

Review

Ecophysiology of *Aspergillus* Section *Nigri* Species Potential Ochratoxin A Producers

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Abstract: After aflatoxins, ochratoxin A (OTA) is the most studied mycotoxin due to the toxicological significance in human and animal diets. OTA presence has been extensively reported worldwide in the last decade in several agricultural products. The main OTA producer in tropical and temperate climates is *Aspergillus carbonarius* followed by species belonging to *A. niger* aggregate. Currently, many scientists worldwide have studied the influence of water activity and temperature for growth and biosynthesis of OTA by these species on synthetic media. This article reviews ecophysiological studies of *Aspergillus* section *Nigri* strains on synthetic media and natural substrates. The results of these investigations suggest that significant amounts of OTA can be produced in only five days and that the use of different storage practices, such as a_w and temperature levels below 0.930 and 15 °C, respectively, allow controlling fungal contamination and minimizing the OTA production in several products as peanuts, corn, dried grapes and derived products for human consumption.

Keywords: *Aspergillus niger* aggregate; *Aspergillus carbonarius*; ochratoxin A; water activity; temperature

1. Introduction

The *Aspergillus* genus is distributed worldwide but is most commonly isolated from latitudes 26°–35° north or south of the Ecuador. Therefore, these fungi are common in warm and temperate climates. Their ability to develop under conditions of high temperature and relatively low a_w allows them to adapt and colonize a diverse array of cereals and dried fruits [1].

Some of the species belonging to this genus, *A. niger*, *A. sojae* and *A. oryzae*, are known as producers of industrial enzymes and metabolites able to give flavor to food. However, there are other species capable to infect plant tissue and produce mycotoxins [2]. Ochratoxins—the second mycotoxin group in importance after aflatoxins—includes at least nine metabolites that are similar in structural terms, of which ochratoxin A (OTA) is the most studied metabolite due to its occurrence in food and feed and toxicological significance in human and animal diets. This toxin is known to have nephrotoxic, immunotoxic, teratogenic and carcinogenic effects on animals. The International Agency for Research of Cancer (IARC) has classified OTA as a group 2B carcinogen based on toxicity on rats [3,4]. For these reasons, the European Commission has fixed maximum limits for OTA in several agricultural products destined for human and animal consumption [5].

OTA presence has been extensively reported worldwide in the last decade in several products, such as coffee [6,7], wines [8–14], beers [15–17], grapes [18–20], dried grapes [7,21–24], cereals and derivatives destined for humans and animals [25–34], oilseeds and derivatives products [35] and occasionally, in body fluids, plasma, meat and kidneys of several animal species [36,37].

In the 90s, this toxin was considered to be only produced by *Penicillium verrucosum* in temperate and cold climates, and *A. ochraceus* and related species in warm and tropical climates. Due to their physiological differences, each of these species occupies a particular ecological niche. In the last years, several studies reported the presence of potential OTA producers *Aspergillus* section *Nigri* species in food and feed; considering *Aspergillus carbonarius* as the main OTA producer followed by species belonging to the *A. niger* aggregate [38].

In Argentina, as in other temperate and tropical countries, *P. verrucosum* and *A. ochraceus* have not been reported as frequent colonizers in agricultural products, while *A. carbonarius*, *A. niger* aggregate species and monoseriate *Aspergillus* species such as *A. aculeatus* and *A. japonicus* have been frequently isolated from several agricultural products, e.g., coffee beans, red wine, dried grapes, corn and peanut kernels and feeds. OTA was detected in some of these substrates except in coffee beans and corn kernels [10,13,21,25,35,39–43]. In others South American countries, several authors have also informed the presence of this species in cocoa and coffee beans, grapes and poultry feed [44–48].

Crops contaminated with mycotoxins reflect a loss of income for agricultural producers. The strategies that prevent the entry of these metabolites in the food chain protect public health and reduce economic losses caused by contaminated agricultural products.

The knowledge of the ecophysiology of OTA producing fungi and ecological factors that influence OTA production is essential to optimize the implementation of preventive strategies aimed at controlling the sanitary quality of raw materials and/or products susceptible to fungal colonization.

There are multiple factors significantly involved in the development of section *Nigri* species and secondary metabolites biosynthesis, e.g., humidity, temperature, presence of oxygen and carbon dioxide, incubation time, substrate composition, loss of grain integrity caused by insects or

mechanical/thermal damage, fungal inoculum, and the interaction/competition between other contaminated fungal species. Of these factors, water activity (a_w) and temperature have shown the greatest effects on growth and OTA production. In general, the toxigenic species are not aggressive pathogens, but are often well adapted to substrates with low humidity, and they can easily colonize cereal grains and oilseeds that are stored under inappropriate environmental conditions.

In recent years, scientific studies have focused mainly on the influence of environmental factors on OTA production by *P. verrucosum* and *A. ochraceus* since they are recognized as the main producer species. Several ecophysiological studies on *A. ochraceus* were reported in the two last decades [49–55] as well as studies on *P. viridicatum*, nowadays classified as *P. verrucosum* [56–59].

Currently, many scientists worldwide have studied the influence of water activity and temperature for biosynthesis of OTA by other ochratoxigenic species belonging to section *Nigri* such as *A. niger* aggregate and *A. carbonarius* [60–79], although only a few studies have reported the effect of incubation time on the amount of OTA produced by some strains belonging to the genera *Aspergillus* section *Nigri* [80–82].

Besides environmental factors, biological factors have a noticeable influence on growth and OTA production. Some of these biological factors are intrinsic; hence, depend only on the genetic basis of the fungal strain. The strains vary in their ability to produce OTA as well as the quantity produced. Recently, several studies have allowed the detection of some genes that may be involved in the biosynthesis of this toxin. Initially, genes encoding a polyketide synthase of *A. ochraceus* [83,84], *P. verrucosum* [85] and *P. nordicum* [86] were characterized; but these genes had low homology between the last two species. The simultaneous presence of different bacteria or other fungi in the substrates also influences the growth of these ochratoxigenic species and OTA production.

2. Ecophysiological Studies of *Aspergillus* Section *Nigri* Strains on Synthetic Media

Several authors have shown minima and optima conditions for mycelial growth of ochratoxigenic species belonging to *Aspergillus* section *Nigri* on different culture media [60,64–72,76,77,79,80,82] (Table 1). In these studies, growth rates of *Aspergillus* section *Nigri* species showed a marked decrease with the reduction of temperature and water activity level, and were not higher than 3 mm day^{−1} at 15 °C at the lowest a_w . The highest growth rates of these species found at optimal conditions differ depending on the agar media used. The highest growth rates achieved by *A. carbonarius* species was found on CYA by Romero *et al.* [79] at 0.95 a_w and 30 °C (17.46 mm day^{−1}) with a strain isolated from Argentinean dried vine fruits and Leong *et al.* [77] reported a maximum growth rate (11.35 mm day^{−1}) for an *A. niger* aggregate strain at 0.98 a_w and 35 °C on a simulated grape juice medium. However, the literature shows that in most combinations of temperature and a_w tested, *A. niger* grew more rapidly than *A. carbonarius*. The reproducibility of these optima for growth indicates that *A. niger* aggregate has a higher optimum temperature for growth than *A. carbonarius* and possibly also takes greater advantage of high water activities for rapid growth. In general, the optimum conditions for growth of assayed strains belonging to the *A. niger* aggregate and *A. carbonarius* strains were similar than that reported by the former authors. These optimal conditions for growth by *Aspergillus* section *Nigri* strains are in agreement with previous European works [60,63,70,81], which reported optimal growth rates for *A. carbonarius* strains at temperatures between 30–35 °C

and 0.950–0.980 a_w . The environmental conditions of growth inhibition observed by Astoreca *et al.* [64] agree with those obtained previously on synthetic media with strains of section *Nigri* isolated from maize grains [87] and from grapes [60,70,81]. In another study from Argentina, Romero *et al.* [79] evaluated the growth rate of *A. carbonarius* strains isolated from dried grapes at different a_w and temperature conditions and the results showed that the inhibition of growth occurred at 15 °C among 0.825 to 0.900 a_w , and 25 °C at 0.825 a_w .

Ochratoxin A production in relation to ecophysiological factors by *Aspergillus* section *Nigri* strains was evaluated by several authors on synthetic media [62,63,65–71,73,81,82] (Table 2).

Astoreca *et al.* [65] evaluated the influence of ecophysiological factors on OTA production by six *A. niger* aggregate strains isolated from peanut seeds, corn kernels and coffee beans; and two *A. carbonarius* strains isolated from dried grapes in Argentina. These authors observed that the optimum temperature for OTA production was 25 °C for all strains belonging to the *A. niger* aggregate; while the *A. carbonarius* strains produced the highest OTA levels at 25 and 30 °C, without significant differences in production profiles between these strains. These OTA production patterns agree with those previously reported by Bellí *et al.* [72]. In this study, the results of OTA production by *Aspergillus* section *Nigri* species on a medium similar to grape composition were modeled by a multiple linear regression and predictive models were obtained. The results showed that high levels of a_w (0.960 and 0.995) favored OTA production by *A. carbonarius* and *A. niger* aggregate strains as Bellí *et al.* [81], Esteban *et al.* [61,62], Joosten *et al.* [6] and Romero *et al.* [78] reported. On the other hand, Oueslati *et al.* [80] evaluated the effect of three alternating temperatures cycles (20/30, 20/37 and 25/42 °C) and photoperiod on growth and OTA production of six strains of *A. carbonarius* from Tunisian grapes on synthetic nutrient medium. The different temperature regimes assayed affected significantly both the mycelial growth and the OTA production. The best growth and OTA production were recorded with the 20/30 °C cycle.

Several studies have detected similar minimum a_w level, 0.90–0.94 for OTA production by *Aspergillus niger* aggregate and *A. carbonarius* strains on different synthetic media (Table 2), whereas, Romero *et al.* [78] reported *A. carbonarius* strains able to produce traces amounts of OTA at lowest a_w level (0.87) on CYA medium. The results present in the literature shows that different substrates significantly affect the optimum and the minimum a_w conditions for OTA synthesis.

3. Ecophysiological Studies of *Aspergillus* Section *Nigri* Strains on Natural Substrates

Results obtained on synthetic substrates may not accurately represent the real fungal ability to produce OTA on a natural substrate. Therefore, studies on OTA production have been frequently carried out on natural and common substrates for ochratoxigenic *A. ochraceus* and *P. verrucosum*, such as cereal grains, green coffee beans and grapes, but there is little information about the influence of a_w and temperature on growth parameters and OTA production of *Aspergillus* section *Nigri* strains on natural substrates.

Table 1. Optima and minima conditions (temperature and water activity) for *Aspergillus* section *Nigri* species growth on different culture media.

Fungal species	Medium	Tested temperature range (°C)	Optimum temperature (°C)	Optimum a_w	Minimum a_w	References
<i>A. section Nigri</i>	Grains and fruits based	15–30	30	0.97	0.85	Astoreca <i>et al.</i> [64]
	Synthetic nutrient	10–37	30–37	0.98	-	Bellí <i>et al.</i> [72]
	CYA-YES	5–45	30–35	-	-	Esteban <i>et al.</i> [82]
<i>A. niger</i> aggregate	Corn grains	15–30	30	0.95	0.91	Astoreca <i>et al.</i> [66]
	Peanut seeds	15–30	30	0.99	0.91	Astoreca <i>et al.</i> [67]
	Coffee beans	15–30	25	0.99	0.93	Astoreca <i>et al.</i> [68]
	Synthetic nutrient	10–37	25	0.95	-	Selouane <i>et al.</i> [76]
	Simulated grape juice	15–35	35	0.98	0.92 (15 °C)	Leong <i>et al.</i> [77]
<i>A. carbonarius</i>	Synthetic grape juice	10–40	35	0.98	0.88	Mitchell <i>et al.</i> [60]
	Dried grapes	15–30	25–30	0.99	0.91	Astoreca <i>et al.</i> [64]
	Synthetic grape	15–37	30	0.95–0.99	0.90 (15 °C)	Bellí <i>et al.</i> [70]
	Synthetic nutrient	20–28	28	-	-	Bellí <i>et al.</i> [71]
	Synthetic nutrient	10–37	25	0.95	-	Selouane <i>et al.</i> [76]
	Simulated grape juice	15–35	30	0.965	0.92 (15 °C)	Leong <i>et al.</i> [77]
	CYA	15–35	30	0.95	0.85	Romero <i>et al.</i> [79]
		20/30				
	Synthetic nutrient	20/37	20/30	-	-	Oueslati <i>et al.</i> [80]
		25/42				

CYA: Czapek Yeast Autolysate agar;

YES: Yeast Extract Sucrose agar;

-: Data not reported.

Table 2. Optima and minima conditions for ochratoxin A (OTA) production by *Aspergillus* section *Nigri* species on different culture media.

Fungal species	Medium	Tested temperature range (°C)	Optimum temperature (°C)	Optimum a_w	Minimum a_w	References
<i>A. section Nigri</i>	Grains and fruits based	15–30	25–30	0.97–0.99	0.89–0.90	Astoreca <i>et al.</i> [65]
	CYA-YES	5–45	25	-	-	Esteban <i>et al.</i> [82]
<i>A. niger</i> aggregate	CYA-YES	-	-	0.99	0.94	Esteban <i>et al.</i> [61]
		15–30	15	0.98	0.90	Esteban <i>et al.</i> [62]
	Corn grains	15–30	25	0.97	0.91–0.93	Astoreca <i>et al.</i> [66]
	Peanut seeds	15–30	25	0.97 (25°C)	0.91	Astoreca <i>et al.</i> [67]
	Coffee beans	15–30	30	0.99	0.95	Astoreca <i>et al.</i> [68]
	Synthetic nutrient	10–37	30–37	0.90–0.95	-	Selouane <i>et al.</i> [76]
	Simulated grape juice	15–35	15	0.95	-	Leong <i>et al.</i> [77]
	Synthetic nutrient	10–37	30	0.995	-	Bellí <i>et al.</i> [81]
<i>A. carbonarius</i>	Coffee cherries	15–35	25	0.99	0.94	Joosten <i>et al.</i> [6]
	Synthetic grape juice	10–40	15–20	0.95–0.98	-	Mitchell <i>et al.</i> [60]
	Dried grapes	15–30	30	0.99	0.91	Astoreca <i>et al.</i> [69]
	Synthetic grape	15–37	20	0.95–0.99	0.90	Bellí <i>et al.</i> [70]
	Synthetic Nutrient	20–28	28	-	-	Bellí <i>et al.</i> [71]
	Table grapes	20–30	30	0.99	-	Bellí <i>et al.</i> [73]
	Synthetic nutrient	10–37	25–30	0.95–0.99	-	Selouane <i>et al.</i> [76]
	Simulated grape juice	15–35	15	0.95–0.98	-	Leong <i>et al.</i> [77]
	CYA	15–35	15	0.95	0.87	Romero <i>et al.</i> [78]
		20/30				
	Synthetic nutrient	20/37	20/30	-	-	Oueslati <i>et al.</i> [80]
		25/42				
	Synthetic nutrient	10–37	25	0.96	-	Bellí <i>et al.</i> [81]

CYA: Czapek Yeast Autolysate agar;

YES: Yeast Extract Sucrose agar;

-: Data not reported.

Most of the studies were done on solid synthetic and different substrate based media. The first study carried out with OTA producing strains belonging to *Aspergillus* section *Nigri* on natural substrate was reported by Joosten *et al.* [6], who studied the effect of environmental factors on growth parameters and OTA production by *A. carbonarius* strains on coffee cherries in Thailand. These authors showed that the optimal conditions of OTA production by *A. carbonarius* were 25 °C and 0.990 a_w . In other study, four ochratoxigenic *A. carbonarius* strains isolated from wine grapes were used to inoculate artificially damaged and undamaged table grapes [73]. Grapes were stored at three levels of relative humidity (80, 90 and 100%) and at two temperatures (20 and 30 °C). The results showed that temperature and relative humidity significantly influenced both infection and toxin content. At 30 °C, the detected OTA amount was higher than at 20 °C in most of the treatments. The highest relative humidity (100%) led to maximum OTA amounts while no significant differences were found between 90% and 80% in the OTA content.

Recently, Astoreca *et al.* [66–69] reported on the influence of environmental factors (a_w : 0.995, 0.973, 0.951, 0.928 and 0.910 and temperature: 15, 25 and 30 °C) on lag phase, growth rate, and OTA production by strains belonging to *A. niger* aggregate on irradiated corn, peanuts, dried grapes and coffee beans at 7, 14 and 21 days of incubation. The observed general pattern of *in vitro* growth [64,65] was different from those obtained on the natural substrates. The assayed strains on irradiated peanut seeds showed the shortest lag phases at the optimum conditions (7 h at 30 °C and 0.995) while the other strains reached the exponential phases only after around 35.3, 13.2 and 94.8 h at the same condition on irradiated corn, dried grapes and coffee beans, respectively. Under optimal conditions (0.973 a_w , 25 °C and seven days of incubation), the OTA production in irradiated corn, peanut and dried grapes were significantly greater than the concentrations obtained by these strains on the medium based on each substrate.

Marín *et al.* [88] developed a suitable validated model to predict the growth and OTA production boundaries by an *A. carbonarius* strain in the function of moisture content and storage temperature of pistachios. These authors showed that OTA accumulation was mainly a function of the temperature of storage, with a sharp increase at <15–20 °C; this value was very different from 30 to 35 °C, which was optimum for growth. This suggests that the substrate combined with certain environmental conditions on which strains are developed affects OTA production. According to these results, significant amounts of OTA on natural substrates could be produced in only five days at $a_w \geq 0.970$ in a wide temperature range. These studies of predictive models represent an essential improvement to the quality and safety of food, allowing the prediction of toxin contamination.

4. Conclusions

In general, temperature and a_w range for OTA production is more restricted than fungal growth, both on natural substrate but also on the culture media based substrate. The knowledge of the ecophysiology of strains belonging to the genera *Aspergillus* section *Nigri* is critical in the development and prediction of risk models for contamination of both raw materials and finished foods with these species under the interaction of several environmental parameters. The use of different storage practices, such as a_w and temperature levels below 0.930 and 15 °C, respectively, allow

controlling fungal contamination and minimizing OTA production in several products as peanuts, corn, dried grapes and derived products for human consumption.

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