, for *A. steynii* 22339 and *A. westerdijkiae* 10738 this was at 35°C, for *A. ochraceus* 11952 this was 25°C. Table 2 also shows that there were statistically significant differences based on species, temperature, a_w and their interactions (Table 3). OTB production followed the same pattern at all a_w levels and temperatures examined. However, significantly less OTB than OTA g^{-1} medium was produced, especially by the *A. ochraceus* strain (Figure 3b, Table 3b).

4. Discussion

This is the first study comparing the influence of a_w and temperature on growth, sporulation and ochratoxin A and B production by a new grouping of strains of the *A. ochraceus* group representing three species (*A. ochraceus, A. steynii* and *A. westerdijkiae*). Maximum growth occurred at 0.99-0.95 a_w and 30°C for all three *A. ochraceus* group strains were examined. *A. ochraceus* has been described as a fungus which grows between 8°C and 37°C, with an optimum at 24-30°C (ICMSF, 1996). Ramos *et al*. (1998) studied growth of three isolates of *A. ochraceus on* barley extract agar medium, optimal a_w for growth was found at 0.98-0.96 a_w with temperature optima of 30°C for two isolates and 25-30°C for the other isolate and the highest growth rate was at 0.95 a_w.

No work has been carried out on sporulation of the *A. ochraceus* group in relation to physiological factors. This is important as it may help understand which species may be present in a contaminated food matrix because of the relative differences in sporulation. We found that higher temperatures and water stress (0.95-0.90 a_w) were more suitable for asexual spore production especially at 30°C for *A. westerdijkiae* 10738 and *A. ochraceus* 11952 and

35°C for *A. steynii* 22339. The highest production of spores was registered by *A. westerdijkiae* 10738 and the lowest spore production by *A. ochraceus* 11952. Previous studies by Gervais *et al*. (1988, 2003) with *P. roquefortii* strains from cheese grew optimally at 0.97-0.98 a_w, while maximum spore production was at 0.96 a_w. Parra *et al*. (2004) showed that the highest amount of spores produced by a genetically engineered *A. niger* strain was at 0.95 a_w when this was modified with glycerol at 35°C, and by a wild-type strain of *A. niger* at 0.97 a_w and 35°C. Giorni *et al.* (2008) found that *A. flavus* produced 1.8 x 10^7 conidia cm^2 at 0.98 a_w and that this was reduced to practically no sporulation at 0.90 when an ionic solute was used (NaCl). In contrast, with a non-ionic solute (glycerol) sporulation still occurred at 0.85 a_w (4.7x10^5 conidia cm^2). They also showed that differences of 5°C and 0.005 a_w from optimal conditions for *A. flavus* (25°C; 0.995 a_w) can produce a 10-15% reduction in fungal extension, a significant reduction in AFB_1 production and sporulation (65-80% and 55% respectively). These differences could be explained by results reported by Brodhagen & Keller (2006) regarding the regulation of both sporulation and mycotoxin production in mycotoxigenic fungi by G protein signalling pathways. The relationship between mycotoxin production and sporulation were also found by Mostafa *et al.* (2005) who demonstrated that most of the toxins were produced after the fungus has completed its initial growth phase and began the development stage, represented by sporulation, and for *A. flavus*, sclerotial formation.

Little attention has been paid to the influence of incubation temperature, changes in a_w on ochratoxin A & B production by *A. ochraceus* strains. Maxima for OTA production were observed at high water stress (0.95-0.90 a_w) and high temperature 30-35°C for *A. steynii* 22339 and *A. westerdijkiae* 10738, 25-30°C for *A. ochraceus* 11952. Ramos *et al*. (1998) demonstrated that *A. ochraceus* strains produced the maximum amounts of OTA at the highest a_w treatment (0.98 a_w) and 25–30°C on barley extract malt agar and on barley grains.

Maximum OTA accumulation on synthetic grape juice medium by two *A. carbonarius* strains isolated from grapes was obtained at 0.96 a_w and 25°C (Mitchell *et al*., 2004, Belli *et al*., 2004). Optimal conditions for OTA production on coffee cherries were 25°C and 0.99 a_w using one strain isolated from green coffee (Joosten *et al*., 2001). This data should be useful to scientists who need to differentiate between these closely related mycotoxigenic species and to enable effective comparisons between isolated ochratoxingenic *Aspergillus* species in different food matrices.

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References

Abarca, M.L., Bragulat, M.R., Castellá, G., Cabañes, F.J., 1994. Ochratoxin A production by strains of *Aspergillus niger* var. *niger*. Applied and Environmental Microbiology. 60:2650-2652.

Aldred, D., Magan, N., Lane, B.S., 1999. Influence of water activity and nutrient on growth and production of squalestatin S_1 by a *Phoma sp*. Journal of Applied Microbiology. 87:842-848.

Bayman, P., Baker, J.L., Doster, M.A., Michailides, T.J., Mahoney, N.E., 2002. Ochratoxin production by the *Aspergillus ochraceus* group and *Aspergillus alliaceus*. Applied and Environmental Microbiology. 68:2326-2329.

Belli, N., Marin, S., Sanchis, V., Ramos, A.J., 2004. Influence of water activity and temperature on growth of isolates of *Aspergillus* section *Nigri* obtained from grapes. International Journal of Food Microbiology. 96:19-27.

Brodhagen M, Keller NP, 2006. Signalling pathways connecting mycotoxin production and sporulation. Molecular Plant Pathology **7**: 285-301.

Frisvad JC, Samson RA, Smedsgaard, J., 2004. *Emericella astellata*, a new producer of aflatoxin B_1, B_2 and sterigmatocystin. Letters in Applied Microbiology. 38:440-445.

Gervais, P., Molin, P., 2003. The role of water in solid-state fermentation. Biochemical Engineering Journal. 13:85-101.

Gervais, P., Molin, P., Bensoussan, M., 1988. Influence of water activity of a solid substrate on the growth rate and sporogenesis of filamentous fungi. Biotechnology and Bioengineering. 31: 457-463.

Giorni, P. Battilani, P., Magan, N., 2008. Effect of solute, matric potential and temperature on *in vitro* development of *Aspergillus flavus* strains from Italy**.** Fungal Ecology 1: 102-108.

International Commission on Microbiological Specification for Foods (ICMSF), 1996. Toxigenic fungi: *Aspergillus* in ICMSF, Microorganisms in foods. Characteristics of Food Pathogens, London, Blackie Academic and Professional. 347-81.

Joosten, H.M., Goetz, L.J., Pittet, A., Schellenberg, M., Bucheli, P., 2001. Production of ochratoxin A by *Aspergillus carbonarius* on coffee cherries. International Journal of Food Microbiology. 65:39-44.

Magan, N., Hope, R., Cairns, V., Aldred, D., 2003. Post-harvest fungal ecology: impact of fungal growth and mycotoxin accumulation in stored grain. European Journal of Plant Pathology. 109:723-730.

Mitchell, D., Parra, R., Aldred, D., Magan, N., 2004. Water and temperature relations of growth and ochratoxin a production by *Aspergillus carbonarius* strains from grapes in Europe and Israel. Journal of Applied Microbiology. 97:439-445.

Mostafa ME, Barakat A, El Shanawany AA, 2005. Relationship between aflatoxin synthesis and *Aspergillus flavus* development. African Journal of Mycology and Biotechnology 13: 35-51.

Parra, R., Aldred, D., Archer, D., Magan, N., 2004. Water activity, solute and temperature modify growth and spore production of wild type and genetically engineered *Aspergillus niger* strains. Enzyme and Microbial Technology. 35:232-237.

Patriarca, A., Vaamonde, G., Fernandez, V., Comerio, R., 2001. Influence of water activity and temperature on the growth of *Wallemia sebi*: application of a predictive model. International Journal of Food Microbiology. 68:61-67.

Ramos, A.J., Laberia, N., Marin, S., Magan, N., 1998. 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. International Journal of Food Microbiology. 44:133-140.

Ramos, A.J., Sanchis, V., Magan, N., 1999. Osmotic and matric potential effects on growth, sclerotia and partitioning of polyols and sugars in colonies and spores of *Aspergillus ochraceus*. Mycological Research. 103:141-147.

Sinha, R. N., 1995. The stored-grain ecosystem. In Jayas D. S., White, N. D. G., Muir, W. E. (Eds.) Stored Grain Ecosystems, New York, Marcell Dekker, pp. 1-32.

Stormer, F.C., 1992. Ochratoxin A—a mycotoxin of concern. In: Bhatnagar, D., Lillehoj, E.B., Arora, D.K. (Eds.), Handbook of Applied Mykology: Mycotoxins in Ecological Systems, vol. 5. Marcel Dekker, New York, Basel, Hong Kong, pp. 403–432.

Tsubouchi, H., Terada, H., Yamamoto, K., Hisada, K., Sakabe, Y., 1995. Caffeine degradation and increased ochratoxin production by toxigenic strains of *Aspergillus ochraceus* isolated from green coffee beans. Mycopathologia. 90: 181-186.

Table 1 Analysis of variance of the effect of $a_{w,}$ temperature, strains and their interaction on growth rate of *A. ochraceus* strains (*A. steynii*, *A. westerdijkae*, *A. ochraceus*).

	DF	MS	F	P
Factor				
Temperature	3	19.8876	5059.4	0.00
a_w	2	21.7285	5527.8	0.00
Strain	2	6.5418	1664.2	0.00
Interaction factors				
Temperature x a_w	6	2.2493	572.2	0.00
Temperature x Strain	6	1.4970	380.8	0.00
a_w x Strain	4	2.4640	626.8	0.00
Temperature x a_w x Strain	12	0.4436	112.9	0.00
Error	72	0.0039		

DF: Degree of freedom, MS: mean square, P: Probability

Table 2 Analysis of variance of the effect of $a_{w,}$ temperature, strains and their interaction on spore production of *A. ochraceus* strains (*A. steynii*, *A. westerdijkae*, *A. ochraceus*).

Effect	**DF**	**MS**	**F**	**P**
Factor				
Temperature	3	587.869	5679.34	0.00
a_w	2	349.918	3380.52	0.00
Strain	2	371.819	3592.10	0.00
Interaction factors				
Temperature x a_w	6	53.475	516.62	0.00
Temperature x Strain	6	119.425	1153.75	0.00
a_w x Strain	4	272.594	2633.51	0.00
Temperature x a_w x Strain	12	96.268	930.04	0.00
Error	72	0.104		

DF: Degree of freedom, MS: mean square, P: Probability

Table 3 Analysis of variance of the effect of $a_{w,}$ temperature, strains and their interaction on (a) ochratoxin A and (b) ochratoxin B production by *A. ochraceus* strains (*A. steynii*, *A. westerdijkae*, *A. ochraceus*).

(a)

	DF	MS	F	P
Factor				
Temperature	3	108.844	368.25	0.000000
a_w	2	66.608	225.35	0.000000
Strain	2	241.112	815.75	0.000000
Interaction factors				
Temperature x a_w	6	24.262	82.09	0.000000
Temperature x Strain	6	11.014	37.26	0.000000
a_w x Strain	4	7.022	23.76	0.000000
Temperature x a_w x Strain	12	1.187	4.01	0.000094
Error	72	0.296		

(b)

	DF	MS	F	P
Factor				
Temperature	3	96.501	251.404	0.000000
a_w	2	70.078	182.566	0.000000
Strain	2	295.264	769.220	0.000000
Interaction factors				
Temperature x a_w	6	8.064	21.008	0.000000
Temperature x Strain	6	14.051	36.605	0.000000
a_w x Strain	4	7.600	19.799	0.000000
Temperature x a_w x Strain	12	1.102	2.870	0.002785
Error	72	0.384		

DF: Degree of freedom, MS: mean square, P: Probability

Figure legends

Figure 1 Comparison of growth rate of *A. ochraceus* strains in relation to water activity (a_w) and temperature on a YES medium. Vertical bar indicates 95% confidence limits for all three strains.

Figure 2 Effect of water activity (a_w) and temperature on asexual conidial spore production by *A. ochraceus* strains in relation to water activity (a_w) and temperature on YES medium. Vertical bar indicates the 95% confidence limits.

Figure 3 Comparison of effects of water activity (a_w) and temperature on (a) ochratoxin A (log_{10}) and (b) ochratoxin B (log_{10}) by strains of the *A.ochraceus* on a YES medium. The 95% confidence limits were: OTA, at 0.99, 0.95 and 0.90 a_w: 0.5, 0.43, 0.63, OTA B: 0.54, 0.5, 0.55 respectively.

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