Black aspergilli and ochratoxin A in foods.

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Abstract

Ochratoxin A (OTA) is a potent nephrotoxin and carcinogen which is found in a wide variety of common foods and beverages. The black aspergilli are distributed worldwide and are regarded as common food spoilage fungi. These fungi are one of the more difficult groups concerning classification and identification. New molecular approaches have shown that there is a high biodiversity, but that species are occasionally difficult to recognise based solely on their phenotypic characters. Only few species have been confirmed to be OTA producers in this group and fewer are known to contaminate foods with this mycotoxin as a natural occurring contaminant. In this paper, the OTA-producing species included in the *Aspergillus* section *Nigri* and the foods that they are able to contaminate are reviewed in depth.

Introduction

Ochratoxin A (OTA) is a potent nephrotoxin and carcinogen which is found in a wide variety of common foods and beverages [1]. Cereals are considered the major source of human exposure to OTA. For this reason many countries have set a limit for OTA in cereals [2] However, human exposure to OTA is most likely coming from low level contamination of a wide range of different foods. In order to prevent or minimize this risk, the European Union have established maximum OTA levels for many foodstuffs such as cereals, coffee, dried vine fruit, liquorice, spices, wheat gluten and wine [3]. Nonetheless, this regulatory status of OTA lacks consensus in other countries [4].

More than a half century has elapsed since OTA was discovered as a metabolite of *Aspergillus ochraceus* [5••]. A few years later, *Penicillium verrucosum* (as *Penicillium viridicatum*) was reported to produce also this mycotoxin [6•, 7]. Traditionally, OTA contamination of food and feeds was believed to be restricted to *A. ochraceus* and *P. verrucosum*, which affect mainly dried stored foods and cereals respectively, in different regions of the world [8]. Currently, the list of OTA-producing species has expanded and, consequently, the list of foods which can be contaminated with this mycotoxin has also increased. However, although a high number of additional *Aspergillus* species are able to produce OTA, few of them are known to contaminate foods with this mycotoxin [9].

Of these latter species, those include in the *Aspergillus* section *Nigri* (black aspergilli) are becoming of interest because some of them are able to colonize foods other than cereals and cereal by-products and to contaminate them with OTA. In this paper, the OTA-producing species included in this section and the foods that they are able to contaminate are reviewed in depth.

The black aspergilli (*Aspergillus* section *Nigri*)

The black aspergilli are distributed worldwide and are regarded as common food spoilage fungi. Some species are widely used in the biotechnolgy industry and a few of them are involved in animal or human mycoses. These fungi are one of the more difficult groups concerning classification and identification, and several taxonomic schemes have been proposed [10•]. In the last revision of the genus *Aspergillus*, 27 species were accepted in the *Aspergillus* section *Nigri* [11••]. Basic information about these taxa based on the last taxonomic proposals [11••,12, 13, 14•,15] are summarized in Table1. Some of these species are rare or recently described, and only a few strains of them have been studied. Consequently, the distribution of these species it is not well known.

The main morphological character of the most common species (e.g. *A. niger*) included in this section is the colour of the conidial head, which is black or some shade of black. For this reason, these fungi form generally characteristic black or dark brown colonies (Figure 1). However, the colour of the colonies varies not only in different species, but in the same strain, depending upon the culture medium, the age of the culture, the abundance of sporulation or the production of sclerotia, among other factors. Consequently, depending on these factors, some species can form purple-brown, reddish-brown, dark greenish, yellow or orange colonies [16•]. In the conidial head, some species have a single layer of phialides on the vesicle and are named uniseriate species (Table 1 and Figure 1). However, most of them have biseriate conidiophores (Figure 1), showing a layer of metulae on the vesicle and a second layer of phialides over them. These are named biseriate species (Table 1).

New molecular approaches have shown that there is a high biodiversity in this group. However, these species are difficult to recognise based solely on their phenotypic characters [16•]. For example, the taxa related to *A. niger* have always been extremely difficult to distinguish by morphological means. In these fungi, the differences between the described species and varieties in the proposed classifications are very subtle and the number of taxa varies from one author to another [10•]. For this group of fungi, it was proposed the *A. niger* aggregate. The term aggregate is used in mycology for groups of closely related morphospecies only distinguishable with difficultly. Nowadays, this group comprise ten species [11••], including *A. brasiliensis*, *A. niger* and *A. tubingensis* which are the most common species isolated from foods. Although there is a substantial call for searching for new molecular and physiological markers that are usable in the classification of black aspergilli, the concept of several recently described species was only based on genetic differences at one locus [17].

Nevertheless, new polyphasic taxonomic approaches have been proposed for the classification and identification of these fungi [11••, 16•]. These polyphasic studies include sequence analysis of some genes such as ITS-5.8S rRNA region (ITS), and β-tubulin (BenA) and calmodulin (CaM) genes, morphological analyses and characterization of extrolite profiles, among other characters. Although ITS is considered the universal DNA barcode of fungi, these sequences do not contain enough variation for distinguishing among all species in the *Aspergillus* section *Nigri*. A secondary barcode or identification marker usually is needed to identify a black aspergilli culture to species level with confidence [11••]. For example, the uncommon species *A. lacticoffeatus* is characterized by its hair brown to dark blonde colonies which is an important distinguishing feature in its description [18] (Figure 2). However, *A. lacticoffeatus* is very close molecularly to *A. niger* sensu stricto [18]. In fact, this species has been considered a colour mutant of *A. niger* [12]. They have identical ITS sequences and can not be separated by their BenA sequences but can be distinguished using CaM sequence data and have also a different extrolite profile [16•].This new taxonomic approach has also allowed to determine that some black aspergilli which are used in the production of Asian fermented foods and beverages, such as *A. luchuensis*, *A. coreanus*, *A. kawachii* and *A.*

acidus were the same species. *A. luchuensis* was selected as the correct name based on priority [11••, 12, 14•].

In the last ten years, following these taxonomical approaches, the number of described uniseriate species has dramatically increased from two to eleven species (see Table 1). Only, *A. aculeatus* and *A. japonicus* were recognized previously [10•, 18]. Consequently, nowadays, in order to accurately identify black aspergilli, in addition to characterize the micromorphology (e.g. conidial head, conidia) of an isolate is necessary to compare ITS and some protein-coding gene sequences such as BenA and CaM with published sequences in curated International Databases [11••, 19••]. The use of CaM as a temporary secondary identification marker in *Aspergillus* has been suggested [11••]. Using these markers, these taxa can be divided into five main molecular clades (e.g. *A. aculeatus, A. carbonarius, A. heteromorphus, A. homomorphus* and *A. niger* aggregate clades) (see Table 1).

OTA producing species in the black aspergilli

Only a few black aspergilli have been confirmed to be OTA producers and fewer are known to contaminate foods with this mycotoxin as a natural occurring contaminant. In the biseriate species, only five species are considered to be able to produce OTA. In the *A. niger* aggregate, the phylogenetically close species *A. niger* and *A. welwitschiae* are considered OTA producers (Figure 3). They are black aspergilli morphologically indistinguishable.

The common species *A. niger* has a worldwide distribution and the reported percentage of OTA-producing strains in this species is usually very low [10•]. It is one the most commonly reported species from foods (e.g. sun dried products, fresh fruits, nuts, cereals) [1]. Nevertheless, since the first description of OTA production by *A. niger* [20•] this species is achieving a greater significance regarding OTA content in some food commodities such as grapes, raisins and wine, and also in coffee. Interestingly, this species is perhaps the most important mold used in biotechnology. It is worth noting that *A. niger* products hold the GRAS (Generally Regarded as Safe) status from the Food and Drug Administration and is a widely applied industrial species for large-scale biotechnological production of organic acids and enzymes in the food industry. However, some of the most frequently used strains in industry were able to produce this toxin on media suggested for citric acid production [21•]. Among other things, due to their potential for mycotoxin production, no filamentous fungi has the QPS (Qualified Presumption of Safety) status proposed by the European Food Safety Authority [22•].

Regarding *A. welwitschiae*, which was previously called *A. niger* or *A. niger* var. *phoenicis*, most strains produce occasionally OTA. This species has not yet been reported frequently because this old species name has been reintroduced in a recent study about the elucidation of the taxonomic position of some black aspergilli involved in the awamori fermentation [14•]. Consequently, *A. welwitschiae* is expected to be more frequently reported in a near future. It has been found on Welwitschia plants, grapes, dried fruits, coffee, cocoa, and other sources and it has also a worldwide distribution from all the continents [14•].

On the other hand, the rare species *A. lacticoffeatus* which is located in the same molecular subclade as *A. niger* and *A. welwitschiae* (*A. niger*/*welwitschiae* subclade) [11••] is also able to produce OTA over a wide range of temperatures [18, 23] (Figure 3). However, in this case, *A. lacticoffeatus* which was isolated from coffee beans, forms distinctive coffee-with-milk coloured colonies (Figure 2).

Outside this group, *A. carbonarius* is very consistent in producing this mycotoxin, and non-OTA-producing strains in this species are very rare [9, 24, 25•] (Figure 3). In some cases, the suspected non-OTA producing isolates previously identified as *A. carbonarius* belonged actually to other different black aspergilli species [26, 27]. The conidia of *A. carbonarius* are much larger (> 6μm) than those of most of the species in section *Nigri*. A large number of studies have shown that *A. carbonarius* is the main responsible source of OTA in wine or dried vine fruits from main viticultural regions worldwide [28•, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39]. It is also considered a potential source of OTA in coffee. This species has been reported from coffee beans from various coffee-producing countries such as Brazil [40], Philippines [41], Thailand [42, 43] and Vietnam [44]. However, *A. ochraceus* and *A. westerdijkiae* are considered the most significant source of OTA contamination in some coffee varieties [40, 43]. Although it is not so common as *A. niger*, *A. carbonarius* has been also reported from other foods such as figs, maize, paprika, peanuts among others [1].

On the other hand, *A. sclerotioniger* which is phylogenetically close to *A. carbonarius,* has been also confirmed as an OTA producer. This species has also large conidia and produce sclerotia. Nevertheless, *A. sclerotioniger* is known from only one strain (CBS 115572) which was isolated from green Arabica coffee beans in India [18].

Natural occurrence of OTA in maize and maize-based products is a worldwide problem. The available information on the ochratoxigenic mycobiota and OTA presence in corn, corn based food and feed is limited. Several surveys have been shown that *A. niger* and *A. ochraceus* could be the main source of OTA [45]. It is well known that *P. verrucosum* is the major producer of OTA in cereals such as wheat and barley in temperate and cold climates, and , although *A. ochraceus* has been isolated from a wide range of cereals, records are rather infreqüent [46, 47]. However, *A. niger* is frequently isolated from maize [1, 48, 49, 50] and a high incidence of *A. carbonarius* has been also reported in this product [49]. It has been speculated that both species could be a source of OTA in maize and other food products in both tropical and subtropical zones of the world [51]. In fact, both species were able to produce OTA in maize kernels from the fifth day of incubation over a wide range of temperatures and water availabilities [52].

OTA-producing strains of both species have been also reported from cocoa beans. Several studies have shown that *A. carbonarius* and *A. niger* are important fungal sources of OTA in cocoa [53, 54, 55]. However, ochratoxigenic strains of *Aspergillus melleus*, *A. westerdijkiae* and *A. ochraceus* have also been reported [55]. Different molds may contaminate many stages in cocoa processing, and poor practices may have a strong influence on the quality of the beans. OTA is found at all stages of cocoa processing, with the major incidence during drying and storage. In fact, the contamination of cocoa by OTA-producing fungi can already take place in the fermentation, but a considerable increase in the numbers of these species, as well in ochratoxin A contamination is observed during drying and storage [55, 56•]. More systematic surveys on different stages of cocoa production in other important cocoa producing countries are needed in order to confirm the OTA fungal sources in these products.

In addition to cereals, coffee, dried vine fruit and wine, the European Union have established maximum OTA levels also on other foodstuffs such as liquorice and spices, including dried spices, *Piper* spp. (including white and black pepper), *Myristica fragrans* (nutmeg), *Zingiber officinale* (ginger), *Curcuma longa* (turmeric), *Capsicum* spp. (including chillies, chilli powder, cayenne and paprika) and mixtures of spices containing one of the above mentioned spices [3].

At present, the source of OTA in liquorice is not known. In recent studies in China [57, 58], some *Penicillium* spp., such as *P. polonicum* and *P. chrysogenum,* among other fungal species have been reported to be a possible source of OTA in this product. These authors claimed that *P. chrysogenum* derived from surrounding environments was likely to be a stable contributor to high OTA level in liquorice. However, none of these species are considered OTA producers [47, 59]. This should be confirmed using proper chromatographic detection of OTA and accurate identification of the fungi.

Currently, the source of OTA in spices is also unknown. However, *A. niger* has been reported as the most frequently isolated species in *Piper* spp [60, 61], *Myristica fragrans* [62], *Zingiber officinale* [61], *Curcuma longa* [61] and *Capsicum* spp. [61, 63, 64, 65]. Some other potential OTA-producing species such as *A. ochraceus* and *A. westerdijkiae* were also reported in some of these studies, but less frequently. Interestingly, solid-state fermentation with *A. niger* is used in the process of pepper peeling. So, some OTA-producing starter strains of this species could be the OTA source in this product. This kind of fermentation is widely used in traditional Chinese food fermentation due to its easy operation, low cost, and wide feasibility on farms and in the countryside [60].

Some strains of other black aspergilli such as *A. awamori*, *A. usamii* and *A. foetidus* have been also cited as OTA producers [10, 66]. However, their identity has been questioned [14•, 18]. Most *A. awamori* strains isolated from oriental food fermentation process could be accommodated into *A. niger* group, such as *A. luchuensis*, *A. niger*, *A. tubingensis* or *A. welwitschiae*. In fact, the neotype of *A. awamori* (CBS 557.65) did not originate from awamori fermentation and it was shown to be identical with *A. welwitschiae* [14•].

The ability of *A. tubingensis* to produce OTA remains a controversial issue. Some strains of this species have been cited as OTA producers [67, 68, 69]. However, *A. tubingensis* is not considered an OTA producer [10•, 18, 21] (Figure 3). In fact, a high number of strains of this species tested by other authors were not able to produce OTA [28•, 29, 37, 70, 71, 72, 73, 74]. Very recently, none of the 261 *A. tubingensis* strains isolated from wine grapes was found to be ochratoxigenic when they were analysed with UPLC-MS/MS [74]. Nevertheless, some of these isolates were initially considered to be able to produce the toxin when they were screened by HPLC-FLD. Consequently, OTA production by strains of *A. tubingensis* and their taxonomical identity, should be confirmed using proper techniques.Similarly, none of the uniseriate species are considered to be able to produce OTA [66, 71, 73, 75, 76, 77]. Although the ability of some of these species to produce OTA has been mentioned [30, 78, 79, 80], this fact needs to be confirmed.

As an unusual OTA-producing species is proposed, an accurate identification of the isolate and a proper detection of the OTA production must be carried out in different culture media and conditions in order to confirm that is a new OTA fungal source (Figure 1). OTA confirmation by mass spectrometry is recommended (Figure 4). In order to avoid the chronic problem of misidentification of mycotoxigenic fungi some useful recommendations have been proposed [9, 72, 81].

Conclusions

Aspergillus section *Nigri* is one of the more difficult fungal groups concerning classification and identification. Only a few of these fungi are able to produce OTA. Some of these OTA-producing species can contaminate a wide variety of foods and beverages. Nowadays, we know that *A. carbonarius* is the main responsible source of OTA in wine or dried vine fruits from main viticultural regions worldwide. Although there is clear evidence of the participation of *A. carbonarius* and *A. niger* on the OTA contamination of cocoa and coffee, their exactly role have not been stated. On the other hand, it is not always clear which black aspergilli species are responsible for OTA contamination in other foods. While *A. niger* and *A. welwitschiae* are usually reported from a wide variety of foods, their role as a source of OTA is not well known. In fact, *A. niger* is frequently isolated from some EU regulated foods such as liquorice and spices. Nevertheless, the source of OTA in these foods has not been identified yet. More systematic research is needed to confirm which black aspergilli species are responsible for the OTA contamination in these and other commodities.

References

[1] Pitt JI, Hocking AD (Eds). *Fungi and food spoilage*. Springer; 2009.

[2] Food and Agriculture Organization of the United Nations: **Worldwide regulations for mycotoxins in food and feed 2003**; *FAO Food and Nutrition Paper* 2004, No. 81.

[3] Commission Regulation (EC) No. 1881/2006 with ammendments.

[4] Duarte SC, Lino CM, Pena A: **Mycotoxin food and feed regulation and the specific case of ochratoxin A: a review of the worldwide status.** *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* 2010, **27**:1440-1450.

[5] van der Merwe KJ, Steyn PS, Fourie L, Scott DB, Theron JJ: **Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus* Wilh.** Nature 1965, **205**:1112-1113.

•• In this seminal paper, the isolation and characterization of ochratoxin A was reported for the first time.

[6] Van Walbeek W, Scott PM, Harwig J, Lawrence JW: ***Penicillium viridicatum* Westling: a new source of ochratoxin A.** *Can J Microbiol* 1969, **15**:1281-1285.

• In this paper, the production of ochratoxin A by a species of *Penicillium* is reported for the first time.

[7] Pitt JI: ***Penicillium viridicatum*, *Penicillium verrucosum*, and production of ochratoxin A.** *Appl Environ Microbiol* 1987, **53**: 266-269.

[8] Pitt JI, Hocking AD (Eds). *Fungi and food spoilage*. Blackie Academic and Professional; 1997.

[9] Cabañes FJ, Bragulat MR: **Ochratoxin A in profiling and speciation.** *In* Aspergillus *in the genomic era*. Edited by Varga J, Samson RA. Wageningen Academic Publishers; 2008: 57-70.

[10] Abarca ML, Accensi F, Cano J, Cabañes FJ. **Taxonomy and significance of black aspergilli.** *Antonie Van Leeuwenhoek* 2004, **86**: 33-49.

• This paper provides an overview of the significance of black aspergilli focusing on all the approaches made in the taxonomy of such fungi.

[11] Samson RA, Visagie CM, Houbraken J, Hong SB, Hubka V, Klaassen CHW, Perrone G, Seifert KA, Susca A, Tanney JB *et al*. **Phylogeny, identification and nomenclature of the genus *Aspergillus*.** *Stud Mycol* 2014, **78**:141-173.

•• In this paper, the last revision on the phylogeny, identification and nomenclature of the genus *Aspergillus* is presented.

[12] Varga J, Frisvad JC, Kocsubé S, Brankovics B, Tóth B, Szigeti G, Samson RA: **New and revisited species in *Aspergillus* section *Nigri*.** *Stud Mycol* 2011, **69**:1-17. doi: 10.3114/sim.2011.69.01.

[13] Jurjević Z, Peterson SW, Stea G, Solfrizzo M, Varga J, Hubka V, Perrone G. **Two novel species of *Aspergillus* section *Nigri* from indoor air.** *IMA Fungus*, 2012, **3**: 159-173.

[14] Hong SB, Lee M, Kim DH, Varga J, Frisvad JC, Perrone G, Gomi K, Yamada O, Machida M, Houbraken J, Samson RA. ***Aspergillus luchuensis*, an industrially important black *Aspergillus* in East Asia.** PLoS One. 2013 May 28;8(5):e63769. doi: 10.1371/journal.pone.0063769.

• In this paper, the taxonomical situation of some black aspergilli used for awamori, shochu, makgeolli and other food and beverage fermentations is revised.

[15] Fungaro MHP, Ferranti LS, Massi FP, da Silva JJ, Sartori D, Taniwaki MH, Frisvad JC, Iamanaka BT: ***Aspergillus labruscus* sp. nov., a new species of *Aspergillus* section *Nigri* discovered in Brazil.** *Sci Rep* 2017 Jul 24;7(1):6203. doi: 10.1038/s41598-017-06589-y.

[16] Samson RA, Noonim P, Meijer M, Houbraken J, Frisvad JC, Varga J: **Diagnostic tools to identify black aspergilli.** *Stud Mycol* 2007, **59**: 129-145. doi: 10.3114/sim.2007.59.13.

• In this paper, a pictorial overview of phenotypic and molecular methods to identify black aspergilli is presented.

[17] Hubka V, Kolarick M: **β-tubulin paralogue *tubC* is frequently misidentified as the *benA* gene in *Aspergillus* section *Nigri* taxonomy: primer specificity testing and taxonomic consequences.** *Persoonia* 2012, **29**: 1-10.

[18] Samson RA, Houbraken JAMP, Kuijers AFA, Frank JM, Frisvad J. **New ochratoxin A or sclerotium producing species in *Aspergillus* section *Nigri*.** *Stud Mycol* 2004, **50**: 45-61.

[19] Hibbett D, Abarenkov K, Kõljalg U, Öpik M, Chai B, Cole J, Wang Q, Crous P, Robert V, Helgason T *et al*. **Sequence-based classification and identification of Fungi.** *Mycologia* 2016, **108**:1049-1068.

•• This paper provides an interesting perspective on the potential of sequence-based classification and identification of fungi and identify its conceptual challenges.

[20] Abarca ML, Bragulat MR, Castella G, Cabañes FJ. **Ochratoxin A production by strains of *Aspergillus niger* var. *niger*.** *Appl Environ Microbiol* 1994, **60**: 2650-2652.

• In this paper, the production of ochratoxin A by *A. niger* is reported for the first time.

[21] Frisvad JC, Larsen TO, Thrane U, Meijer M, Varga J, Samson RA, Nielsen KF. **Fumonisin and ochratoxin production in industrial *Aspergillus niger* strains.** PLoS One. 2011;6(8):e23496.

• In this paper, the production of fumonisins and ochratoxin A by some of the most frequently used *A. niger* strains in industry is confirmed.

[22] European Food Safety Authority (EFSA). **Introduction of a qualified presumption of safety (QPS) approach for assessment of selected microorganisms referred to EFS (Question No EFSA-Q-2005-293).** *The EFSA Journal* 2007, 587: 1-16.

•This EFSA scientific opinion introduces the qualified presumption of safety (QPS) approach to assess the safety of a broad range of biological agents in the context of notifications for market authorisation as sources of food and feed additives, enzymes and plant protection products.

[23] Alborch L, Bragulat MR, Abarca ML, Cabañes FJ. **Temperature and incubation time effects on growth and ochratoxin A production by *Aspergillus sclerotioniger* and *Aspergillus lacticoffeatus* on culture media.** *Let Appl Microbiol* **52**, 208-212.

[24] Frisvad JC, Larsen TO, de Vries R, Meijer M, Houbraken J, Cabañes FJ, Ehrlich K, Samson RA. **Secondary metabolite profiling, growth profiles and other tools for species recognition and important *Aspergillus* mycotoxins.** *Studies in Mycology* 2004, **59**: 31-37.

[25] Cabañes FJ, Bragulat MR, Castellá G. **Characterization of nonochratoxigenic strains of *Aspergillus carbonarius* from grapes.** Food Microbiol 2013, **36**: 135-141.

• In this paper, nonochratoxigenic wild strains of *A. carbonarius* were characterized in deep for the first time.

[26] Bau M, Castellá G, Bragulat MR, Cabañes FJ. **DNA based characterization of ochratoxin A-producing and non producing *Aspergillus carbonarius* strains from grapes.** *Res Microbiol* 2005, **156**: 375-381.

[27] Serra R, Cabañes FJ, Perrone G, Castellá G, Venâncio A, Mulè G, Kozakiewicz Z. ***Aspergillus ibericus*: a new species of the section Nigri isolated from grapes.** Mycologia 2006, **98**: 295-306.

[28] Cabañes FJ, Accensi F, Bragulat MR, Abarca ML, Castellá G, Mínguez S and Pons A. **What is the source of ochratoxin A in wine?** *Int J Food Microbiol* 2002, **79**: 213-215.

•In this paper, a strong evidence of the contribution of *A. carbonarius* in the OTA contamination in wine is reported for the first time.

[29] Abarca ML, Accensi F, Bragulat MR, Castella G, Cabañes FJ. ***Aspergillus carbonarius* as the main source of ochratoxin A contamination in dried vine fruits from the Spanish market.** *J Food Prot* 2003, **66**: 504-506.

[30] Battilani P, Pietri A, Bertuzzi T, Languasco L, Giorni P, Kozakiewicz Z. **Occurrence of ochratoxin A-producing fungi in grapes grown in Italy.** *J Food Prot* 2003, **66**: 633-636.

[31] Bau M, Bragulat MR, Abarca ML, Mínguez S, Cabañes FJ. **Ochratoxigenic species from Spanish wine grapes.** *Int J Food Microbiol* 2005, **98**: 125-130.

[32] Serra R, Braga A, Venâncio A. **Mycotoxin-producing and other fungi isolated from grapes for wine production, with particular emphasis on ochratoxin A.** *Res Microbiol* 2005, **156**: 515-521.

[33] Bejaoui H, Mathieu F, Taillandier P, Lebrihi A. **Black aspergilli and ochratoxin A production in French vineyards.** *Int J Food Microbiol* 2006, **111** (Suppl. 1), S46-S52.

[34] Tjamos SE, Antoniou PP, Tjamos EC. ***Aspergillus* spp., distribution, population composition and ochratoxin A production in wine producing vineyards in Greece.** *Int J Food Microbiol* 2006, **111** (Suppl. 1): S61-S66.

[35] Chulze SN, Magnoli CE, Dalcero AM. **Occurrence of ochratoxin A in wine and ochratoxigenic mycoflora in grapes and dried vine fruits in South America.** *Int J Food Microbiol* 2006, **111** (Suppl. 1): S5-S9.

[36] Gómez C, Bragulat MR, Abarca ML, Mínguez S, Cabañes FJ. **Ochratoxin A-producing fungi from grapes intended for liqueur wine production.** *Food Microbiol* 2006, **23**: 541-545.

[37] Leong SL, Hocking AD, Scott ES. ***Aspergillus* species producing ochratoxin A: isolation from vineyard soils and infection of Semillon bunches in Australia.** *J Appl Microbiol* 2007, **102**: 124-133.

[38] Díaz GA, Torres R, Vega M, Latorre BA. **Ochratoxigenic *Aspergillus* species on grapes from Chilean vineyards and Aspergillus threshold levels on grapes.** *Int J Food Microbiol* 2009, **133**:195-199.

[39] Palumbo JD, O'Keeffe TL, Vasquez SJ, Mahoney NE. **Isolation and identification of ochratoxin A-producing *Aspergillus* section *Nigri* strains from California raisins.** Lett Appl Microbiol 2011, **52**: 330-336.

[40] Taniwaki MH, Pitt JI, Teixeira AA, Iamanaka BT. **The source of ochratoxin A in Brazilian coffee and its formation in relation to processing methods.** *Int J Food Microbiol* 2003, **82**: 173-179.

[41] Alvindia DG, de Guzman MF. **Survey of Philippine coffee beans for the presence of ochratoxigenic fungi.** *Mycotoxin Res* 2016, **32**: 61-67.

[42] Joosten HM, Goetz J, Pittet A, Schellenberg M, Bucheli P. **Production of ochratoxin A by *Aspergillus carbonarius* on coffee cherries.** *Int J Food Microbiol* 2001, **65**: 39-44.

[43] Noonim P, Mahakarnchanakul W, Nielsen KF, Frisvad JC, Samson RA. **Isolation, identification and toxigenic potential of ochratoxin A-producing *Aspergillus* species from coffee beans grown in two regions of Thailand.** *Int J Food Microbiol* 2008, **128**:197-202.

[44] Leong SL, Hien LT, An TV, Trang NT, Hocking AD, Scott ES. **Ochratoxin A-producing aspergilli in Vietnamese green coffee beans.** *Lett Appl Microbiol* 2007, **45**: 301-306.

[45] Magnoli CE, Astoreca AL, Chiacchiera SM, Dalcero AM. **Occurrence of ochratoxin A and ochratoxigenic mycoflora in corn and corn based foods and feeds in some South American countries.** *Mycopathologia* 2007, **163**: 249-260.

[46] Pitt JI. **Toxigenic fungi: which are important?** *Med Mycol* 2000, **38** Suppl 1:17-22.

[47] Cabañes FJ, Bragulat MR, Castellá G. **Ochratoxin A producing species in the genus *Penicillium*.** *Toxins* 2010, **2**: 1111-1120.

[48] Magnoli C, Hallak C, Astoreca A, Ponsone L, Chiacchiera S, Dalcero AM. **Occurrence of ochratoxin A-producing fungi in commercial corn kernels in Argentina.** *Mycopathologia* 2006, **161**: 53-58.

[49] Shah HU, Simpson TJ, Alam S, Khattak KF, Perveen S. **Mould incidence and mycotoxin contamination in maize kernels from Swat Valley, North West Frontier Province of Pakistan**. *Food Chem Toxicol* 2010, **48**: 1111-1116.

[50] Wicklow DT. **Patterns of fungal association within maize kernels harvested in North Carolina.** *Plant Dis* 1988, **72**: 113-115.

[51] Palencia ER, Hinton DM, Bacon CW. **The black *Aspergillus* species of maize and peanuts and their potential for mycotoxin production.** *Toxins* 2010, **2**: 399-416.

[52] Alborch L, Bragulat MR, Abarca ML, Cabañes FJ. **Effect of water activity, temperature and incubation time on growth and ochratoxin A production by *Aspergillus niger* and *Aspergillus carbonarius* on maize kernels.** *Int J Food Microbiol* 2011, **147**: 53-57.

[53] Mounjouenpou P, Gueule D, Fontana-Tachon A, Guyot B, Tondje PR, Guiraud JP. **Filamentous fungi producing ochratoxin a during cocoa processing in Cameroon.** *Int J Food Microbiol* 2008, 121: 234-241.

[54] Sánchez-Hervás M, Gil JV, Bisbal F, Ramón D, Martínez-Culebras PV. **Mycobiota and mycotoxin producing fungi from cocoa beans.** *Int J Food Microbiol* 2008, 125: 336-340.

[55] Copetti MV, Pereira JL, Iamanaka BT, Pitt JI, Taniwaki MH. **Ochratoxigenic fungi and ochratoxin A in cocoa during farm processing.** *Int J Food Microbiol* 2010, **143**: 67-70.

[56] Copetti MV, Iamanaka BT, Pitt JI, Taniwaki MH. **Fungi and mycotoxins in cocoa: from farm to chocolate.** *Int J Food Microbiol* 2014, **178**: 13-20.

•This paper is one of the most detailed reviews regarding the problem of the mycotoxins in cocoa.

[57] Chen AJ, Huang LF, Wang LZ, Tang D, Cai F, Gao WW. **Occurrence of toxigenic fungi in ochratoxin A contaminated liquorice root.** *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* 2011, **28**: 1091-1097.

[58] Chen AJ, Tang D, Zhou YQ, Sun BD, Li XJ, Wang LZ, Gao WW. **Identification of ochratoxin A producing fungi associated with fresh and dry liquorice.** *PLoS One* 2013, Oct 21;8(10):e78285. doi: 10.1371/journal.pone.0078285. eCollection 2013.

[59] Nguyen HDT, McMullin DR, Ponomareva E, Riley R, Pomraning KR, Baker SE, Seifert KA. **Ochratoxin A production by *Penicillium thymicola*.** *Fungal Biol* 2016, **120**: 1041-1049.

[60] Hu Q, Zhang J, Xu C, Li C, Liu S. **The dynamic microbiota profile during pepper (*Piper nigrum* L.) peeling by solid-state fermentation.** *Curr Microbiol* 2017, **74**: 739-746.

[61] Jeswal P, Kumar D. **Mycobiota and natural incidence of aflatoxins, ochratoxin A, and citrinin in Indian spices confirmed by LC-MS/MS.** *Int J Microbiol* 2015, 2015:242486. doi: 10.1155/2015/242486. Epub 2015 Jul 2.

[62] Rajeshwari P, Raveesha K. **Mycological analysis and aflatoxin B1 contaminant estimation of herbal drug raw materials.** *Afr J Tradit Complement Altern Med* 2016, **13**:123-131.

[63] Melo González MG, Romero SM, Arjona M, Larumbe AG, Vaamonde G. **Microbiological quality of Argentinian paprika.** *Rev Argent Microbiol* 2017, May 29. pii: S0325-7541(17)30043-3. doi: 10.1016/j.ram.2017.02.006. [Epub ahead of print]

[64] Santos L, Marín S, Mateo EM, Gil-Serna J, Valle-Algarra FM, Patiño B, Ramos AJ. **Mycobiota and co-occurrence of mycotoxins in *Capsicum* powder.** *Int J Food Microbiol* 2011, **151**: 270-276.

[65] Martín A, Aranda E, Benito MJ, Pérez-Nevado F, Cordoba MG. **Identification of fungal contamination and determination of mycotoxigenic molds by micellar electrokinetic capillary chromatography in smoked paprika.** *J Food Prot* 2005, 68: 815-822.

[66] Téren J, Varga J, Hamari Z, Rinyu E, Kevei F. **Immunochemical detection of ochratoxin A in black *Aspergillus* strains.** *Mycopathologia* 1996, **134**: 171-176.

[67] Medina A, Mateo R, López-Ocaña L, Valle-Algarra FM, Jiménez M. **Study of Spanish grape mycobiota and ochratoxin A production by isolates of *Aspergillus tubingensis* and other members of *Aspergillus* section *Nigri*.** *Appl Environ Microbiol* 2005, **71**: 4696-4702.

[68] Perrone G, Mulè G, Susca A, Battilani P, Pietri A, Logrieco A. **Ochratoxin A production and amplified fragment length polymorphism analysis of *Aspergillus carbonarius*, *Aspergillus tubingensis*, and *Aspergillus niger* strains isolated from grapes in Italy.** *Appl Environ Microbiol* 2006, **72**: 680-685.

[69] Lahouar A, Marin S, Crespo-Sempere A, Saïd S, Sanchis V. **Influence of temperature, water activity and incubation time on fungal growth and production of ochratoxin A and zearalenone by toxigenic *Aspergillus tubingensis* and *Fusarium incarnatum* isolates in sorghum seeds.** *Int J Food Microbiol* 2017, **242**: 53-60.

[70] Accensi F, Abarca ML, Cano J, Figuera L, Cabañes FJ. **Distribution of ochratoxin a producing strains in the *A. niger* aggregate.** *Antonie van Leeuwenhoek* 2001, **79**: 365-370.

[71] Bau M, Castellá G, Bragulat MR, Cabañes FJ. **RFLP characterization of *Aspergillus niger* aggregate species from grapes from Europe and Israel.** *Int J Food Microbiol* 2006, **111**: S18-S21.

[72] Storari M, Bigler L, Gessler C, Broggini GA. **Assessment of the ochratoxin A production ability of *Aspergillus tubingensis*.** Food Addit Contam Part A Chem Anal Control Expo Risk Assess 2012, **29**:1450-1454.

[73] Lamboni Y, Nielsen KF, Linnemann AR, Gezgin Y, Hell K, Nout MJ, Smid EJ, Tamo M, van Boekel MA, Hoof JB, Frisvad JC: **Diversity in secondary metabolites including mycotoxins from strains of *Aspergillus* section *Nigri* isolated from raw cashew nuts from Benin, West Africa.** *PLoS One* 2016, Oct 21;11(10):e0164310. doi: 10.1371/journal.pone.0164310. eCollection 2016

[74] Pantelides IS, Aristeidou E, Lazari M, Tsolakidou MD, Tsaltas D, Christofidou M, Kafouris D, Christou E, Ioannou N. **Biodiversity and ochratoxin A profile of *Aspergillus* section *Nigri* populations isolated from wine grapes in Cyprus vineyards.** *Food Microbiol* 2017, **67**: 106-115.

[75] Parenicova L, Skouboe P, Frisvad J, Samson RA, Rossen L, Ten Hoor-Suykerbuyk M, Visser J. **Combined molecular and biochemical approach identifies *Aspergillus japonicus* and *Aspergillus aculeatus* as two species.** *Appl Environ Microbiol* 2001, **67**: 51-527.

[76] Qi TF, Renaud JB, McDowell T, Seifert KA, Yeung KK, Sumarah MW. **Diversity of mycotoxin-producing black aspergilli in Canadian vineyards.** *J Agric Food Chem* 2016, **64**: 1583-1589.

[77] Garmendia G, Vero S. **Occurrence and biodiversity of Aspergillus section Nigri on 'Tannat' grapes in Uruguay.** *Int J Food Microbiol* 2016, **216**: 31-39.

[78] Dalcero A, Magnoli C, Hallak C, Chiachiera SM, Palacio G, Rosa CAR. **Detection of ochratoxin A in animal feeds and capacity to produce this mycotoxin by *Aspergillus* section *Nigri* in Argentina.** *Food Add Contam* 2002, **19**: 1065-1072.

[79] Ponsone ML, Combina M, Dalcero A, Chulze S. **Ochratoxin A and ochratoxigenic *Aspergillus* species in Argentinean wine grapes cultivated under organic and non-organic systems.** *Int J Food Microbiol* 2007, **114**: 131-135.

[80] Zhang X, Li Y, Wang H, Gu X, Zheng X, Wang Y, Diao J, Peng Y, Zhang H. **Screening and identification of novel ochratoxin A-producing fungi from grapes.** *Toxins* 2016, **8**, 333; doi:10.3390/toxins8110333.

[81] Frisvad JC, Nielsen KF, Samson RA. **Recommendations concerning the chronic problem of misidentification of mycotoxigenic fungi associated with foods and feeds.** *Adv Exp Med Biol* 2006, **571**: 33-46.

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Conflict of interests

None.

Table 1. Current accepted species in the *Aspergillus* section *Nigri*, their main ecological characteristics and OTA production.

Species, authorities and year of the description	Molecular clade	Conidial head	Ecology, main habitats and distribution	OTA production [a] / OTA source [b]
A. aculeatinus, Noonim *et al.*, 2008	*A. aculeatus*	Uniseriate	Coffee beans, Thailand	-
A. aculeatus, Iizuka, 1953	*A. aculeatus*	Uniseriate	Tropical soil, unknown, air, USA, *Lactuca sativa*, Indonesia, dead branches, Papua	-
A. brasiliensis, Varga *et al.*, 2007	*A. niger* aggregate	Biseriate	Soil, wine grapes, worldwide	-
A. brunneoviolaceus, Batista & Maia, 1957	*A. aculeatus*	Uniseriate	Air, Trinidad and Tobago, and USA	-
A. carbonarius, (Bainier) Thom, 1916	*A. carbonarius*	Biseriate	Soil, wine grapes, coffee, figs, maize, paprika, peanuts, worldwide	+ / Wine, dried vine fruits and other grape products (Cocoa, coffee) [b]
A. costaricaensis, Samson & Frisvad, 2004	*A. niger* aggregate	Biseriate	Soil, Costa Rica	-
A. ellipticus, Raper & Fennell, 1965	*A. heteromorphus*	Biseriate	Soil, Costa Rica	-
A. eucalypticola, Varga *et al.*, 2011	*A. niger* aggregate	Biseriate	Leaves of *Eucalyptus* sp. , Australia	-
A. floridensis, Jurjević *et al.*, 2012	*A. aculeatus*	Uniseriate	Air, USA and Martinique, soil, Japan, almonds USA	-
A. heteromorphus, Batista & Maia, 1957	*A. heteromorphus*	Biseriate	Fungal culture contaminant, Brazil	-
A. homomorphus, (Steiman *et al.*) Samson & Frisvad, 2004	*A. homomorphus*	Biseriate	Soil of death sea area, Israel	-
A. ibericus, Serra *et al.*, 2006	*A. carbonarius*	Biseriate	Wine grapes and raisins, Portugal and Spain	-
A. indologenus, Frisvad *et al.*, 2011	*A. aculeatus*	Uniseriate	Soil, India	-
A. japonicus, Saito, 1906	*A. aculeatus*	Uniseriate	Soil, Brazil	-
A. labruscus, Fungaro *et al.*, 2017	*A. homomorphus*	Uniseriate	Grapes for juice production, Brazil	-
A. lacticoffeatus, Frisvad & Samson, 2004	*A. niger* aggregate	Biseriate	Coffee beans, Indonesia and Venezuela	+/ (coffee) [b]
A. luchuensis, Inui, 1901	*A. niger* aggregate	Biseriate	East Asia, food fermentation environment	-
A. neoniger, Varga *et al.*, 2011	*A. niger* aggregate	Biseriate	Mangrove water, Venezuela, and desert sand, Namibia	-
A. niger, van Tieghem, 1867	*A. niger* aggregate	Biseriate	Worldwide, cosmopolitan fungus	+ / (many foods) [b]
A. piperis, Samson & Frisvad, 2004	*A. niger* aggregate	Biseriate	Grounded black pepper of tropical origin	-
A. saccharolyticus, Sørensen *et al.*, 2011	*A. homomorphus*	Uniseriate	Oak wood, Denmark	-
A. sclerotiicarbonarius, Noonim *et al.*, 2008	*A. carbonarius*	Biseriate	Coffee beans, Thailand	-
A. sclerotioniger, Samson & Frisvad, 2004	*A. carbonarius*	Biseriate	Green Arabica coffee, India	+ / (coffee) [b]
A. trinidadensis, Jurjević *et al.*, 2012	*A. aculeatus*	Uniseriate	Air, Trinidad and Tobago, and USA	-
A. tubingensis, Mosseray, 1934	*A. niger* aggregate	Biseriate	Worldwide, cosmopolitan fungus	-
A. uvarum, Perrone *et al.*, 2008	*A. aculeatus*	Uniseriate	Wine grapes, Europe and Israel , air, USA	-
A. vadensis, Samson *et al.*, 2005	*A. niger* aggregate	Biseriate	Air, Egypt	-
A. welwitschiae, (Bresadola) Hennings apud Wehmer, 1907	*A. niger* aggregate	Biseriate	Grapes, dried fruits, coffee, cocoa, worldwide	+ / (many foods) [b]

[a] OTA production: +, OTA producing species; -, non-OTA producing species

[b] (potential source)

Data are from Cabañes & Bragulat [9], Samson *et al.* [11], Varga *et al.* [12], Jurjević *et al.* [13], Hong *et al.* [14], Fungaro *et al.* [15], Samson *et al.* [18], Taniwaki *et al.* [40], Copetti *et al* [56].

Legends

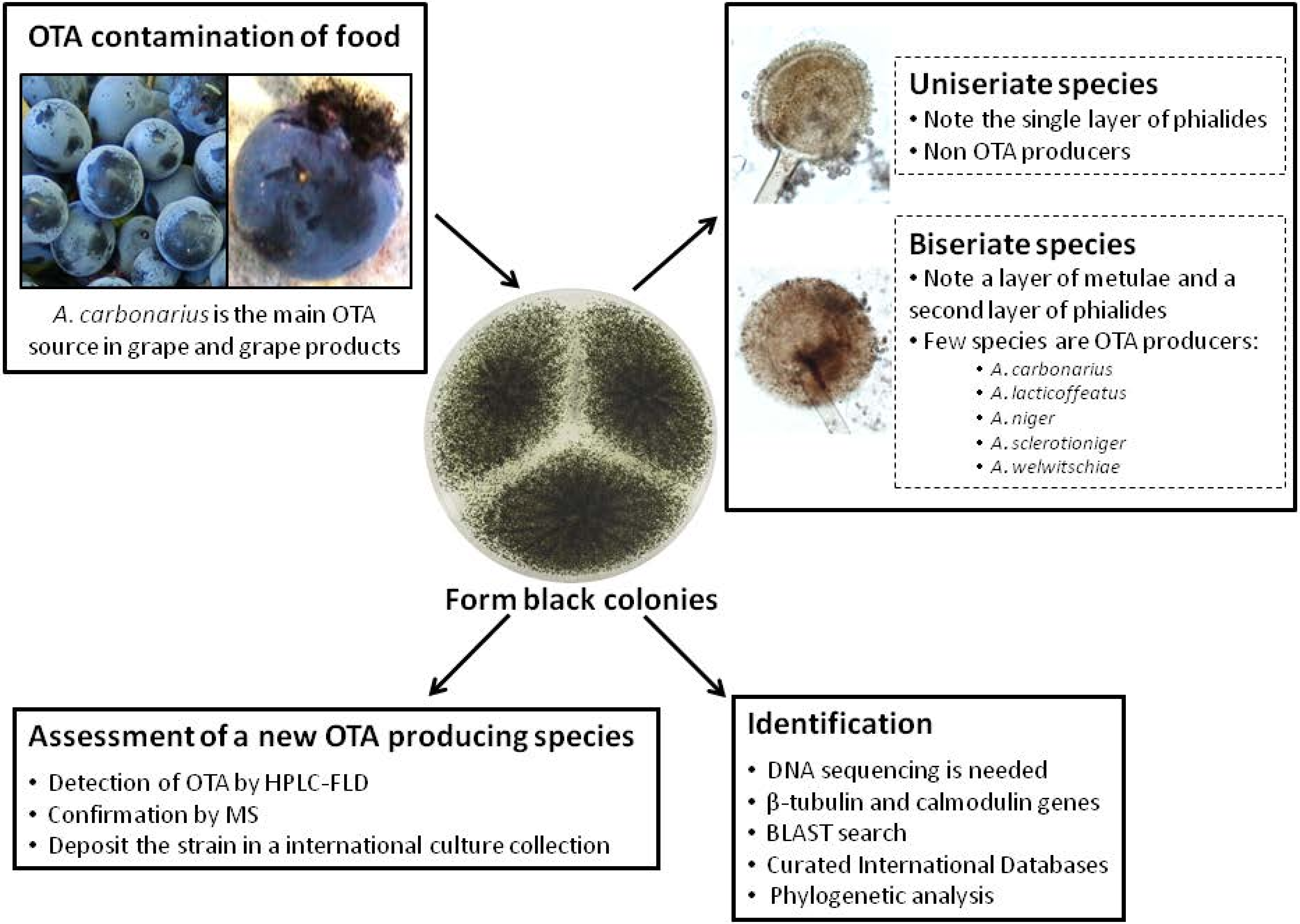
Figure 1. Importance, detection and identification of black aspergilli in foods.

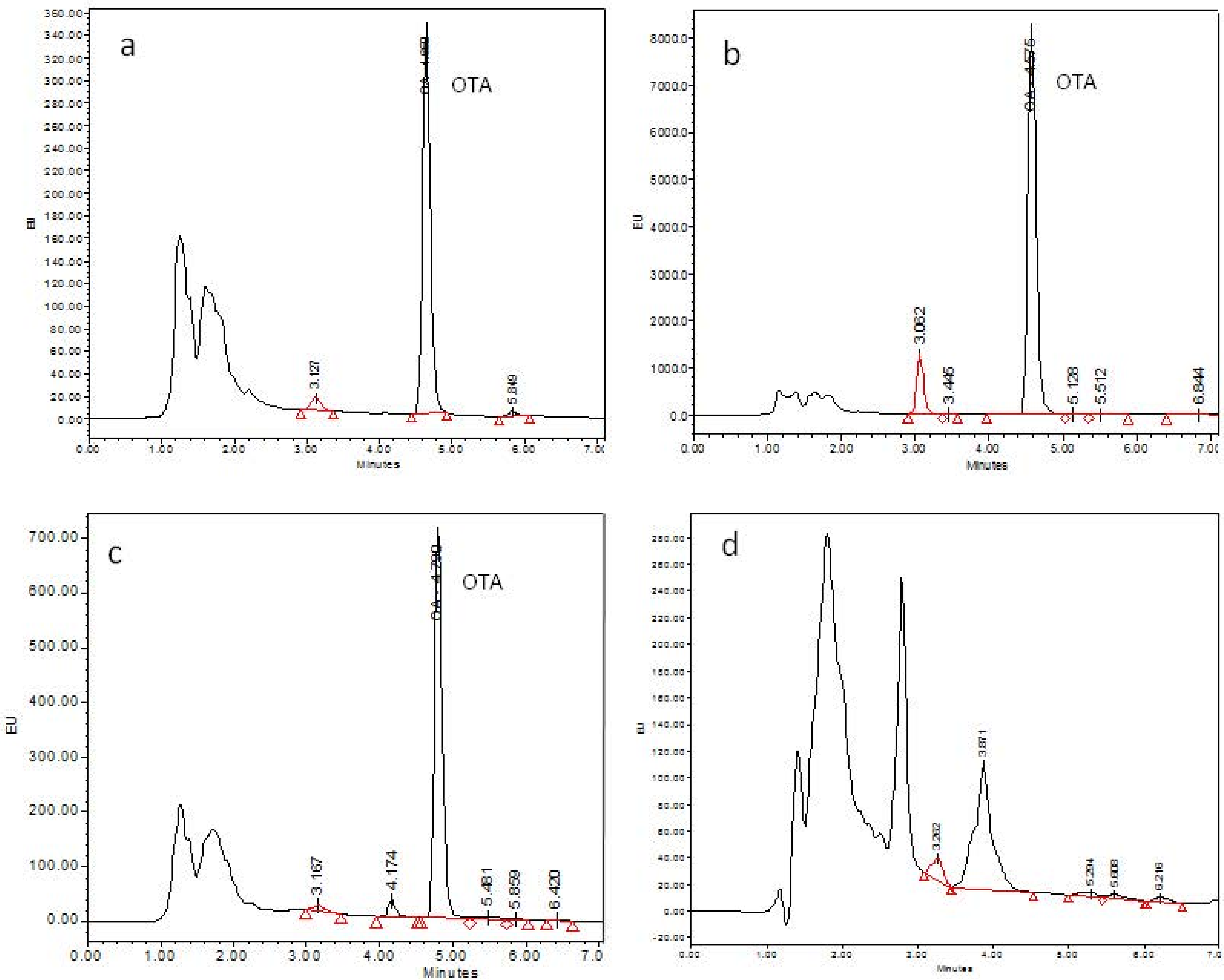
(This figure summarizes the main concepts discussed)

Figure 2. Colonial morphology of *A. lacticoffeatus* (CBS 101883), grown on Czapek Yeast extract Agar at 30ºC for 10 days. Note the distinctive coffee-with-milk colour of the colony.

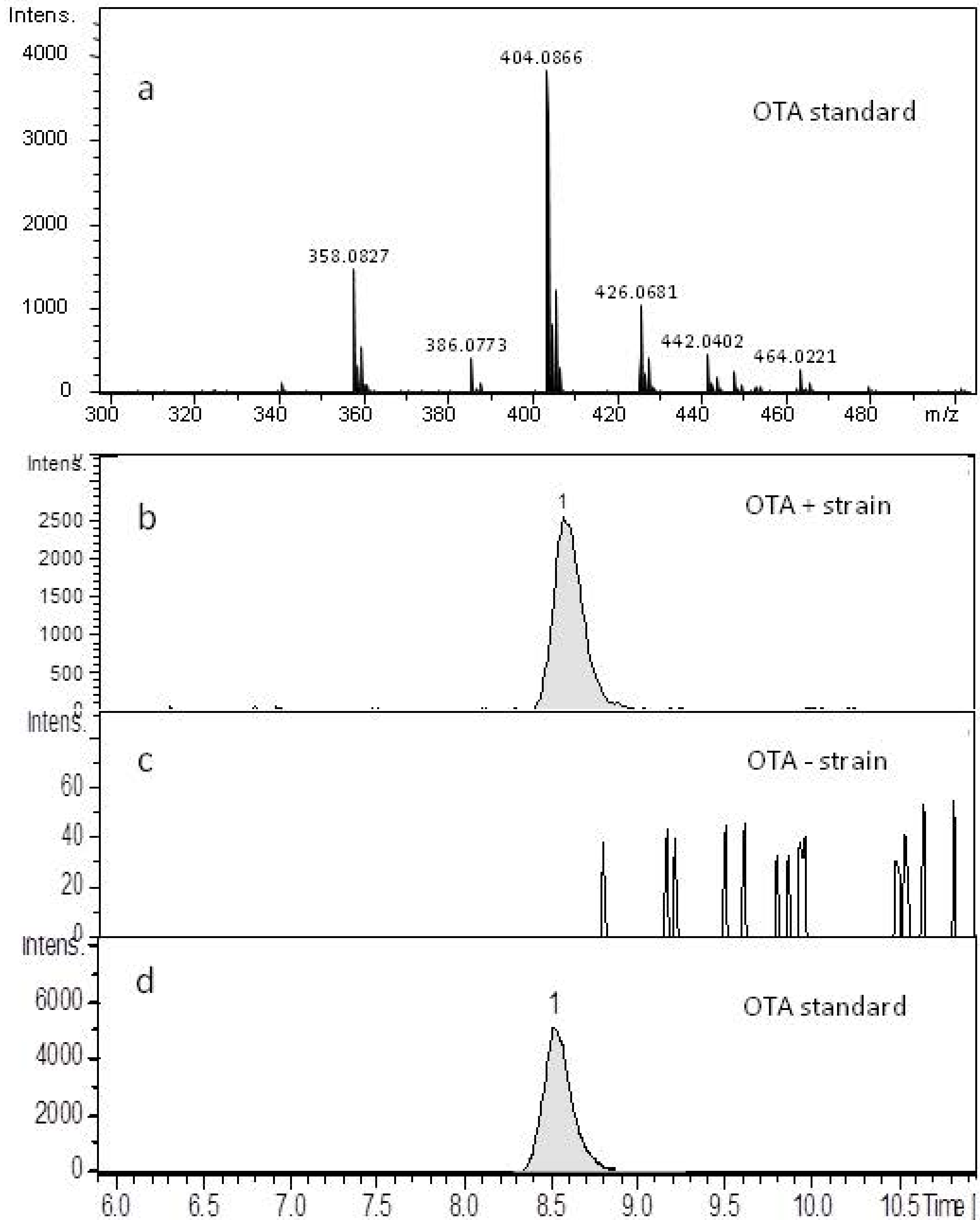
Figure 3. Selected chromatograms of fungal extracts analysed using HPLC coupled to a fluorescence detector of (a) an OTA-producing strain of *A. carbonarius*, (b) an OTA-producing strain of *A. niger*, (c) an OTA-producing strain of *A. lacticoffeatus* and (d) a non-OTA-producing strain of *A. tubingensis*.

Figure 4. Electrospray ionisation–mass spectrometry spectrum of OTA (a) (major ions:*m*/*z* 358.08 [MH-HCOOH]+, *m*/*z* 404.09 [MH]+ and*m*/*z* 426.07 [MNa]+) and selected extracted ion chromatograms of fungal extracts of an OTA-producing strain of *A. carbonarius* (b) and a non-OTA-producing strain of *A. carbonarius* (c) analysed using HPLC-MS. OTA standard (d).


OTA contamination of food
A. carbonarius is the main OTA source in grape and grape products
Uniseriate species
• Note the single layer of phialides
• Non OTA producers
Biseriate species
• Note a layer of metulae and a second layer of phialides
• Few species are OTA producers:
• A. carbonarius
• A. lacticoffeatus
• A. niger
• A. sclerotioniger
• A. welwitschiae
Form black colonies
Assessment of a new OTA producing species
• Detection of OTA by HPLC-FLD
• Confirmation by MS
• Deposit the strain in a international culture collection
Identification
• DNA sequencing is needed
• β-tubulin and calmodulin genes
• BLAST search
• Curated International Databases
• Phylogenetic analysis



a
OTA
3.127
5.849
EU
Minutes
b
OTA
3.062
3.445
4.576
5.128
5.512
6.844
EU
Minutes
c
OTA
3.167
4.174
4.790
5.481
5.859
6.420
EU
Minutes
d
3.262
3.871
5.294
5.608
6.216
EU
Minutes


Intens.
4000
3000
2000
1000
0
a
OTA standard
404.0866
358.0827
386.0773
426.0681
442.0402
464.0221
300
320
340
360
380
400
420
440
460
480
m/z
Intens.
2500
2000
1500
1000
500
0
b
1
OTA + strain
Intens.
60
40
20
0
c
OTA - strain
Intens.
6000
4000
2000
0
d
1
OTA standard
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
10.5
Time