Aspergillus steynii and *Aspergillus westerdijkiae* as potential risk of OTA contamination in food products in warm climates

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Abstract

Aspergillus steynii and *A. westerdijkiae* are the main ochratoxin A (OTA) producing species of *Aspergillus* section *Circumdati*. Due to its recent description, few data are available about the influence of ecophysiological factors on their growth and OTA production profiles. In this work, the effect of temperature (20, 24 and 28 ºC) and water activity (a_w) (0.928, 0.964 and 0.995) on growth, sporulation and OTA production by these fungi was examined in CYA and media prepared from paprika, green coffee, anise, grapes, maize and barley. Growth was positively affected by the highest temperature and a_w values indicating that both species might be expected in warm climates or storage conditions. However, optimal growth conditions showed differences depending on the medium. OTA production was markedly affected by substrate and showed qualitative and quantitative differences. Both species, especially *A. steynii*, represent a great potential risk of OTA contamination due to their high production in a variety of conditions and substrates, in particular in barley and paprika-based media. Additionally, neither growth nor sporulation did result good indicators of OTA production by *A. steynii* or *A. westerdijkiae;* therefore, specific and highly-sensitive detection methods become essential tools for control strategies to reduce OTA risk by these species.

Keywords

Aspergillus steynii, *Aspergillus westerdijkiae*, ecophysiological factors, ochratoxin A

1. Introduction

Mycotoxins are toxic secondary metabolites produced by different fungal species. These compounds are considered a high risk to human diet representing the larger group of alert notifications which arrived to the European Rapid Alert System for Food and Feed (RASFF) in 2011 (RASFF, 2012). One of the most important mycotoxins is ochratoxin A (OTA) due to its high toxicity towards both animals and humans and presents nephrotoxic, inmunotoxic and teratogenic properties (Pfohl-Leszkowicz and Manderville, 2007). The International Agency for Research on Cancer (IARC, 1993) classified this toxin as a possible human carcinogen (group 2B). Traditionally, OTA has been considered an important contaminant in different dietary products commonly consumed by humans like cereals and cereal products, coffee, grapes and grape-products (Duarte et al., 2010; Romani et al., 2000; Varga and Kozakiewicz, 2006). However, recent studies regarding OTA incidence have reported its presence in an increasing number of substrates among which nuts and dried fruits, spices, cocoa and chocolate or liquorice are considered the most relevant (Pietri et al., 2010; Serra, 2004; Zaied et al., 2010). The maximum OTA levels in all these products are strongly regulated in the European Union (European Commission, 2006, 2010).

The majority of OTA-producing species are included in the genus *Aspergillus*. Several species of *Aspergillus* section *Circumdati* are capable of producing OTA. Although *A. ochraceus* used to be considered the most important OTA producer, particularly in warm climates, new species of *Aspergillus* section *Circumdati* have been described and they are also able to produce OTA, in particular *A. steynii* and *A. westerdijkiae* (Frisvad et al., 2004). In a recently published work carried out in our group (Gil-Serna et al., 2011), *A. steynii* has been reported as the main OTA-producing species within the section with a 90% of producing strains and toxin levels 1000 times higher than *A.*

ochraceus, followed by *A. westerdijkiae* with a 75% of producing isolates and production levels 100 times higher than *A. ochraceus*. Regarding to the percentage of producing strains, *A. steynii* is also the most important species (90%), followed by *A. westerdijkiae* (75%) and finally *A. ochraceus* with only a 22% of producing isolates (Gil-Serna et al., 2011). Due to their recent description, the presence of these species has been reported in few food matrices although currently they seem to be more frequent than *A. ochraceus*. *A. westerdijkiae* and *A. steynii* occurrence has been described in different products including coffee (Leong et al., 2007; Noonim et al., 2008), grapes (Díaz et al., 2009), paprika (Santos et al., 2011) or barley (Mateo et al., 2011).

Mycotoxin contamination of foodstuffs is very difficult to predict because it depends on a wide range of ecophysiological factors either intrinsic or extrinsic. D'Mello & MacDonald (1997) and Khalesi and Khatib (2011) suggested that the main factor affecting fungal growth and OTA production is water activity (a_w) followed by temperature, although the effect of substrate should not be disregarded. All these factors are always interacting in the environment; therefore, fungal ability to grow and produce mycotoxins is affected by a complex combination of parameters. Moreover, optimum conditions for fungal growth are usually different from those for mycotoxin production.

The effect of ecophysiological factors on *A. ochraceus* has been extensively studied. Both a_w and temperature have a great influence on this fungus and its optimal intervals to grow and produce OTA were established between 25-30 ºC and a_w 0.95-0.99 (Pardo et al., 2005, 2006a, 2006b; Ramos et al., 1998). The ability of *A. ochraceus* to produce OTA depends considerably on the substrate on which it is growing. For instance, Pardo et al. (2006b) indicated that its capacity of producing OTA was higher on green coffee beans than on barley.

Up to date, there are few data available regarding the effect of ecophysiological factors on *A. westerdijkiae* and *A. steynii*. Abdel-Hadi and Magan (2009) studied the effect of a_w and temperature on growth, sporulation and OTA production by these species in YES medium. In a recently published work, Wawrzyniak et al. (2013) studied the effect of storage conditions on *A. westerdijkiae* growth in stored barley.

The results obtained from culture media prepared from infusions of selected food products have been considered so far a reasonable and good approximation to the growth and toxin production patterns obtained in natural substrates (Pardo et al., 2005). Furthermore, Ramos et al. (1998) found similar patterns of OTA production in media prepared from infusions of barley and directly on cereal grains.

The aim of this work was to examine the effect of substrate composition, temperature and a_w on fungal growth and OTA production by *A. steynii* and *A. westerdijkiae*. For this purpose, two strains of each species from different origin were cultured in a commonly used medium (CYA) and on matrix-based media prepared from paprika, grapes, coffee, barley, anise and maize.

2. Materials and Methods

2.1. Fungal strains

Two strains of *A. westerdijkiae* were used in this study; the type species CECT 2948 and 3.58 isolated from grapes. Likewise, two isolates of *A. steynii* were evaluated in this work Aso2 and 3.53, isolated from grapes and coffee, respectively. These strains were kindly provided by Dr Sanchis (University of Lleida, Spain) and Dr Jimenez (University of Valencia, Spain). The correct identification of all the isolates 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±1ºC for

4-5 days and then stored at 4 ºC until required and as spore suspension in 15% glycerol at –80ºC.

2.2. Media

The assays were carried out in CYA medium (Czapek Yeast Agar) and using different matrix-based media. Paprika (*Capsicum annuum*, Spain), green coffee (*Coffea arabica*, Brazil), white grape (*Vitis vinifera*, var. Vinalopo, Spain), anise (*Pimpinella anisum*, Spain), barley (*Hordeum vulgare*, Spain) and maize (*Zea mays*, Spain) were used to prepare the media. In all these food products, *A. westerdijkiae* and/or *A. steynii* have isolated so far or these species have been detected directly on them by our group using specific PCR protocols (Díaz et al., 2009; Leong et al., 2007; Mateo et al., 2011; Noonim et al., 2008; Santos et al., 2011).

The matrix-based media used contained 3% (w/v) of each matrix with 20 g/l of agar (Pronadisa, Spain). They were prepared by boiling 30 g of dry grounded matrix 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 0.928, 0.964 and 0.995 (Dallyn & 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 Nº 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 20, 24 and 28 ºC. Each strain was inoculated in two independent plates in each medium, a_w and temperature condition.

The diameter of the colonies was measured in two directions at right angles to each other after 10 days of incubation and the average of these two values was used as indicator of fungal growth.

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 µm, 4.6 mm x 250 mm; Teknokroma, Barcelona, Spain) at 45 ºC 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, Steinheim, Germany).

2.5. Sporulation analysis

Three plugs (diameter 5 mm) adjacent of those used for OTA evaluation were removed from each plate and introduced in 2 ml tubes containing 1 ml of saline solution (9 g/l sodium chloride). Spores were separated mechanically from the medium by vortexing and spore concentrations in the extracts were measured by microscopy using a Thoma counting chamber. Spore concentrations were estimated by calculating the number of

spores/cm^2 taking into account that 1 ml of the spore suspensions in saline solution came from three agar plugs (diameter 5 mm) with a total surface of 5.9x10^{-1} cm^2 of fungal colony.

2.6. Statistical analysis

SPSS 19 was used for statistical treatment of the results. 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 the three parameters considered was studied by analysing Pearson correlation index using pairwise comparisons (growth-OTA, growth-spores, OTA-spores). In all cases, statistically significance was established at p±0.05.

3. Results

The Kruskal-Wallis test analysis performed did not reveal significant differences between the two strains analyzed for each species in any of the experiments considered; therefore, the results obtained for each species and treatment are represented as the average of four values corresponding to the two replicates per strain.

3.1. Effects of ecophysiological factors on fungal growth

The results of fungal colony diameter of *A. westerdijkiae* and *A. steynii* in all media and all conditions of temperature and a_w tested after 10 days of incubation are shown in figure 1. The results of the statistical analyses are also displayed in table 1.

3.1.1. Effect of temperature

Fungal growth in CYA medium was affected significantly by temperature in both species. *A. westerdijkiae* growth increased with temperature, although no significant differences were detected between 24 and 28 ºC. Similarly, *A. steynii* growth increased with temperature. In this case, 28 ºC had a higher permissive effect on growth than 24 ºC and this higher than 20 ºC.

The same statistical analysis was performed with the results of all matrix-based media and the factor temperature and it was confirmed the overall positive effect of temperature on growth in both *A. westerdijkiae* and *A. steynii*. Indeed, in general, the shift from 20 ºC to either 24 or 28 ºC seemed to be critical to reach optimal values for fungal growth in both species. However, when the results from each medium were analyzed using the posthoc U-Mann Whitney test, some differences were detected (table 1).

3.1.2. Effect of water activity

Water activity significantly affected fungal growth in CYA medium in both species. Fungal growth was significantly reduced at the lowest a_w (0.928), whereas no differences were found between colony diameters at a_w 0.964 and 0.995 in both species. Similar results were obtained for all matrix-based media when they were analyzed either as a whole or independently using the posthoc test and, in all cases, the statistical differences were only found between the lowest a_w tested and the two highest (table 1).

3.1.3. Effect of substrate

Both species showed similar growth pattern in CYA mediums. In general, and for both species, CYA medium supported the highest growth values in comparison with any of the matrix-based media tested. However, their optimal conditions for growth showed

differences depending on the medium considered. Both species were able to grow in all the media tested with similar pattern, although appearance of the colonies varied depending on the media (for instance, in pigmentation of mycelium and/or medium).

Significant differences in growth depending on the medium were observed in the case of *A. steynii*. This species produced lower growth values in maize and anise based media, significantly different from those obtained in paprika. *A. westerdijkiae* showed no significant differences in growth in any of the media tested and colony diameter reached similar values in all the cases.

The statistical analysis of all matrix-based media results as a whole suggested that *A. westerdijkiae* grew significantly better than *A. steynii*. However, post hoc tests indicated that this was only true in the case of anise-based medium, where their growth markedly differed. Indeed, both species showed no differences in any of the other matrix-based medium.

3.2. Effects of ecophysiological factors on OTA production

The results of OTA concentration values obtained measured by HPLC in both CYA and matrix-based media and all conditions of temperature and a_w tested are shown in figure 2. Statistical analyses are also summarized in table 2. OTA production profiles were very different in both species. *A. steynii* was able to produce the toxin in a wider set of conditions and at significant higher levels than *A. westerdijkiae* in all media and conditions tested.

3.2.1. Effect of temperature

A. westerdijkiae ability to produce OTA was not statistically affected by temperature in CYA medium although a tendency was observed regarding an increased OTA

concentration at the highest level of temperature tested. On the other hand, *A. steynii* reduced significantly OTA production at the lowest (20 ºC) (figure 2 and table 2). Temperature showed a significant influence on the ability to produce OTA in both species but the effect varied widely along the media. Generally speaking, the lowest temperature (20 ºC) had a negative effect on OTA production in both species (figure 2 and table 2).

3.2.2. Effect of water activity

The ability to produce OTA in CYA medium by both species was significantly affected by a_w. In both cases, the lowest a_w value (0.928) negatively affected toxin production. Additionally, the effect of a_w varied widely in both species depending on the matrix-based media used, suggesting the existence of an a_w-substrate interaction (figure 2). In general, OTA production increased with temperature and a_w, particularly in the case of *A. steynii*.

3.2.3. Effect of substrate

OTA production was markedly affected by substrate and showed qualitative and quantitative differences between both species (figure 2).

OTA production by *A. westerdijkiae* was detected in all the media except in grape-based medium, showing significant lower values than *A. steynii.* The highest production by *A. westerdijkiae* was found in CYA, paprika and barley-based media. *A. steynii* was able to produce OTA in all the media at markedly higher values than *A. westerdijkiae*. CYA, paprika and barley were also the substrates which induced higher OTA production by *A. steynii.* The lowest values were obtained from anise and particularly grape-based medium.

The optimal conditions for OTA production in CYA medium were different for each species and resulted in highly different values. At the optimal conditions for this medium (28 ºC and a_w=0.995), the maximum OTA concentration obtained for *A. steynii* was 128.66 µg/g whereas 2.36 µg/g were obtained for *A. westerdijkiae* (24 ºC and a_w=0.995). Maximum OTA levels in matrix-based media differed from those obtained in CYA medium. Optimal conditions (28 ºC and a_w 0.995) for OTA production by *A. steynii* in matrix-based media were obtained in paprika (43.32 µg/g). The highest OTA production in matrix-based medium by *A. westerdijkiae* were also obtained in paprika (10.28 µg/g) in this case at 24 ºC and a_w 0.964.

3.3. Effects of ecophysiological factors on sporulation

The influence of abiotic factors (temperature, a_w and substrate) on sporulation was analyzed in both species as spores/cm^2. Sporulation analysis was only carried out at the highest a_w levels (0.964 and 0.995) due to insufficient amount of mycelium grown at the lowest a_w value. The results of spore concentration obtained for both species on the matrix-based media are shown in table 3. No data were obtained in CYA medium since sporulation was poor or not detected at all. The statistical analysis indicated that there were no differences between both species, although in all cases *A. westerdijkiae* produced higher spore concentrations than *A. steynii*.

Both species showed similar pattern in relation with the substrates. The spore concentration was highest in paprika, followed by coffee, anise and barley. The lowest concentration was found in maize and particularly in grape-based medium. Generally speaking, spore concentration was positively affected by increasing temperature in *A. westerdijkiae* whereas no effect of a_w was found except for maize-based medium. On the other hand, a positive effect of increasing a_w values on sporulation was significant in

299 the case of *A. steynii* and temperature did not affect sporulation in any case but in
300 paprika-based medium.
301
302 *3.4. Correlation analysis among growth, OTA production and sporulation*
303 Single correlation among the three factors analyzed in pairs was studied using Pearson
304 index. Correlations were studied globally and in each media separately. In any case,
305 correlation values were not higher than 0.3; therefore, no correlation does exist between
306 growth-OTA production, growth-sporulation or OTA production-sporulation.
307
308 **4. Discussion**
309 The effect of temperature, a_w and substrate on growth, OTA production and sporulation
310 rate was examined in *A. westerdijkiae* and *A. steynii*. All the information previously
311 available on the relative importance of these ecophysiological factors on both growth
312 and OTA production in *Aspergillus* Section *Circumdati* species is practically limited to
313 *A. ochraceus*, and published prior to the description of *A. westerdijkiae* and *A. steynii* as
314 new species. Furthermore, it is important to note that many works previously performed
315 used the strain NRRL 3174 (=CECT 2948), considered for a long time as the type
316 species of *A. ochraceus*. However, Frisvad et al. (2004) re-classified this isolate and
317 nowadays is considered the type species of *A. westerdijkiae*. Several authors have
318 reported high variability among isolates of *A. ochraceus* in ecophysiological studies
319 (Pardo et al., 2004, 2006b). However, this could be explained by assuming that the
320 group of isolates of *A. ochraceus* used by these authors could also include members of
321 any of the other two species (*A. westerdijkiae* or *A. steynii*). In our study, no
322 intraspecific variability has been found. Furthermore, the performance of the isolates in

323 the different matrix-based media had no relationship with the matrix from where the
324 strains tested were isolated.
325 In order to facilitate discrimination among species within Section *Circumdati*, specific
326 PCR-based assays were developed by our group and they have been used to confirm the
327 identification of the isolates from *A. westerdijkiae* and *A. steynii* used in this study (Gil-
328 Serna et al., 2009). Therefore, this is the first work to our knowledge with these two
329 recently described species in food matrix-based substrates. There is only a similar report
330 on the influence of temperature and a_w on these species performed in YES medium
331 (Abdel-Hadi and Magan, 2009). When growth profiles regarding temperature and a_w
332 from this study were compared with those obtained in our case in CYA medium, similar
333 permissive and optimal conditions were observed. Similarly, OTA production by both
334 species was optimal at higher temperatures and a_w and decreased at lower temperature
335 and a_w. In our study, *A. steynii* produced higher OTA levels than *A. westerdijkiae* in all
336 conditions whereas Abdel-Hadi and Magan (2009) reported that OTA production by *A.*
337 *westerdijkiae* was higher than by *A. steynii* at low temperature and a_w.This might be
338 caused by substrate or strain differences. Influence of substrate has been reported by
339 Pardo et al. (2005). These authors observed large variations in OTA values among
340 different culture media. Wawrzyniak et al. (2013) studied the effect of ecophysiological
341 factors (temperature and a_w) on *A. westerdijkiae* growth in stored barley and also
342 suggested that a_w was a critical factor regarding fungal growth in this product.
343 In general, optimal ecophysiological conditions for growth and OTA production are
344 similar and basically coincident in both species. These conditions predict higher risk for
345 both fungal and OTA contamination at temperatures higher than 24 ºC and higher a_w
346 (0.96-0.99). Risk would decrease at temperatures close to 20 ºC and a_w lower than 0.92.
347 On the other hand, the high OTA production by *A. steynii* observed in this study

indicated that the presence of this species even at very low levels would represent a much higher risk of OTA contamination than *A. westerdijkiae.*

The importance of food matrix on growth and OTA production was studied in a wide sample of matrix-based culture media. These products were selected because both species had been previously detected or isolated from those food matrices in our group or reported by other authors (Díaz et al., 2009; Mateo et al., 2011; Noonim et al., 2008; Santos et al., 2011). Our results indicated that the effect on growth and OTA production depended on the food matrix considered.

In the case of growth profiles, in general, both *A. westerdijkiae* and *A. steynii* species were similar in all the media although *A. westerdijkiae* showed higher values than *A. steynii*. However, differences between both species were found in the case of OTA production. On the other hand, although these species were able to grow in all the substrates tested, OTA production was highly variable among the substrates and between both species. This fact was in agreement with Lai et al. (1970) who described that the composition of the medium had little effect on fungal growth, although it was very important for their ability to produce OTA. Moreover, it has been suggested that OTA production and growth might not be directly correlated, and that toxin biosynthesis did not occur in all the conditions that the fungus was able to grow (Mühlencoert et al., 2004, Pardo et al. 2006a). In our work no significant correlation was found between OTA production and fungal growth. Therefore, fungal growth could not be used as an indicator of OTA contamination by these species in food products. The different influence of the food matrices tested on growth and especially on OTA production might be due to their different composition as suggested by some authors. These have reported that the predominant carbon source in the medium have a slight effect on fungal growth whereas it is critical for the OTA production ability by

373 *Aspergillus* species (Medina et al., 2008; Mühlencoert et al., 2004). These authors
374 pointed out that *Aspergillus* section *Circumdati* species produce higher amounts of toxin
375 when the carbon source in the medium is sucrose and lower amounts when the sugar
376 available is fructose (Medina et al., 2008; Mühlencoert et al., 2004). In grapes, besides
377 water, sugars are the most abundant compounds being fructose by far the most abundant
378 (Dharmadhikari, 1994). In agreement with these reports, our results indicate that the
379 grape-based medium was suitable for both species to grow but they hardly produce
380 OTA (very low levels by *A. steynii*, and no production by *A. westerdijkiae)*. The results
381 obtained in this work also agree with other authors (Kapetanakou et al., 2009; Pardo et
382 al., 2006b) who observed a very limited OTA production by species of *Aspergillus*
383 section *Circumdati* in grapes or derivatives when compared with the production of the
384 isolates in other commodities.
385 On the contrary, the *Aspergillus* species studied in this work achieved the highest values
386 of both growth and OTA production in paprika-based medium. *Capsicum annuum* fruits
387 (from which paprika is obtained) contained high amounts of sucrose and glucose
388 (Prabha et al., 1998) which, as said above, might positively affected OTA production by
389 these species.
390 Several authors have demonstrated the ability to produce OTA by *A. ochraceus* in
391 cereals (Pardo et al., 2006b; Ramos et al., 1998). In our study, both *A. westerdijkiae* and
392 *A. steynii* showed high growth and OTA production on cereal-based media (particularly
393 in barley). Carbon source might also be critical to explain these results. Different studies
394 have shown that several toxigenic fungi are able to use starch as unique carbon source
395 to grow which is also suitable for toxin production (Bluhm and Woloshuk, 2005;
396 Roussos et al., 2006). These authors performed these works using different aflatoxins
397 and fumonisin-producing species; therefore, more studies should be conducted to

confirm if these previous results would be also applied to OTA-producing species, and particularly to *A. westerdijkiae* and *A. steynii*.

As mentioned above, *A. ochraceus* was considered for a long time the main source of OTA in coffee (Batista et al., 2003; Taniwaki et al., 2003; Urbano et al., 2001) and several works have studied OTA production by this fungus in this matrix (Mantle and Chow, 2000, Pardo et al., 2005, 2006b). The optimal conditions for OTA production by *A. ochraceus* reported by these authors are in agreement with those obtain in our work for *A. westerdijkiae* and *A. steynii* (28 ºC and a_w 0.99). However, toxin production levels of all isolates tested had lower OTA production capacity in this coffee-based medium than in others such as paprika or barley, even when fungal growth was similar in all cases. Several studies have shown that one component of coffee, caffeine, can reduce or even completely inhibit OTA production by ochratoxigenic *Aspergillus* of section *Circumdati* (Buchanan et al., 1982; Suárez-Quiroz et al., 2004). Therefore, the reduction in OTA production in green coffee-based medium could be due to the presence of this compound and *A. westerdijkiae* seemed to be more susceptible than *A. steynii*. According to the results obtained, the presence of *A. steynii* in coffee might represent a greater risk of OTA contamination than if the fungus detected is *A. westerdijkiae*.

The results obtained in this work suggest that *A. steynii* and *A. westerdijkiae* would be considered as a great potential risk of OTA contamination due to their high capacity of producing OTA in a variety of conditions and commodities. These species are expected particularly in warm climates or storage conditions although in some cases their growth is not a good indicator of OTA production. The most relevant substrates regarding their expected OTA contamination and/or their dietary importance are cereals (mainly barley) and paprika. On the other hand, since their high ability to produce OTA (both levels of OTA production and percentage of OTA producing strains), particularly in the case of

A. steynii, a highly sensitive assay for specific detection of these species on food products might be a crucial point in control and prevention strategies. Currently, there are a number of simple, fast and sensitive tools based on PCR to detect them, even directly in food matrices (Gil-Serna et al., 2009).

Brodhagen and Keller (2006) reported that sporulation and mycotoxin production might be related because their synthesis routes are regulated similarly. However, the results obtained in this work indicated that *A. steynii* is capable of producing higher levels of OTA, although it is not able to produce more spores than *A. westerdijkiae*. Moreover, no significant correlation was found between OTA production and sporulation rate; therefore, sporulation could not be considered as a good indicator of OTA production in these species.

This study provides interesting data on the ability to grow and produce OTA by *A. steynii* and *A. westerdijkiae*, the most important ochratoxigenic species included in *Aspergillus* section *Circumdati*. The results obtained in this work could be applied to predict the risk of OTA contamination in a variety of food products.

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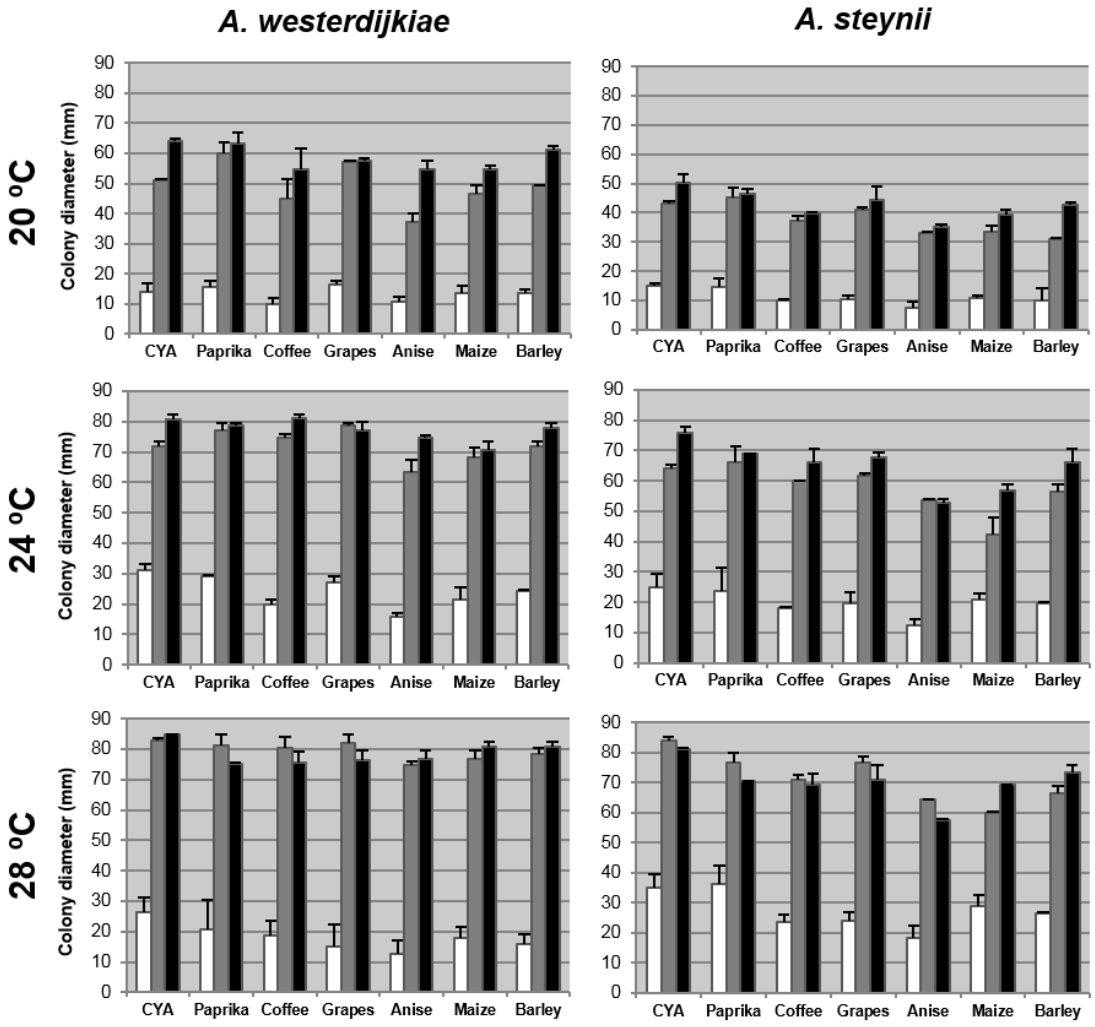
A. westerdijkiae
A. steynii
20 ºC
24 ºC
28 ºC
Colony diameter (mm)
0
10
20
30
40
50
60
70
80
90
CYA
Paprika
Coffee
Grapes
Anise
Maize
Barley

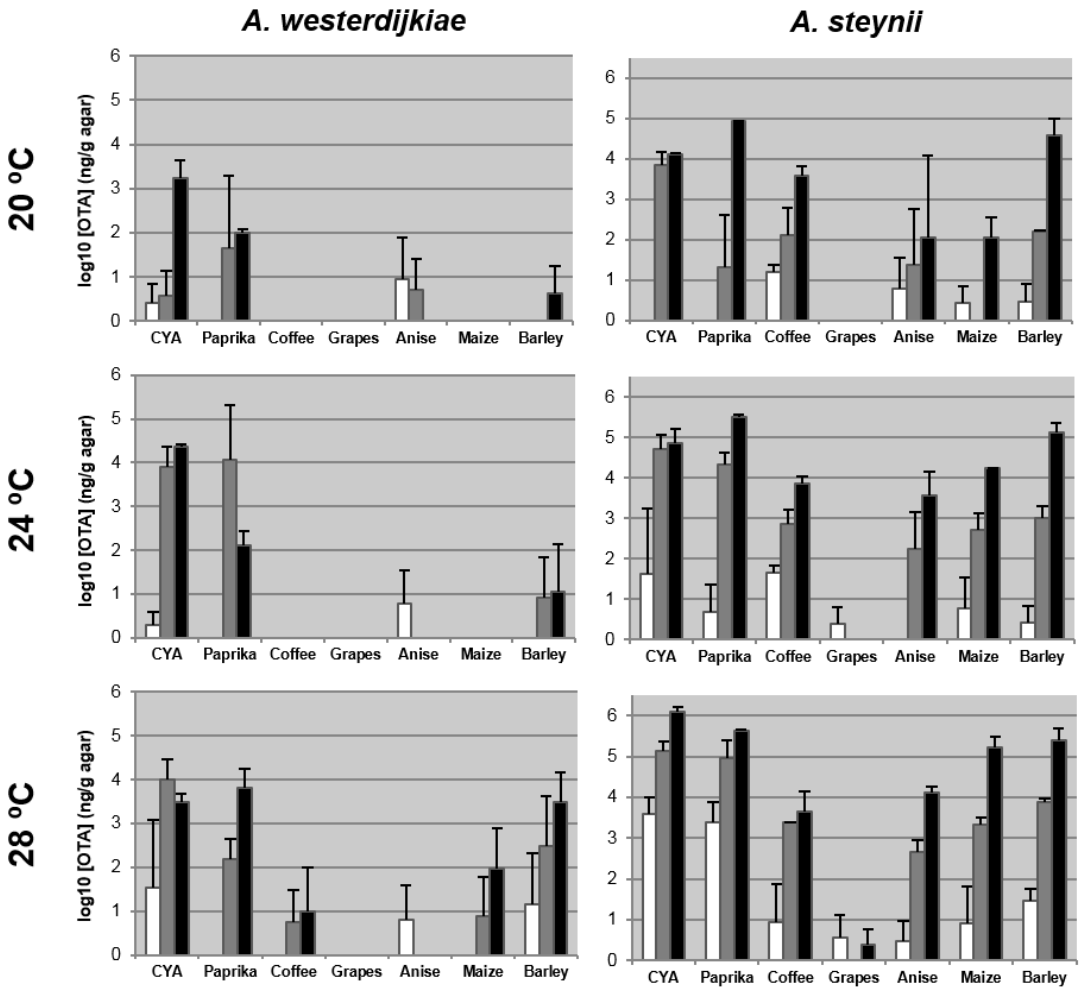
A. westerdijkiae
A. steynii
20 ºC
24 ºC
28 ºC
log10 [OTA] (ng/g agar)
0
1
2
3
4
5
6
CYA
Paprika
Coffee
Grapes
Anise
Maize
Barley

Figure 1. Evaluation of fungal growth by *A. westerdijkiae* (left) and *A. steynii* (right) in different media at all temperatures and a_w assayed (white bars a_w=0.928; grey bars a_w=0.964; black bars a_w=0.995). In all the cases, the results are the average of both replicates of the two strains analyzed and their standard deviation is indicated as vertical thin lines.

Figure 2. OTA production by *A. westerdijkiae* (left) and *A. steynii* (right) in CYA and matrix-based media at different temperatures and a_w (white bars a_w=0.928; grey bars a_w=0.964; black bars a_w=0.995). To facilitate graphs interpretation, $\log_{10}$ of OTA concentration (ng/g) is represented. In all the cases, the results are the average of both replicates of the two strains analyzed and their standard deviation is indicated as vertical thin lines.

A. westerdijkiae		**CYA**	**Paprika**	**Coffee**	**Grapes**	**Anise**	**Maize**	**Barley**
	Media	A	A	A	A	A	A	A
a_w	0.928	a	a	a	a	a	a	a
	0.964	b	b	b	b	b	b	b
	0.995	b	b	b	b	b	b	b
Temperature	20ºC	1	1	1	1	1	1	1
	24ºC	2	2	2	1	1	2	2
	28ºC	2	1 2	2	1	1	2	2

A. steynii		**CYA**	**Paprika**	**Coffee**	**Grapes**	**Anise**	**Maize**	**Barley**
	Media	C	C	ABC	BC	A	AB	ABC
a_w	0.928	a	a	a	a	a	a	a
	0.964	b	b	b	b	b	b	b
	0.995	b	b	b	b	b	b	b
Temperature	20ºC	1	1	1	1	1	1	1
	24ºC	2	2	2	2	2	1	2
	28ºC	3	2	2	2	3	2	2

Table 1. Summary of the statistical analyses of fungal growth obtained for *A. westerdijkiae* (above) and *A. steynii* (below) in the 7 culture media at different conditions of temperature and a_w. The effect of the medium was evaluated including all the data obtained in the different conditions tested for each medium and the results are indicated by capital letters. The effect of temperature and a_w was independently evaluated for each medium and the results are indicated by small letters (a_w) and numbers (temperature). The same letter or number indicates no statistically significant differences among groups and the lower the number or the letter (alphabetical order), the lower the growth value (average).

A. westerdijkiae		**CYA**	**Paprika**	**Coffee**	**Grapes**	**Anise**	**Maize**	**Barley**
	Media	D	D	B	A	BC	B	CD
a_w	0.928	a	a	a	a	a	a	a
	0.964	b	b	a	a	b	a	a
	0.995	b	b	a	a	b	a	a
Temperature	20ºC	1	1	1	1	1	1	1
	24ºC	1	1	1	1	1	1	1
	28ºC	1	1	2	1	1	2	2

A. steynii		**CYA**	**Paprika**	**Coffee**	**Grapes**	**Anise**	**Maize**	**Barley**
	Media	E	DE	CD	A	B	BC	DE
a_w	0.928	a	a	a	a	a	a	a
	0.964	b	b	b	a	ab	b	b
	0.995	b	c	c	a	b	c	c
Temperature	20ºC	1	1	1	1	1	1	1
	24ºC	2	1 2	1	1	1	2	1
	28ºC	2	2	1	1	1	2	1

Table 2. Summary of the statistical analyses of OTA production obtained for *A. westerdijkiae* (above) and *A. steynii* (below) in the 7 culture media at different conditions of temperature and a_w. The effect of the medium was evaluated including all the data obtained in the different conditions tested for each medium and the results are indicated by capital letters. The effect of temperature and a_w was independently evaluated for each medium and the results are indicated by small letters (a_w) and numbers (temperature). The same letter or number indicates no statistically significant differences among groups and the lower the number or the letter (alphabetical order), the lower the OTA production value (average).

	A. westerdijkiae		Sporulation (million of spores/cm^2)					
		a_w	**20°C**		**24°C**		**28°C**	
D	**Paprika**	0.964 [a] 0.995 [a]	89.3 ± 38.9 129.0 ± 42.1	1	144.0 ± 70.0 139.0 ± 6.5	1	227.0 ± 131.0 195.0 ± 47.5	1
C	**Coffee**	0.964 [a] 0.995 [a]	43.4 ± 1.0 41.9 ± 11.7	1	71.5 ± 2.7 80.3 ± 9.2	2	107.0 ± 17.0 104.0 ± 45.8	2
A	**Grapes**	0.964 [a] 0.995 [a]	4.2 ± 2.7 14.4 ± 2.8	1	6.6 ± 4.8 15.4 ± 13.3	2	10.4 ± 8.7 24.3 ± 23.6	2
C	**Anise**	0.964 [a] 0.995 [a]	44.0 ± 11.9 35.1 ± 8.2	1	37.7 ± 15.8 43.0 ± 29.3	1	62.7 ± 26.8 104.0 ± 16.1	2
B	**Maize**	0.964 [a] 0.995 [b]	21.1 ± 4.6 23.5 ± 5.1	1	25.5 ± 17.5 24.4 ± 6.2	1	44.5 ± 27.3 49.7 ± 1.5	1
C	**Barley**	0.964 [a] 0.995 [a]	43.8 ± 6.1 34.6 ± 5.0	1	57.3 ± 26.1 49.1 ± 17.0	1	97.3 ± 42.0 97.1 ± 13.3	2

	A. steynii		Sporulation (million of spores/cm^2)					
		a_w	**20°C**		**24°C**		**28°C**	
E	**Paprika**	0.964 [a] 0.995 [b]	77.4 ± 15.9 102.0 ± 21.2	1	111.0 ± 25.2 171.0 ± 4.2	2	134.0 ± 16.5 144.0 ± 27.6	2
CD	**Coffee**	0.964 [a] 0.995 [b]	22.4 ± 2.8 52.4 ± 8.1	1	32.1 ± 2.7 62.3 ± 6.5	1	36.7 ± 7.3 57.3 ± 9.4	1
A	**Grapes**	0.964 [a] 0.995 [b]	0.8 ± 0.0 10.6 ± 0.4	1	0.8 ± 0.3 12.5 ± 5.0	1	0.8 ± 0.6 6.1 ± 0.3	1
D	**Anise**	0.964 [a] 0.995 [b]	25.4 ± 1.5 44.6 ± 6.9	1	33.5 ± 5.6 54.9 ± 4.6	1	36.7 ± 1.5 90.1 ± 6.6	2
B	**Maize**	0.964 [a] 0.995 [b]	10.5 ± 0.2 41.5 ± 0.3	1	9.9 ± 3.8 40.5 ± 5.3	1	32.8 ± 15.0 40.8 ± 3.8	1
C	**Barley**	0.964 [a] 0.995 [b]	19.3 ± 3.4 55.9 ± 2.0	1	32.1 ± 10.9 54.9 ± 7.2	1	37.9 ± 0.5 52.7 ± 2.9	1

[1, 2] Differences among temperatures in each media. Different numbers indicate statistical differences among groups.

[a, b] Differences among water activity levels in each media. Different letters indicate statistical differences among groups.

[A, B, C, D, E] Differences among media in each species. Different letters indicate statistical differences among groups.

Table 3. Effects of substrate, temperature and a_w on sporulation by *A. westerdijkiae* (above) and *A. steynii* (below) in different culture media. In all the cases, results are the average of both replicates of the two strains analyzed ± standard deviation. The results of statistical analysis are also displayed. The effects of a_w and temperature were independently evaluated in each medium