

Evaluation of growth and ochratoxin A production by Aspergillus steynii and Aspergillus westerdijkiae in green-coffee based medium under different environmental conditions

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Abstract

Aspergillus steynii and *A. westerdijkiae* are important ochratoxin A (OTA)-producing species frequently found in coffee. Although the processing of green coffee beans reduce markedly OTA contamination, levels exceeding the legal limits might remain in the final product. Environmental conditions are a crucial factor affecting growth and OTA production in fungal species; therefore, in this work, we analyzed the effect of different levels of temperature (23, 28, 32, 37, 42 °C) and water activity (a_w) (0.89, 0.91, 0.93, 0.95, 0.97, 0.99) on growth and toxin production by *A. steynii* and *A. westerdijkiae* in green coffee-based medium. *A. steynii* was able to grow and produce OTA in a wider set of conditions than *A. westerdijkiae*. A new index (OTA risk index) has been described to integrate both fungal growth and OTA production and, according to it *A. steynii* would pose a higher risk of OTA contamination in coffee than *A. westerdijkiae* at all the conditions tested.

Neither *A. steynii* nor *A. westerdijkiae* were able to grow at the lowest value of a_w (0.89) and OTA production was extremely low at 0.91. Therefore, the application of good practices during storage aimed to maintain low humidity levels might be essential to prevent OTA contamination in coffee. The optimal conditions of both species to grow and produce OTA were established at warm temperatures (28-32 °C) and high a_w levels. Therefore, these species could be considered well-adapted in predicted climate change scenarios resulting in a potential high risk source of OTA contamination for this product.

Keywords: *Aspergillus steynii*, *Aspergillus westerdijkiae*, ochratoxin A, green coffee, environmental conditions

1. Introduction

Ochratoxin A (OTA) is a widespread mycotoxin produced by several *Aspergillus* and *Penicillium* species. It presents a broad range of toxic properties towards animals and humans including its classification as a possible human carcinogen (group 2B) by the International Agency for Research on Cancer (IARC, 1993 and Pfohl-Leszkowicz and Manderville, 2007).

The occurrence of OTA in green coffee beans has been extensively reported since its first description in 1974 (Levi et al., 1974) as well as its presence in roasted coffee since a few years after (Tsubouchi et al., 1988). The origin of green coffee samples is not relevant regarding OTA occurrence since the toxin has been reported in different continents. However, African and Asian coffee seems to be the most contaminated (Romani et al., 2000 and Taniwaki, 2006).

The processing of this product usually involves treatments at high temperatures necessary to roast the coffee resulting in a drastic, although not complete, decrease in OTA content (Romani et al., 2003, Suarez-Quiroz et al., 2005 and Oliveira et al., 2013). However, the temperature applied should be carefully controlled since it can also affect the organoleptic properties of the product and can cause the occurrence of several toxic degradation metabolites of OTA (Cramer et al., 2008 and Ferraz et al., 2010). These might be the reasons why the occurrence of OTA in processed retailed coffee is frequently reported worldwide both in ground or instant coffee (Taniwaki, 2006).

In the last few decades, several strategies were undertaken to improve agricultural and processing practices leading to a decrease on the incidence of OTA in coffee within the European Market (Jørgensen, 2005). Additionally, more restrictive legislation by the

European Union was approved regarding the levels of this toxin in roasted beans, roasted ground coffee and instant coffee (European Commission, 2006). In spite of this tendency of OTA reduction in coffee, the number of alerts distributed among the EU countries regarding OTA in coffee has risen dramatically during the three first terms of 2013 with respect to former years (RASFF, 2013).

Since the first report on the occurrence of OTA in coffee, *Aspergillus ochraceus* was considered the main source of the toxin in this product (Batista et al., 2003, Taniwaki et al., 2003 and Urbano et al., 2001). However, the taxonomy of the *A. ochraceus* group has been revised and new important species capable of producing OTA were described such as *A. steynii* and *A. westerdijkiae* (Frisvad et al., 2004). In our group, it has been demonstrated that these recently described species are far more important OTA producers than *A. ochraceus* because they are able to produce higher levels of the toxin and their relative number of OTA-producing isolates is also higher (Gil-Serna et al., 2011). Both *A. steynii* and *A. westerdijkiae* have been reported to occur in green coffee beans (Leong et al., 2007 and Noonim et al., 2008). Moreover, Leong et al. (2007) hypothesized that the ochratoxigenic isolates of *A. ochraceus* reported in coffee before the description of the new species are likely to belong to *A. steynii* or *A. westerdijkiae*. Indeed, Morello et al. (2007) found that more than 80% of *A. ochraceus* isolated from Brazilian coffee were actually *A. westerdijkiae* strains using molecular methods.

Several authors have studied the ability to produce OTA by *A. ochraceus* in coffee at different environmental conditions (Mantle and Chow, 2000 and Pardo et al., 2005). However, since the description of *A. westerdijkiae* and *A. steynii* there are no works available in relation to the effect of ecophysiological factors on growth and OTA production by these species in this product. In this context, Pardo et al. (2005) showed that fungal growth and production patterns observed in culture medium prepared from

infusions of green coffee beans are a reasonable approximation to that obtained in natural substrates.

Therefore, the aim of this work was to study the effect of water activity (a_w) and temperature on growth and OTA production by *A. steynii* and *A. westerdijkiae* in green coffee-based medium.

2. Materials and Methods

2.1. Fungal strains

Two strains of each species tested, *A. westerdijkiae* and *A. steynii* were used in this work. *A. steynii* 3.53 and Aso2 strains were isolated from coffee and grapes respectively, whereas *A. westerdijkiae* AO-PD16-1 strain, kindly supplied by Dr Ramos (University of Lleida, Spain), was isolated from paprika and CECT 2948 strain was obtained from the Spanish Type Culture Collection. All strains were selected regarding their high ability to produce OTA in CYA medium (Gil-Serna et al., 2011) and their correct identification was confirmed using species-specific PCR assays according to Gil-Serna et al. (2009). They were maintained by regular subculturing on Potato Dextrose Agar (PDA, Pronadisa, Spain) at 25±1°C for 4-5 days and then stored at 4 °C until required and as spore suspension in 15% glycerol at –80°C.

2.2. Medium preparation

The assays were performed in green coffee-based medium. The medium contained 3% (w/v) of green coffee beans with 20 g/l of bacteriological agar (Pronadisa, Spain). It was prepared by boiling 30 g of grounded coffee beans in 1 l of distilled water for 30 min. Subsequently, the mixture was filtered through a double layer of muslin and the volume

was adjusted up to 1 l. Water activity was modified with glycerol, a non-ionic solute, up to 0.89, 0.91, 0.93, 0.95, 0.97 and 0.99 (Dallyn and Fox, 1980).

2.3. Inoculation, incubation and measurement of growth

Fungal conidia suspensions were prepared from sporulating cultures (7 day-old) on Czapek-Dox Modified Agar (Pronadisa, Spain) and filtered through Whatman N° 1 paper. Concentrations were measured by microscopy using a Thoma counting chamber and the suspensions were diluted up to a final concentration of 10^7 spores/ml. Two microliters of these suspensions were placed in the centre of the plates prepared as described above and they were incubated at 23, 28, 32, 37 and 42 °C. Each strain was inoculated in two independent plates in each a_w and temperature condition.

The diameter of the colonies was measured daily during 10 days in two directions at right angles. Fungal colony diameters were plotted against time and a linear regression was applied to obtain the slope of the straight line which represented the growth rate.

2.4. OTA evaluation

OTA was extracted from the plates after 10 days of incubation as described elsewhere (Bragulat et al., 2001). Three agar plugs were removed from different points of the colony and extracted with 1 ml of methanol. OTA was measured in the extracts by High Performance Liquid Chromatography (HPLC) on a reverse phase C18 column (Tracer Extrasil ODS2; 5 μ m, 4.6 mm x 250 mm; Teknokroma, Spain) at 45 °C in a Perkin Elmer Series 200 HPLC system coupled with a fluorescence detector (Perkin Elmer, Massachusetts, USA) at excitation and emission wavelengths of 330 and 470 nm respectively. The mobile phase contained monopotassium phosphate 4 mM pH 2.5 and methanol (33:67) and the flow rate was 1 ml/min. OTA was eluted and quantified by

comparison with a calibration curve generated from OTA standards (OEKANAL[®], Sigma-Aldrich, Germany).

2.5. OTA risk index calculation (ORI)

A new index, OTA risk index (ORI), has been described in this work to relate fungal growth and OTA production in agar plates. The ORI relates fungal colony diameter at the end of the experiment (10 days of incubation) with OTA production ability in each condition. The ORI is calculated from the formulae below:

$$\text{OTA risk index (ORI)} = \log[\text{OTA concentration (ng/mm}^2\text{)} \times \text{Fungal colony surface (mm}^2\text{)} + 1]$$

This index is always represented by a number above zero and indicates the predicted risk of OTA contamination by a change on the environmental conditions. The higher the ORI value, the higher the risk of OTA contamination.

2.6. Statistical analysis

Statistical treatment of the data obtained was performed using SPSS 19 software (IBM, USA). None of the variables studied showed a normal distribution; therefore, the non-parametric Kruskal-Wallis test was used and post-hoc analyses were performed with corresponding U-Mann Whitney tests. Correlation among fungal growth and OTA production was studied by analysing Pearson correlation index. In all cases, statistical significance was established at $p \leq 0.05$.

3. Results

The U-Mann Whitney test performed did not reveal significant differences between the two strains analyzed for each species in any of the experiments considered; therefore, in all cases, the results for each species and condition are represented as the average of four values corresponding to the two replicates per strain.

3.1. Effect of a_w and temperature on fungal growth

The results of fungal growth rate of *A. steynii* and *A. westerdijkiae* in green coffee-based medium at all conditions of temperature and a_w tested are shown in figure 1. The statistical analysis performed regarding growth rate is displayed as well in figure 1.

Both species showed as a whole statistically similar growth rate values in coffee-based media, although it is important to note that *A. steynii* was able to grow in a wider set of conditions than *A. westerdijkiae*. On the other hand, both species showed some differences when the post-hoc analyses were carried out to dissect the interactions between temperature and a_w . Growth rate was significantly modified by temperature in both species. None of them were able to grow at the highest temperature tested (42 °C) and their growth rate was significantly reduced at 37 °C. However, the effect of this parameter was different at the lowest values of temperature tested. *A. westerdijkiae* growth rate was similar in a range of temperature of 23 to 32 °C whereas *A. steynii* seemed to increase its growth rate at intermediate values (28 and 32 °C). On the other hand, the effect of temperature appeared to be strongly influenced by a_w in *A. steynii* and *A. westerdijkiae*, suggesting a clear interaction between a_w and temperature.

The other factor analysed, a_w , also showed a significant effect on fungal growth in both species. Neither *A. westerdijkiae* nor *A. steynii* were able to grow at the lowest a_w level tested (0.89). Growth rate was drastically reduced in both species at lower values (0.91

and 0.93) reaching the optimum at $a_w=0.97$. The highest a_w (0.99) produced different effect on both species: whereas *A. westerdijkiae* showed similar growth rate at a_w 0.99 and 0.97, growth rate was significantly reduced at a_w 0.99 in the case of *A. steynii*.

3.2. Effect of a_w and temperature on OTA production

The results of OTA production by *A. steynii* and *A. westerdijkiae* in green coffee-based medium at all conditions and their corresponding statistical analysis are shown in table 1.

Statistically significant differences between the two species were found for OTA production. *A. steynii* was capable of producing higher levels of OTA than *A. westerdijkiae* in all levels of temperature and a_w tested. Moreover, *A. steynii* produced OTA in a wider range of conditions.

Temperature significantly affected OTA production by both species and either *A. steynii* or *A. westerdijkiae* ability to produce the toxin was reduced at 37 °C. However, these species showed marked differences at other temperature values. OTA production by *A. westerdijkiae* was practically restricted to 28-32°C within a range of a_w values between 0.93-0.99, except at 23°C and 0.99 a_w . In contrast, *A. steynii* was able to produce OTA at all temperatures tested from 23 to 32 °C and a_w values, although production at 0.91 was extremely low. However, strong effect of some combinations of temperature and a_w on OTA production by *A. steynii* were observed. Marginal temperatures (23 °C and 37 °C) in combination with low a_w (0.93) as well as 37 °C and 0.99 produced a strong negative effect on OTA production. The rest of the conditions (0.95-0.99 at 23-37°C) seemed to be highly favourable for OTA production in *A. steynii*.

3.3. Effect of a_w and temperature on OTA risk index

The values of OTA risk index (ORI) obtained for each species in all conditions tested are shown in figure 2. The highest ORI were shown by both species at the highest level of a_w tested (0.99) and 28 °C although the index in *A. steynii* and *A. westerdijkiae* showed qualitative and quantitative differences. In all conditions tested, *A. steynii* showed a higher ORI than *A. westerdijkiae* in green-coffee based medium. Moreover, the presence of *A. steynii* would suppose a risk of OTA contamination in more conditions of a_w and temperature than *A. westerdijkiae*.

It is important to note that *A. steynii* showed a high ORI even at 37 °C at some values of a_w . On the other hand, the ORI values of this species at $a_w=0.95$ were similar regardless temperature.

None of the species would suppose a risk at a_w levels below 0.91 at any of the temperatures tested.

4. Discussion

A. westerdijkiae and *A. steynii* are currently considered important OTA-producing species in green coffee beans (Leong et al., 2007, Morello et al., 2007 and Noonim et al., 2008). However, due to their recent description, there are no studies available which describe their ability to grow and produce OTA under different environmental conditions in this product. In this work, we tested several temperatures and a_w levels and the marginal and optimal conditions to grow and produce OTA were determined in both species.

The comparison of the results of growth rate and OTA production revealed that fungal growth cannot be considered a good indicator of OTA production by any of the two species in green coffee. Indeed, although growth rate patterns were similar in both species, with some slight differences, their patterns of OTA production showed marked

differences, both qualitative and quantitative, in relation with environmental factors. These results agree with those obtained by other authors using this matrix and in other products which indicated that OTA production by *Aspergillus* section *Circumdati* species do not occur in all the conditions at which the fungus is able to grow (Mühlencoert et al., 2004 and Pardo et al., 2006). In that context, the OTA risk index (ORI) described in this work integrating both growth and OTA production might improve the prediction of OTA contamination in coffee at different environmental conditions. The presence of OTA in this product is bound to increase together with a rise of a_w and at warm temperatures (28-32°C); therefore, good practices which control the levels of these parameters might be essential to prevent OTA contamination during different steps of coffee production maintaining low humidity and temperature levels. The application of appropriate measures during harvesting and storage are necessary to prevent OTA contamination of processed coffee (Bucheli and Taniwaki, 2002) as well as controlled environmental levels during transportation (Palacios-Cabrera et al., 2007). Regarding this problem, the ORI is a parameter very simple to calculate and could be of importance to evaluate the effectiveness of prevention methods applied. The identification of the fungus that is responsible for OTA production in green coffee beans is critical to take preventive measures to reduce contamination in the final product. The risk of OTA contamination posed by the presence of *A. steynii* in green coffee is exceedingly important since, as it was mentioned above, it is able to growth and to produce OTA at a wide range of environmental conditions, considered quite extreme for many fungal species, such as high temperatures and low a_w levels (37 °C and 0.95). The risk would increase at high a_w levels and intermediate temperatures and the maximum ORI value was found at 28 °C and a_w =0.99. On the other hand, the risk of OTA contamination when *A. westerdijkiae* is present in green coffee is much lower in

all the environmental conditions tested. This suggests that the identification of the fungus at species level is extremely relevant to predict OTA risk and to devise control strategies. Fortunately, there are fast, sensible and easy to perform analyses based on PCR which are able to discriminate between these two species and other *Aspergillus* species (Gil-Serna et al., 2009).

The results obtained in this work might also help to understand the response of these species towards climate change effects. The Intergovernmental Panel on Climate Change (IPCC) predicts a rise in the mean global temperature in tropical areas up to 5.8 °C by the end of the century as well as a very likely increase in rainfall (IPCC, 2007). Therefore, in those regions where traditionally coffee has been cultured average temperature would be expected to rise up to around 28 °C. This fact, in combination with the high environmental humidity of these regions, might contribute to an increasing potential OTA contamination in coffee contaminated by *A. westerdijkiae* and, particularly, *A. steynii*.

5. Conclusions

A. steynii and *A. westerdijkiae* are able to grow and produce OTA in green coffee-based medium. *A. steynii* is able to produce OTA in a wide set of environmental conditions and would pose a higher risk of OTA contamination in this product. The application of good practices during storage to maintain low levels of temperature and humidity might be essential to prevent OTA contamination of green coffee beans.

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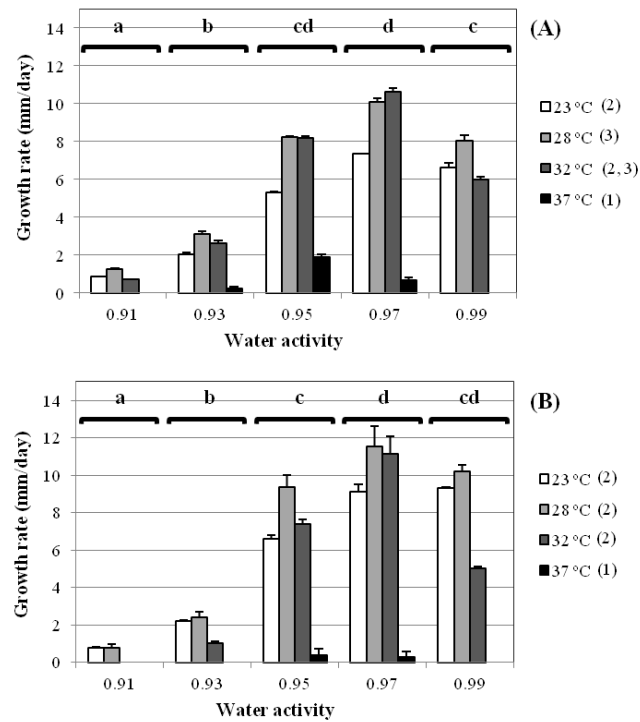


Figure 1. Effect of temperature and a_w on growth rate of *A. steynii* (A) and *A. westerdijkiae* (B). The data are represented as the average of the four values obtained for each species (two strains with two replicates each) and the corresponding standard error, indicated by the thin vertical line are the standard error. Different small letters indicate significant differences among a_w treatments, whereas different numbers in the side legend indicate significant differences among the temperature treatments tested

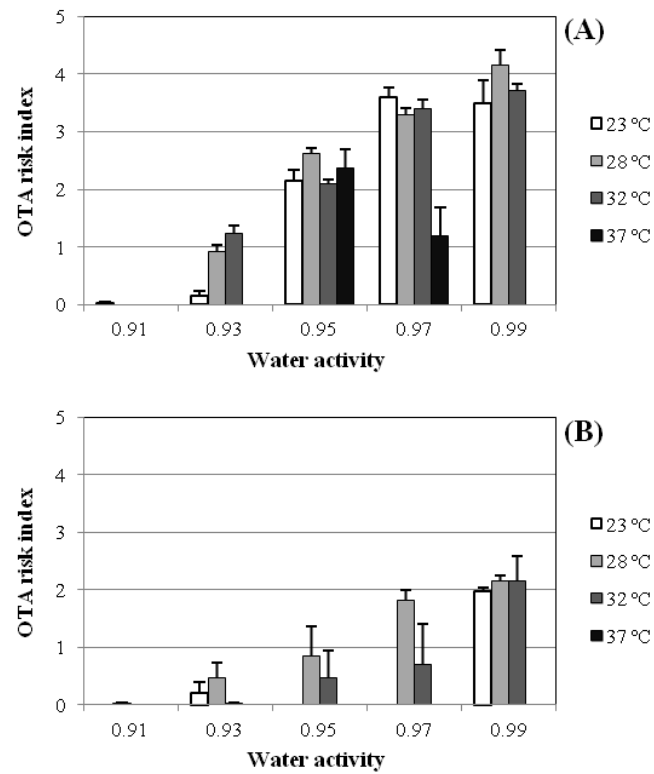


Figure 2. Effect of temperature and a_w on OTA risk index (ORI) of *A. steynii* (A) and *A. westerdijikiae* (B). The data for both species are represented as the average of four values corresponding to the two strains and two replicates per strain, and the standard errors, indicated as vertical thin lines

<i>A. steynii</i>		Temperature			
		23 °C ⁽²⁾	28 °C ⁽²⁾	32 °C ⁽²⁾	37 °C ⁽¹⁾
Water activity	0.91 ^(a)	0.89±0.33	0.00±0.00	0.00±0.00	0.00±0.00
	0.93 ^(b)	0.92±0.48	7.85±2.65	23.20±6.04	0.00±0.00
	0.95 ^(c)	56.15±21.30	56.73±8.83	15.90±2.15	515.28±208.36
	0.97 ^(d)	776.97±291.62	227.92±71.13	315.77±126.08	311.62±170.05
	0.99 ^(d)	1374.27±714.83	2095.31±808.30	1223.68±239.48	0.00±0.00

<i>A. westerdijkiae</i>		Temperature			
		23 °C ⁽²⁾	28 °C ⁽³⁾	32 °C ^(2,3)	37 °C ⁽¹⁾
Water activity	0.91 ^(a)	0.00±0.00	0.89±0.39	0.00±0.00	0.00±0.00
	0.93 ^(a)	0.00±0.00	6.08±3.51	0.31±0.31	0.00±0.00
	0.95 ^(a)	0.00±0.00	3.70±2.80	2.93±2.93	0.00±0.00
	0.97 ^(a)	0.00±0.00	8.56±3.78	4.51±4.51	0.00±0.00
	0.99 ^(b)	9.78±1.65	15.56±3.71	66.21±49.19	0.00±0.00

^{1, 2, 3} Differences among temperature levels. The same number corresponds to no statistically significant differences among groups.

^{a, b, c, d} Differences among water activity levels. The same letter corresponds to no statistically significant differences among groups.

Table 1. Effect of temperature and a_w on OTA production (ng/g agar) by *A. steynii* (above) and *A. westerdijkiae* (below). The data indicate the average of the four values obtained for each species (two strains and two replicates each) \pm standard error.