# CHAPTER 7

# ORIGIN OF OTA

## 7.1. METHODS FOR FUNGAL ENUMERATION, ISOLATION AND IDENTIFICATION

### 7.1.1. Sampling

Sampling is a very important step for obtaining precious results from mycological assays of foods. The discrete nature of fungal and hence mycotoxin contamination in food supplies has long been recognized as a major source of the overall analytical error. The European Commission has regulated the sampling methods in some bulk foods, but not in grapes. There is a need for further sampling plans for mycotoxins in a variety of matrices. The specific sampling plan used in this study for the isolation and identification of the mycoflora responsible for the OTA levels in grapes and wine is described in section 7.5.

### 7.1.2. Enumeration techniques

According to Pitt and Hocking (1997), methods for quantification of filamentous fungi could be divided in two:

- **Direct plating**

This is a good method for detecting, enumerating and isolating fungi from particulate foods. Food particles are placed directly on solidified agar media. In most situations, particles should be surface disinfected before plating, as this removes the inevitable surface contamination arising from dust and other sources, and permits recovery of the fungi actually growing in the particles. Results from direct plating analyses are usually expressed as percentage of infected particles. This was the method used in most of the studies included in this thesis.

- **Dilution plating**

This is the appropriate method for mycological analysis of liquid or powdered foods. For sample preparation, stomaching or blending techniques could be used. The sample size should be as large as possible in both techniques. Usually, the recommended diluent is aqueous 0.1% peptone. Serial dilutions are carried out by the same procedures as those

used in bacteriology, and later, they should be spreat on the surface of solid plated media, incubated and later enumerated.

### 7.1.3. Isolation techniques

One of the problems in the isolation of moulds from infected material is that single species rarely occur alone. There will usually be a mixture of different species of moulds as well as yeasts and bacteria (Smith and Moss, 1985).

Visual assessment of infected plants or plant products is often insufficient to diagnose the causal agent of the disease, particularly where different organisms can induce similar symptoms. Conventional methods for the identification into species generally involve isolation of the fungus. Isolation is the preparation of a pure culture, free from any contaminant and ready for identification.

Isolation techniques can be divided into two broad categories: direct methods and selective methods. Both are routinely used in mycology laboratories and can be further divided into a number of subtypes.

- **Direct isolation**

The term ‘direct’ is applied to techniques involving the simple transfer of a mould from its natural habitat to a pure culture in the laboratory. Isolation of filamentous fungi usually consists in picking a small sample of hyphae or spores and placing it on a fresh plate as a point inoculum, preferably near the centre of the plate as this will allow the best colony development and sporulation in most fungi.

- **Selective methods**

In some instances, selective methods have been developed to enable the target species to be isolated from material where it is only a relatively minor component of the mycoflora present (Booth, 1971). This could be achieved by surface sterilisation of a particulate food, exposition to stress conditions, use of selective nutrients, selective temperatures, etc., techniques focussed on favouring the development of the target mould among the total mycoflora.

### 7.1.4. Incubation conditions

The standard incubation conditions specified by the International Commission on Food Mycology (ICFM) are 25 ºC for 5 days (King et al., 1986; Pitt et al., 1992). In tropical regions, incubation at 30 ºC is recommended, as it is a more realistic temperature for

enumerating fungi from commodities stored at ambient temperatures. In cool temperate regions such as Europe, 22 ºC had also been recommended. Other temperatures could be used in some circumstances too, like when aiming to isolate a particular microorganism with a particular optimum temperature for growth, different from those proposed.

When incubating fungi, Petri dishes should be stored upright, as inverted dishes can transfer the spores to the lid. Reinversion of the Petri dishes for inspection or removal of the lids may liberate spores into the air or onto the benches and cause serious contamination problems (Pitt and Hocking, 1997). However, in particular situations as in the case of little-sporulating fungi, Petri dishes could be incubated inverted.

### 7.1.5. Identification and classification

Once the mould is isolated, further culturing may be required before the organism can be identified.

Usually, fungal identification is done on the basis of morphological characteristics of the colony, conidia and conidiogenous cells. Moulds are characterized by the development of hyphae, which result in the colony characteristics seen in the laboratory. Hyphae elongate by a process known as apical elongation, which requires a careful balance between cell wall lysis and new cell wall synthesis. Because moulds are often differentiated on the basis of conidiogenesis, structures such as conidiophores and conidiogenous cells must be carefully evaluated. Some moulds produce special sac-like cells called sporangia, the entire protoplasm of which becomes cleaved into spores called sporangiospores. Sporangia are typically formed on special hyphae called sporangiophores.

In most modern classifications, fungi are ranked, like plants and animals, as a separate kingdom. A cluster of related species is grouped in a genus, of related genera in families, of families in orders, orders in classes, and classes in subkingdoms. Zygomycotina, Ascomycotina and Deuteromycotina are the three subkingdoms of the kingdom Fungi that include the most significant genera in food spoilage. Fungi from each of these subkingdoms have quite distinct properties, shared with other genera and species from the same subkingdom (Pitt and Hocking, 1997).

The name applied to any fungus is binomial: first appears a capitalised genus name followed by a lower case species name, both written in italics or underlined. The classification of organisms in genera and species was a concept introduced by Linneaus in 1753 and it is the keystone of biological science (Pitt and Hocking, 1997).

### 7.1.6. Pictures of some common genera of moulds

Figure 36 shows 24 of the most common genera of moulds infecting food, and often or occasionally infecting grapes, as it will be shown in the results from field sampling studies of grape mycoflora. Pictures were obtained from the Mycological Research group web page of the St. George Campus, University of Toronto (Canada): www.botany.utoronto.ca/ResearchLabs/MallochLab/Malloch/Moulds/IDPlateI.html,

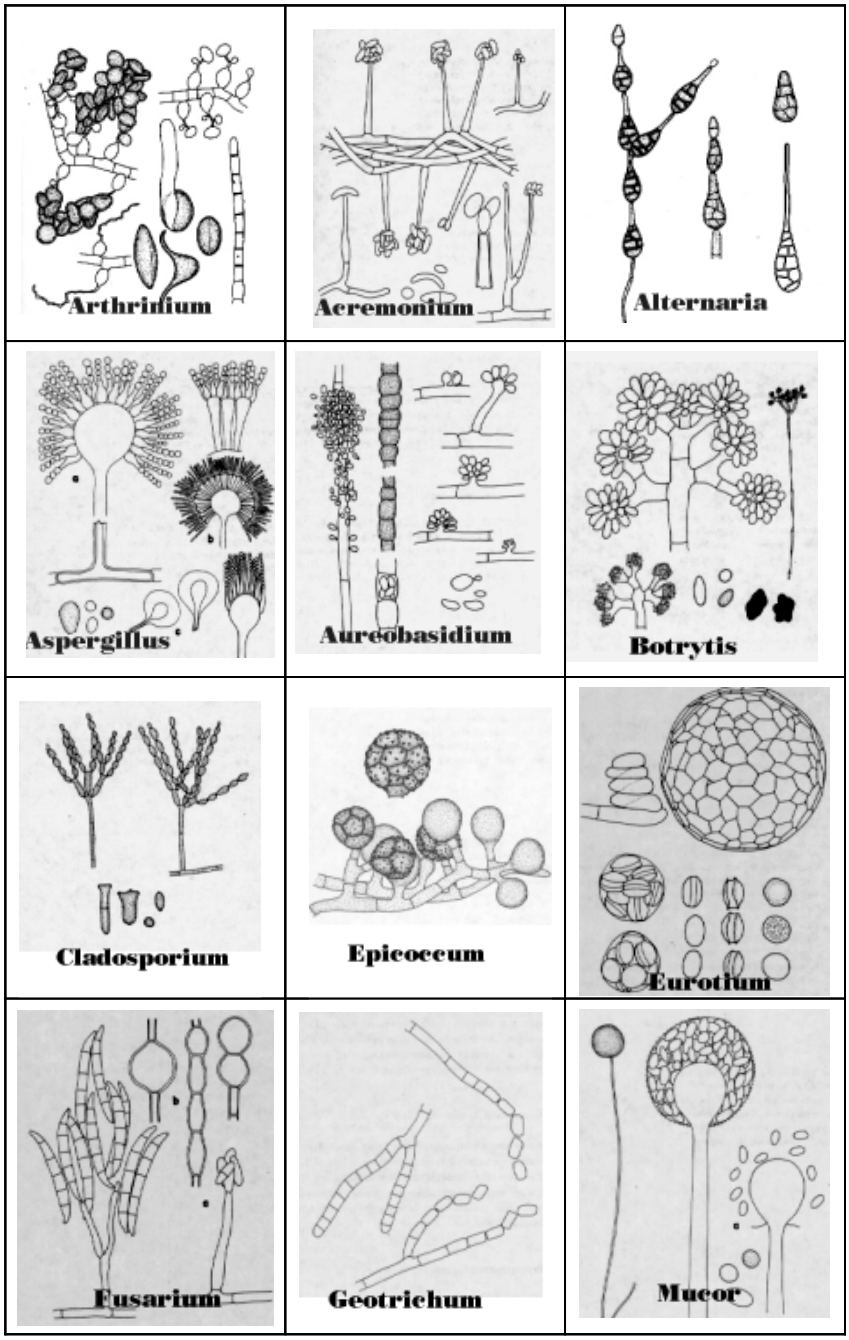


**Figure 36. Common genera isolated from food (Mycological Research Group, University of Toronto, Canada).**

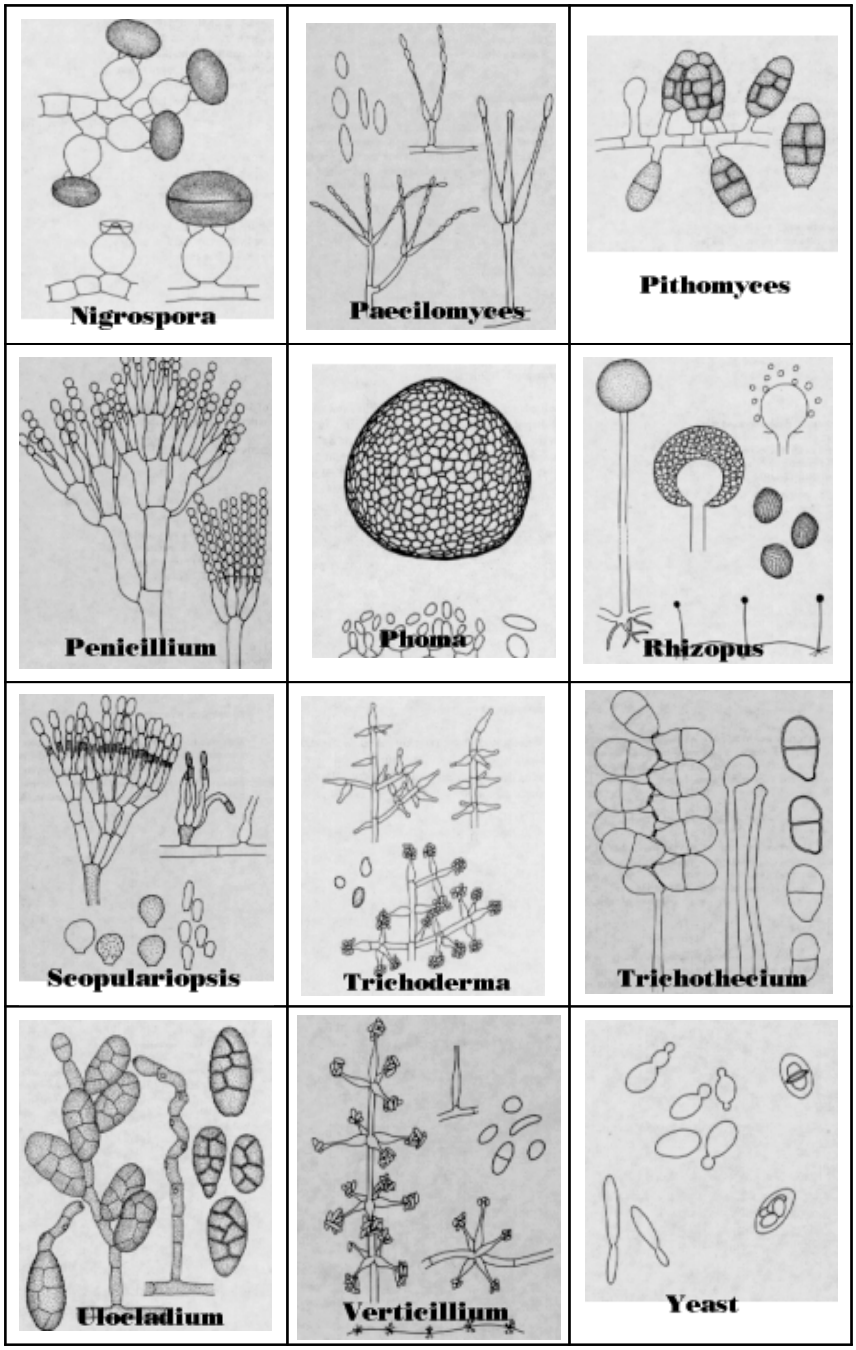


**Figure 36 (continuation). Common genera isolated from food.**

## 7.2. MEDIA COMPOSITION AND PREPARATION

There are some aspects to take into account when selecting a medium for a specific application (Pitt and Hocking, 1997):

- Food object of the study: there are media suitable for high water activity ($a_w$) foods and media that suit better for dried foods.

- Microoganisms studied: media will be different in case the object of the study are moulds, yeasts or both.

- Presence or absence of preservatives.

- Study of mycotoxin production: there are media available for specific mycotoxigenic fungi.

- etc.

### 7.2.1. General enumeration media

The ideal general enumeration medium should (Pitt and Hocking, 1997):

- Inhibit bacterial growth completely, without affecting growth of foodborne fungi.

- Be nutritionally adequate and support the growth of difficult-growing fungi.

- Suppress the growth of rapidly spreading fungi, but not prevent their entirely growth.

- Slow radial growth of all fungi, to permit counting of a reasonable number of colonies per plate.

- Promote growth of relevant fungi and suppress growth of soil fungi or others generally irrelevant in food spoilage.

The general enumeration medium used in the studies included in the studies comprised in this thesis was:

- **Dichloran Rose Bengal Chloramphenicol agar (DRBC)** (Pitt and Hocking, 1997). This medium is recommended for both moulds and yeasts. It is particularly suited to fresh and high $a_w$ foods. This medium contains both dichloran (2,6-dichloro-4-nitroaniline) and

rose bengal to restrict colony size, especially of *Rhizopus* spp., which would otherwise quickly overgrow the whole isolation plate.

### 7.2.2. Selective media

The availability of effective media can greatly simplify the isolation and identification of significant food spoilage and mycotoxigenic fungi. The selective media used in the studies included in this thesis were:

- **Czapek Yeast extract agar (CYA)** (Pitt and Hocking, 1997). This medium was frequently used to test the ability of different species to produce OTA.

- **Czapek Dox agar (CZ)** (Pitt and Hocking, 1997). Many moulds produce very characteristic colonies on it and may also exude pigmented substances. Aerial growth is often suppressed and sporulation may be enhanced. Thus, CZ medium was used for the identification of ochratoxigenic fungi, mainly black aspergilli.

- **Natural media** are so called because they are partly or completely composed of natural materials. In the studies presented here, natural media have been used sometimes, as grape juice or grape berries. The greatest disadvantage of using these media is that they may differ considerably from batch to batch and could modify slightly the experimental results. Therefore, in most of the studies where grape juice was needed as a substrate, a synthetic medium was used instead.

- **Synthetic Nutrient Medium (SNM)**. SNM is a medium that simulates grape composition between veraison and ripeness (Delfini, 1982).

### 7.2.3. Water activity adjustments

Water activity ($a_w$) of the media used in the ecophysiological experiments of this thesis was determined with a water activity meter (AquaLab, Decagon CX-2, Pullman, Washington, USA). Figure 37 shows the experimental curves for two solutes, glycerol and glucose, obtained by plotting different concentrations of each solute versus the resulting $a_w$ of the medium. The adjusted equations and the percentage of variance explained by the model ($R^2$) were also presented for each solute. The amounts of solute (glucose or glycerol) necessary to adjust 100 ml of SNM medium calculated from these graphs are shown in Table 21.

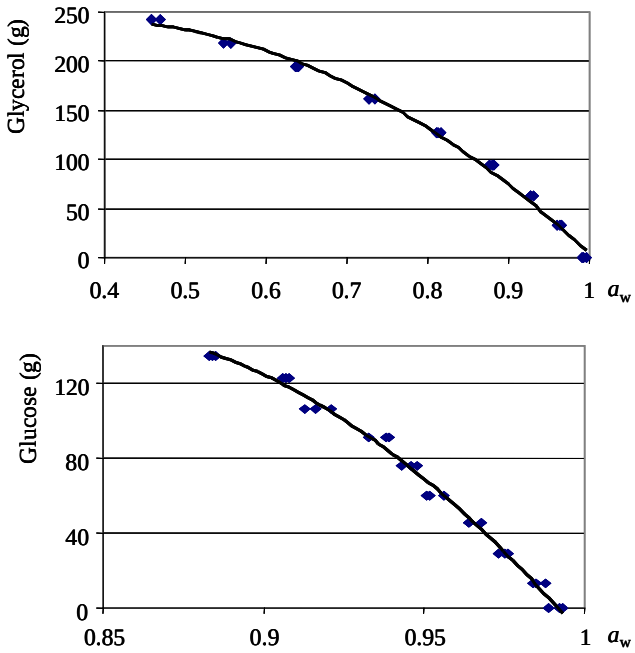


**Figure 37. Amounts of solute (glycerol or glucose) necessary to make up 250 ml of SNM medium at different $a_w$.** Adjusting equations are: [g glycerol = -606.52 $a_w^2$ + 453.35 $a_w$ + 157.43; $R^2$ = 0.995]; [g glucose = -5935.7 $a_w^2$ + 9869 $a_w$ – 3949.6; $R^2$ = 0.992].

**Table 21. Amounts (g) of solute (glycerol or glucose) necessary to make up 100 ml of SNM medium at the required $a_w$.**

| $a_w$ | Glycerol (g) | Glucose (g) |
|---|---|---|
| 0.90 | 29.6 | 50.0 |
| 0.93 | 21.8 | 38.1 |
| 0.95 | 16.3 | 27.8 |
| 0.98 | 7.7 | 8.9 |
| 0.995 | 3.2 | 0 |

## 7.3. MYCOTOXIGENIC FUNGI

Fungi that produce mycotoxins are referred to as toxigenic or mycotoxigenic fungi. The main mycotoxigenic fungi involved in the human food chain belong to three genera: *Aspergillus*, *Fusarium* and *Penicillium* (Scott, 2004) (Table 22). However, toxins have been detected from many other fungi under certain growth conditions. The kind and amounts of toxin produced depend on the fungal strain, the growing conditions, as well as the presence or absence of other organisms.

**Table 22. Principal mycotoxins produced by *Aspergillus*, *Fusarium* and *Penicillium* species.**

| Fungi | Mycotoxins |
|---|---|
| *Aspergillus* | α-cyclopiazonic acid; aflatoxins; echinulin; flavoglaucin; gliotoxin; ochratoxins; patulin; sterigmatocystin; xanthoascin; xanthocillin X |
| *Fusarium* | 4-acetamido-2-buten-4-olide; apicidin; beauvericin; chlamydospol; deoxynivalenol; enniatins; equisetin; fumonisins; fusaproliferin; fusaric acid; fusarin C; fusariocins, fusarochromanone; moniliformin; sambutoxin; trichothecenes; wortmannin; zearalenone |
| *Penicillium* | citrinin; citreoviridin; luteoskyrin; ochratoxins; patulin; penicillic acid; penitrem; PR toxin; roquefortine C; rubratoxin B; secalonic acid D |

## 7.4. OTA-PRODUCING SPECIES

OTA was originally described as a metabolite of *A. ochraceus* (*Aspergillus* section *Circumdati*) from laboratory experiments (van der Merwe et al., 1965). Later, the production of this toxin was repeatedly reported in Section *Nigri*, mainly by *Aspergillus carbonarius* and a low percentage of isolates of the closely related species *A. niger* (Téren et al., 1996). Even recently, new ochratoxigenic species in this section are emerging (Samson et al. 2004; Medina et al., 2005). Isolates from other subgenera usually produce only small amounts of OTA, or they ability to produce the toxin was not confirmed by other authors (Table 23).

Natural occurrence and practical importance of OTA, however, was first linked with *Penicillium* species (Ciegler et al., 1972; Krogh et al., 1973) (Table 24). Some of these, like *P. viridicatum* and *P. cyclopium*, have been found afterwards to do not produce OTA. Later, Larsen et al. (2001), biochemically characterized several OTA-producing strains of the genus *Penicillium*, and separated them in two large groups: *P. verrucosum* and *P. nordicum*. The last deserve a special attention as they produced more OTA than *P. verrucosum* under laboratory conditions. Moreover, they suggested that *P. nordicum*

could be the source of OTA of *Penicillium* contaminated meat-derived products as all the isolates they examined from these food belonged to this group, whereas *P. verrucosum* was only found in plant-derived material.

In tropical regions, OTA is produced generally by *Aspergillus* species, while in temperate regions, by *Penicillium* species. These two genera are most commonly found as contaminants in stored supplies of grain but also occur in other raw and processed foods and beverages. *Penicillium* species produce OTA over a temperature range of 4-31 ºC, whereas *Aspergillus* species prefer higher temperatures (12-39 ºC) (Stander and Steyn, 2002). However, there is considerable uncertainty about the extent to which ochratoxinogenic ability is expressed in natural conditions as many isolates of ochratoxigenic fungi, sometimes do not produce OTA under laboratory conditions. Whether this reflects a constitutive inability to operate the ochratoxin biosynthetic pathway or is an artefact of pure culture is unknown (Mantle, 2002; Medina et al., 2005).

To sum up, the two main species of moulds that produce OTA are *P. verrucosum* and *A. ochraceus*, but a third major source, *A. carbonarius*, have been recently identified, mainly in grapes (see 7.8.). All three species differ in physiology and ecology, which in turn affect the types of foodstuffs in which these moulds are most commonly found.

**Table 23. OTA-producing *Aspergillus* species** (modified from Varga et al., 2001).

| Species | Section | References |
|---|---|---|
| *A. glaucus* | *Aspergillus* | Chelkowski et al. (1987) |
| *A. repens* | *Aspergillus* | El-Kady et al. (1994) |
| *A. sydowii* | *Aspergillus* | Ueno et al. (1990) |
| *A. albertensis* | *Flavi* | Varga et al. (1996); Peterson (2000) |
| *A. alliaceus* | *Flavi* | Ciegler (1972); Doster et al. (1996); Peterson (2000); Bayman et al. (2002) |
| *A. flavus* | *Flavi* | Atalla and El-Din (1993) |
| *A. auricomus* | *Circumdati* | Varga et al. (1996) |
| *A. melleus* | *Circumdati* | Ciegler (1972) |
| *A. muricatus* | *Circumdati* | Frisvad and Samson (2000) |
| ***A. ochraceus*** | *Circumdati* | van der Merwe et al. (1965); Krivobok et al. (1995); Varga et al. (1996); Mühlencoert et al. (2004); Pardo et al. (2004). |
| *A. ostianus* | *Circumdati* | Ciegler (1972) |
| *A. petrakii* | *Circumdati* | Ciegler (1972) |
| *A. sclerotiorum* | *Circumdati* | Ciegler (1972); Moss (1996); Varga et al. (1996) |
| *A. sulphureus* | *Circumdati* | Ciegler (1972); Madhyasta et al. (1990) |
| *A. clavatus* | *Clavati* | Atalla and El-Din (1993) |
| *A. wentii* | *Cremei* | Varga et al. (1996) |
| *A. fumigatus* | *Fumigati* | Abarca et al. (1997); Atalla and El-Din (1993): Varga et al. (2000) |
| *A. awamori* | *Nigri* | Ono et al. (1995); Téren et al. (1996); Accensi et al. (2001) |
| ***A. carbonarius*** | *Nigri* | Horie (1995); Téren et al. (1996); Wicklow et al. (1996); Heenan et al. (1998); Joosten et al. (2001) |
| *A. foetidus* | *Nigri* | Ueno et al. (1991); Téren et al. (1996); Magnoli et al. (2003) |

| | | |
|---|---|---|
| *A. japonicus* | *Nigri* | Dalcero et al. (2002); Battilani et al. (2003) |
| *A. lacticoffeatus* | *Nigri* | Samson et al. (2004) |
| *A. niger* | *Nigri* | Abarca et al. (1994); Ono et al. (1995); Téren (1996); Nakajima et al. (1997); Heenan et al. (1998) |
| *A. sclerotioniger* | *Nigri* | Samson et al. (2004) |
| *A. tubingensis* | *Nigri* | Medina et al. (2005) |
| *A. usamii* | *Nigri* | Ono et al. (1995); Accensi et al. (2001) |
| *A. vadensis* | *Nigri* | De Vries et al. (2004) |
| *A. terreus* | *Terrei* | Ueno et al. (1991) |
| *A. ustus* | *Usti* | Ueno et al. (1991) |
| *A. versicolor* | *Versicolores* | Abarca et al. (1997) |

**Table 24. OTA-producing *Penicillium* species** (modified from Varga et al., 2001).

| Species | Section | Reference |
|---|---|---|
| *P. cyaneum* | *Aspergilloides* | Ueno et al. (1991) |
| *P. implicatum* | *Aspergilloides* | Ueno et al. (1991) |
| *P. montanense* | *Aspergilloides* | Ueno et al. (1991) |
| *P. sclerotiorum* | *Aspergilloides* | Ueno et al. (1991) |
| *P. spinulosum* (*P. purpurescens*) | *Aspergilloides* | El-Banna et al. (1987); Ciegler et al. (1972) |
| *P. variabile* | *Biverticillium* | Ciegler et al. (1972); Krivobok et al. (1995) |
| *P. purpurogenum* | *Biverticillium* | Ueno et al. (1991) |
| *P. verruculosum* | *Biverticillium* | Ueno et al. (1991) |
| *P. canescens* | *Eladia* | Ueno et al. (1991) |
| *P. corylophilum* | *Eladia* | Ueno et al. (1991) |
| *P. fascum* | *Eladia* | Ueno et al. (1991) |
| *P. hirayamae* | *Eladia* | Ueno et al. (1991) |
| *P. janczewskii* | *Eladia* | Ueno et al. (1991) |
| *P. melinii* | *Eladia* | Ueno et al. (1991) |
| *P. miczynskii* | *Eladia* | Ueno et al. (1991) |
| *P. raistrickii* | *Eladia* | Ueno et al. (1991) |
| *P. simplicissimum* | *Eladia* | Ueno et al. (1991) |
| *P. atramentosum* | *Penicillium* | Bridge et al. (1989) |
| *P. aurantiogriseum* (*P. solitum*) | *Penicillium* | Bridge et al. (1989); Krivobok et al. (1995) |
| *P. commune* | *Penicillium* | Ciegler et al. (1972) |
| *P. expansum* | *Penicillium* | Bridge et al. (1989) |
| *P. nordicum* | *Penicillium* | Larsen et al. (2001) |
| ***P. verrucosum*** | *Penicillium* | Pitt (1987); Larsen et al. (2001) |
| *P. chrysogenum* | *Ramosum* | Turner and Aldridge (1983); Krivobok et al. (1995) |

### 7.4.1. *Penicillium verrucosum*

For half a century or more, nephropathy has been an important disease in Danish pigs. Etiological studies first showed it to be associated with mouldy grain, and then with a fungus identified as *P. viridicatum* (Krogh and Hasselager, 1968), which later was demonstrated to be unlikely involved in this disease in favour of *P. verrucosum*. In 1987, Pitt provided unequivocal evidence that *P. viridicatum* and *P. verrucosum* were distinct species, and that the first did not produce OTA. The most useful characteristics for distinguishing between these two species are the differences in growth rates on Czapek Yeast extract Agar (CYA) and on Malt Extract Agar (MEA), the highest ones corresponding to *P. viridicatum.*

*P. verrucosum* is classified in subgenus *Penicillium*, section *Penicillium*, which includes many mycotoxigenic species of common occurrence in foods. Identification of *P. verrucosum* could be achieved by inoculating it in several media and comparing its characteristics with the ones described in table 25, or with those described in any other recognised identification manual.

The major habitat of *P. verrucosum* is cereal crops in the cool and temperate climates of northern Europe and Canada (JEFCA, 2001). The inability of this fungus to grow above 30 ºC makes its presence in the tropics most unlikely. Consequently, OTA is found in cereal products, especially flour-based products. It is also found in commodities with large amounts of proteins and fats, such as cheese and meat products from animals that eat cereals as a major dietary component (Gareis and Scheuer, 2000).

### 7.4.2. *Aspergillus ochraceus*

*A. ochraceus* is the most commonly occurring species in the '*Aspergillus ochraceus* group' of Raper and Fennell (1965), now correctly referred to as *Aspergillus* Section *Circumdati* (Gams et al., 1985). *A. ochraceus* is associated with warmer and tropical climates. Thus, it is most commonly found in drying or decaying vegetation, dried and stored food, such as smoked and salted dried fish, soya beans, chick peas, nuts, pepper and dried fruit. It has been also isolated from a wide range of cereals. *A. ochraceus* is generally present at low levels and rarely causes spoilage. Key identification for this fungus is shown in table 26.

**Table 25. Main morphological characteristics of *P. verrucosum*** (Pitt and Hocking, 1997).

| Medium, diameter | Morphology | Myce-lium | Conidial formation | Exudate | Reverse |
|---|---|---|---|---|---|
| CYA, 15-25 mm | usually closely sulcate, varying from low and velutinous to deep and fasciculate of floccose | white | light to moderate, greyish green to dull green; clear to pale yellow | exudates produced, copiously by some isolates | yellow brown to deep brown |
| MEA, 12-15/20 mm | sulcate, dense and velutinous or centrally floccose | white | moderate, coloured as on CYA | clear exudates occasionally produced | dull brown or olive |
| G25N, 16-20 mm | plane or more commonly sulcate, velutinous to somewhat floccose; often colonies of 2-4 mm diameter produced. No growth at 37 ºC | white | At 5 ºC, microcolony formation at least | - | pale to yellow |
| CSN, 10-15 mm | growth weak, mostly subsurface | - | - | - | neutral |

| | |
|---|---|
| Conidiophores: | borne from subsurface or surface hyphae |
| Stipes: | robust, 200-500 µm long, with walls finely to conspicuously roughened, bearing terminal penicilli |
| Penicilli: | variable, some isolates producing compact terverticillate and quaterverticillate forms almost exclusively, others predominantly terverticillate and biverticillate, often with elements irregularly disposed |
| Ramifications: | 1-2 per stipe, 10-15/20 µm long, sometimes rough walled |
| Metulae: | 7-10/15 µm long |
| Phialides: | ampulliform, , 7-9 µm long, narrowing abruptly to short collula |
| Conidia: | usually spherical, 2.5-3.0 µm diam., less commonly subpheroidal to ellipsoidal, 3.0-3.5 µm long, with smooth walls, born in disordered chains |

**Table 26. Main morphological characteristics of *A. ochraceus*** (Pitt and Hocking, 1997).

| Medium, diameter | Morphology | Myce-lium | Conidial formation | Exudate | Reverse |
|---|---|---|---|---|---|
| CYA, 40-55 mm | plane or sulcate, low and velutinous or lightly floccose; light yellow to golden yellow sclerotia sometimes produced, white when young, later pink to purple | white | conidial heads closely packed | clear exudates sometimes present, some exuded from stipe walls | greyish orange to brown |
| MEA, 40-55 mm | plane, similar to those on CYA but quite sparse | - | - | - | blonde to dark blonde |
| G25N, 20-30 mm | plane, low and dense to deep and floccose. No growth at 5 ºC; usually no growth at 37 ºC | - | light to moderate, coloured as on CYA | - | pale yellow or brown. |

| | |
|---|---|
| Conidiophores: | borne from surface hyphae |
| Stipes: | 1.0-1.5 mm long, with yellowish to pale brown walls, finely to conspicuously roughened |
| Vesicles | spherical, 25-50 μm diam., bearing tightly packed metulae and phialides over the entire surface |
| Metulae: | 15-20 μm long |
| Phialides: | 9-12 μm long |
| Conidia: | spherical to subspheroidal, 2.5-3.5 μm diam., with smooth to finely roughened walls, borne in radiate heads when young, splitting into two or more broad columns with maturity |

### 7.4.3. *Aspergillus* section *Nigri*

Black aspergilli, -*Aspergillus* classified into the Section *Nigri* by Gams et al. (1985), formerly '*A. niger* species group' by Raper and Fennell (1965)-, present dark colonies, often black, and uniseriate or biseriate conidiophores. They have been isolated from a wide variety of food world-wide distributed and are considered as common fungi causing food spoilage and biodeterioration of other materials. Black aspergilli are commonly present in vineyards and have the ability to cause berry rot, know as *Aspergillus* rot or black mould (Snowdon, 1990). However, some of the members of this section, like *A. niger* and *A. awamori*, are a common source of extracellular enzymes such as amylases or lipases, and organic acids, such as citric and gluconic acid, used as additives in food processing. These products hold the GRAS (Generally Recognised As Safe) status from the Food and Drug Administration (FDA) (Bigelis and Lasure, 1987), despite the ability of these species to produce OTA and other mycotoxins (Table 27). The safety of *A. niger* when used for biotechnological purposes, has been recently reviewed by Schuster et al. (2002).

**Table 27. Mycotoxins and secondary metabolites produced by some species in the Section *Nigri*** (Bau, 2005).

| Specie | Metabolite |
|---|---|
| *A. aculeatus* | Secalonic acid B, D and F; aculeasin; eumodin; endocrocin; neoxalin; okaramin |
| *A. carbonarius* | Naphtopyrones; ochratoxin A; pyranonigrin |
| *A. japonicus* | E-64; festuclavin |
| *A. niger* | Glutaconic acid; 4-hidroximandelic acid; kojic acid; monoglucosiloxioctadecanoic acid; aspergillin; aspereillons; asperrubrol; dehidroflavin; flaviolin; genisterins; malformins; naphtopyrones; neoequinulin A; nigerazins; nigragillin; ochratoxin A; orlandin; oxalic acid; pyranonigrin; tubigensin A and B |

#### 7.4.3.1. Taxonomy of black aspergilli

The taxonomy of the section *Nigri* is the most complex inside the genus *Aspergillus*. The unifying feature of the members of this section is the dark colour of their conidial heads (Figure 38). Mosseray (1934) described 35 black aspergilli species, while Raper and Fennell (1965) reduced this number to 12. Later, Al-Musallam (1980) revised the taxonomy of the *A. niger* group and recognised seven species, based on morphological features. *A. niger* was described as an aggregate consisting of seven varieties and two

*formae*. Gams et al. (1985) classified the Section *Nigri* in the subgenera *Circumdati*. In 1989, Kozakiewicz suggested 17 taxa in the *A. niger* group and distinguished two groups: echinulate and verrucose, depending on their conidial ornamentations.

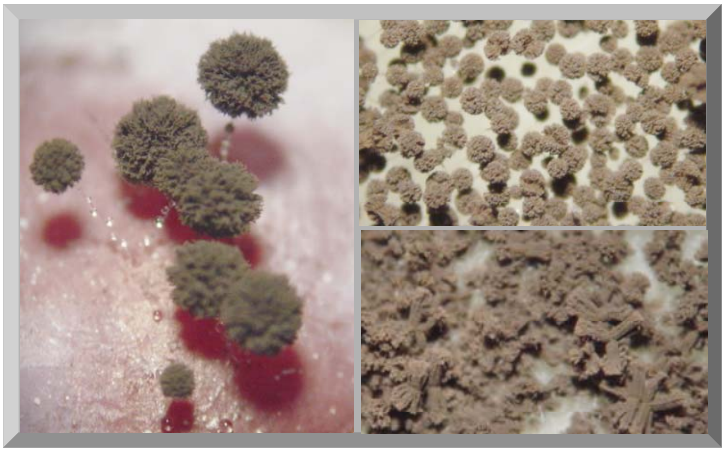

**Figure 38. Conidiophores of *Aspergillus* section *Nigri* under stereomicroscope.**

In the past, it was very common that all *Aspergillus* isolates developing black colonies were identified as *A. niger* by non-taxonomists, because of the similarities in morphology, and sometimes the same isolate was preserved in culture collections under different species names. To solve this problem, a last review in the taxonomy of black aspergilli has been published by Abarca et al. (2004), who proposed an identification key to distinguish the most common taxa (Table 28). Cultures on Czapek agar (CZ), CYA or MEA (Pitt and Hocking, 1997) incubated at 25 ºC for 7 days were recommended. In a first step, *Aspergillus* section *Nigri* isolates were identified as uniseriates or biseriates. Uniseriates were those with uniseriate conidial heads, while biseriates showed biseriates heads, and among these, *A. carbonarius* isolates were identified at species level. This classification was used throughout the mycoflora studies of grapes in this thesis. As it was based on morphological criteria, in some cases, further differentiation at species level was very difficult and required considerable expertise using conventional methods based on morphological features.

A provisonal key of section *Nigri*, based on phenotypic characteristics, extrolites and β-tubulin sequencing, was also proposed by Samson et al. (2004). They accepted 15 taxonomic species in this section: *A. aculeatus*, *A. brasilensis*, *A. carbonarius*, *A. costaricaensis*, *A. ellipticus*, *A. foetidus*, *A. heteromorphus*, *A. homomorphus*, *A. japonicus*, *A. lacticoffeatus*, *A. niger*, *A. piperis*, *A. sclerotioniger*, *A. tubingensis* and *A. vadensis*. Only four of them were recognised as OTA positives: *A. carbonarius*, *A. niger*,

*A. lacticoffeatus* and *A. sclerotioniger*, the first two occurring on grapes and derivates and the last two isolated from coffee and reported as new species of section *Nigri*.

**Table 28. Key for identification of black aspergilli** (Abarca et al., 2004).

| | | |
|---|---|---|
| 1 | Aspergilla uniseriate | *A. japonicus/A. aculeatus* |
| | Aspergilla biseriate | 2 |
| 2 | Conidia more than 6 μm diameter | *A. carbonarius* |
| | Conidia less than 6 μm diameter | *A. niger* aggregate |

As some of the species remain difficult to differentiate using phenotypic methods, other techniques have been described to help in their differentiation: DNA sequences of the cytochrome b gene (Yokoyama et al. 2001), internal transcribed spacers (Parenicová et al., 2001), β-tubulin (De Vries et al., 2004) and RFLP and other fingerprinting methods (Abarca et al., 2004). Furthermore, several methods based on the PCR technique have been recently proposed to discriminate the main species included in the section *Nigri*: *A. carbonarius* and *A. japonicus* (Perrone et al., 2004), *A. japonicus*, *A. heteromorphus*, *A. ellipticus*, *A. niger* and *A. tubingensis* (González-Salgado et al., 2005), etc.

- **Uniseriate species**

*A. japonicus* and *A. aculeatus* are the only species in this section which are uniseriate. Whether *A. japonicus* and *A. aculeatus* are one or two species is still under question. Several authors concluded that both belong to a single species (Yokoyama et al., 2001).

- **Biseriate species**

***Aspergillus niger* aggregate**

The taxa included in the so-called *A. niger* aggregate have always been extremely difficult to distinguish one from each other by morphological means. The number of taxa varies from one author to another. Therefore, in 1991, Kusters van Someren et al. proposed a molecular division of the *A. niger* aggregate into two morphologically indistinguishable species, *A. niger* (N pattern) and *A. tubingensis* (T pattern), based on the restriction fragment length polymorphism (RFLP) analysis of total DNA. Until short time ago, all the OTA-producing isolates from *A. niger* aggregate have been included in the N pattern, whereas none of the isolates classified as *A. tubingensis* were able to produce OTA (Accensi et al., 2001). However, Medina et al. (2005) reported for the first time the ability of *A. tubingensis* to produce OTA. They suggested that failing to detect OTA in cultures of *A. tubingensis* might be due to culture medium and/or incubation time.

***A. carbonarius***

*A. carbonarius* is possibly the most distinct member of this section, as recently it has been suggested that plays an important role in grapes and wine, because of the high percentage of positive strains and the amount of OTA produced. However, because its novelty, it is not described in some currently used identification manuals (Pitt and Hocking, 1997; Samson et al., 2004). Strains of this species can be easily recognised using light microscopy since their conidia are much larger than those of other black aspergilli and have echinulate conidia ornamentation (Abarca et al., 2004).

***A. ibericus***

A possible new *Aspergillus* species was found in section *Nigri*, provisionally designated as *A. ibericus*, for which formal characterization is in progress (Cabañes et al., 2004). Morphological studies carried out by these authors, revealed some differences between the spore size and the OTA production between *A. carbonarius* and this new species. *A. ibericus* species have been identified since now as *A. carbonarius* OTA negatives. The only reported *A. ibericus* isolates were in a survey conducted to assess mycotoxin-producing fungi on Portuguese grapes (Serra et al., 2005). A useful description of the main characteristics of *A. ibericus* and the differences with the other species in section *Nigri*, is found in Serra dissertation (2005).

### 7.4.3.2. Toxicology of black aspergilli other than OTA production

Despite of the second metabolites, black aspergilli are considered the third most common species associated with invasive pulmonary aspergillosis and it is also often a causative agent of aspergilloma (Sharma et al., 1985; Kwon Chung and Bennett, 1992). Moreover, these species have been reported as responsible for a subcutaneous infection (Paldrok, 1965) and they were isolated of the tongue of a patient with respiratory illness (Williams et al., 1984). In immunocompromised patients, pulmonary infections or colonisations may occur, and are often characterized by oxalosis, which is extensive production of microscopically conspicuous oxalic acid crystals in sputum (Kurrein et al., 1975; Staib et al., 1979). Oxalosis may also distinguish *A. niger* in some cases of invasive otomycosis in compromised patients (Landry and Parkins, 1993). Other infections occurring in immunocompromised patients include disseminated and primary cutaneous infections. As with many relatively weak opportunists, *A. niger* causes dialysis-related peritonitis, endocarditis, and other invasions of particularly vulnerable sites (Hoog et al., 2000).

Allergics responses are often common after inhalation of spores of black aspergilli and also of the enzymes they produced. For example, enzymes derived from *A. niger* present in baking additives have been identified as a causative allergen in baker's asthma (Losda et al., 1986; Quirce et al., 2002).

### 7.4.3.3. Black aspergilli in food

Although the main source of black aspergilli is soil (Varga et al., 2004), members of this section have been isolated world-wide in a number of foodstuff (Table 29). They are the cause of several plant diseases originating important economical loses, sometimes to the mycotoxins they produce. The occurrence of OTA in foodstuff is widely reported, but most of the studies only analysed the OTA content, and little is stated about the fungal species producing it. Moreover, *A. ochraceus* and *P. verrucosum* have been contemplated as the responsible for the level of OTA in most of the foods, mainly cereals. Until 1998, they were also believed to be responsible for the production of the toxin in grapes (Ospital et al., 1998).

**Table 29. Presence of black aspergilli in several food.**

| Food | Reference |
|---|---|
| Animal feed | Dalcero et al. (2002); Accensi et al. (2004) |
| Cereals | Accensi et al. (2004); Varga et al. (2004) |
| Cocoa | Varga et al. (2004) |
| Coffee | Téren et al. (1997); Joosten et al. (2001); Bucheli and Taniwaki (2002); Martins et al. (2003) |
| Dried fruits | Özay et al. (1995); El Halouat and Debevere (1997); Arici (2001); Giridhar and Reddy (2001); Abarca et al. (2003); Magnoli et al. (2004) |
| Dried grapes | Abarca et al. (2003); Leong et al. (2004); Magnoli et al. (2004); Valero et al. (2005) |
| Fruits | Varga et al. (2004) |
| Garlic | Varga et al. (2004) |
| **Grapes, must, wine** | see 7.8. |
| Olives | Arici (2001) |
| Onions | Tuffley and Lorbeer (2002) |
| Pulses | Accensi et al. (2004) |

## 7.5. SAMPLING DESIGN OF THE STUDIES OF GRAPE MYCOFLORA

A common sampling design among the participants of the European project was accorded in 2001, in order to identify the common mycoflora in wine grapes, to study their evolution during grape ripening and to find out the ochratoxigenic species present and their capacity to produce OTA. The main points of the sampling are outlined afterwards.

### 7.5.1. Geography

Forty vineyards were selected for the study, contributing to the total 107 vineyards of the European project. Vineyards were distributed in three important regions with long winemaking tradition in Spain: Cataluña (Costers del Segre D.O, n=10; Penedès D.O., n=5; Conca de Barberà D.O., n=5), Comunidad Valenciana (Utiel-Requena D.O., n=10) and La Rioja (Rioja D.O., n=10) (Figure 39) (Table 30). Vineyards were owned by local farmers, wineries, agricultural cooperatives, etc., hence management was done following their criteria in accordance with the established practices of each D.O.

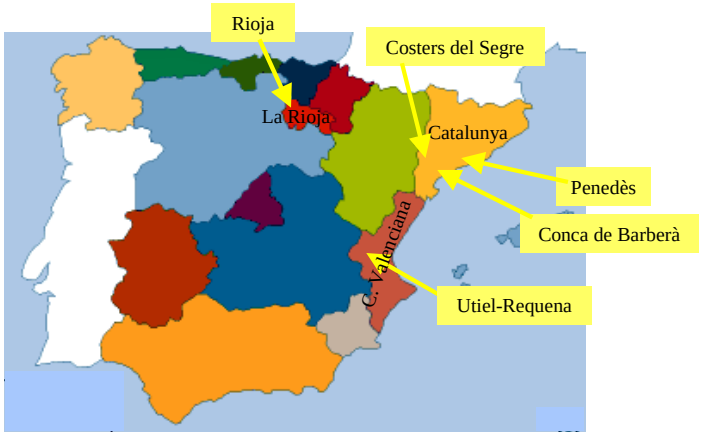


**Figure 39. Location of the sampled areas in Spain.**

**Table 30. Geographical coordinates of the sampled areas in Spain.**

| Region | Latitude | Longitude |
|---|---|---|
| Rioja (Logroño) | 42.30 N | 2.18 W |
| Costers del Segre (Lleida) | 41.37 N | 0.38 E |
| Penedés (Vilafranca del Penedés) | 41.22 N | 1.41 E |
| Conca de Barberà (Montblanc) | 41.23 N | 1.10 E |
| Valencia (Utiel)<br>Valencia (Requena) | 39.34 N<br>39.30 N | 1.10 W<br>1.06 W |

### 7.5.2. Varieties

Wine grape varieties represent only a small portion of the more than 600 kind of grapes. Each grape variety has its own unique combination of characteristics including colour, size, skin thickness, acidity, yield per vine and flavours. While many grape varieties are used to produce wines, only a few grapes have distinguished themselves as being particularly suited for the production of fine wine. These 'noble grape varieties' must still be matched with the right micro-climate and winemaking techniques in order to profit their potential.

The white varieties of grapes account for 61.5 % of the cultivated surface area in Spain, particularly in Castilla-La Mancha, Extremadura, Andalucía and Cataluña regions. The latter two, use this type of grapes for making both sweet wines and cavas, respectively. Red wines make up 43.5 % of Spanish wine production; the Autonomous regions that produce the largest percentage of reds out of their total production are La Rioja, Aragón and Valencia. The most common red grape varieties in Spain are Airén, Tempranillo, Bobal and Garnacha, and the whites, Monastrell, Pardina, Macabeo and Palomino, in order of volume of cultivation.

In the field studies of this thesis, grape varieties were chosen among those of relevance to each country, together with Cabernet Sauvignon, a common international variety chosen by all the partners in the European project (Table 31).

**Table 31. Number of fields of each variety sampled in each region.**

| | Costers del Segre | Penedés/ C. Barberà | Rioja | Utiel-Requena |
|---|---|---|---|---|
| **White varieties** | | | | |
| Chardonnay | 3 | 2 | - | - |
| Macabeo | - | - | 2 | 1 |
| Sauvignon blanc | - | 2 | - | 2 |
| **Red varieties** | | | | |
| Bobal | - | - | - | 3 |
| Cabernet Franc | 1 | - | - | 1 |
| Cabernet Sauvignon | 2 | 2 | - | 1 |
| Garnacha | - | 2 | 1 | 1 |
| Graciano | - | - | 1 | - |
| Merlot | 2 | - | - | - |
| Tempranillo | 2 | 2 | 6 | 1 |
| **Total:** | **10** | **10** | **10** | **10** |

### 7.5.3. Period

Sampling was carried out at four growth stages of the grapes in 2001: fruit setting, one month later, early veraison and harvest time (Figure 40), and at the last three periods in 2002 and 2003 (Table 32). In 2004, only the sampling near harvest was carried out in order to corroborate the results of the previous years.

**Table 32. Dates of the sampling periods of each year of the study (day/month).**

| Sampling[a] | 2001 | 2002 | 2003 | 2004 |
|---|---|---|---|---|
| 1 | 27/5 – 14/6 | - | - | - |
| 2 | 24/6 – 12/7 | 8/7 – 14/7 | 3/7 – 10/7 | - |
| 3 | 5/8 – 20/8 | 7/8 – 18/8 | 4/8 – 12/8 | - |
| 4 | 1/9 – 17/9 | 8/9 – 24/9 | 8/9 – 16/9 | 1/9-16/9 |

[a] 1, Setting; 2, one month later; 3, early veraison; 4, harvest.

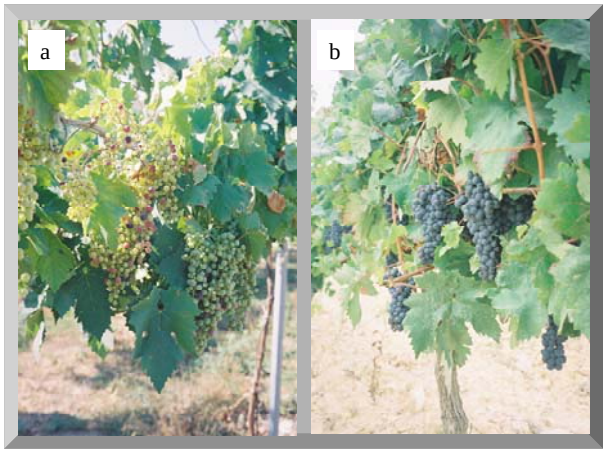


**Figure 40. Grapes at a) veraison and b) near harvest.**

### 7.5.4. Sampling protocol

As stated before, the sampling method is critical to obtain reliable results. In the case of sampling grapes from field, a common protocol to the whole countries participating in the European project was designed, for further comparison of the results. Ten vines where chosen along the diagonals of each vineyard, and at the next sampling date the next plant

in the same row was sampled (Figure 41). A bunch was picked from each plant in a central position. Bunches were collected with care, placed in paper bags and stored in cool conditions, with the shorter transfer time back to the laboratory as possible. They were kept at 4 ºC until laboratory analysis.

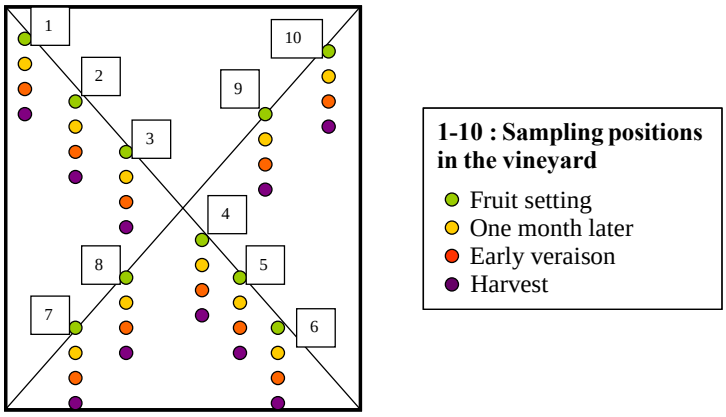


**Figure 41. Sampling plan showing the points in the field along the two diagonals, where grapes were recollected.**

Five berries per bunch were randomly selected and plated directly onto DRBC agar under sterile conditions. Plates were incubated for 7 days at 25 ºC (Figure 42). Wherever possible whole berries were used, otherwise they were aseptically cut in half.

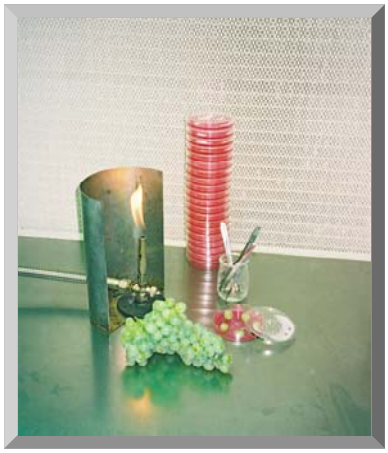

**Figure 42. Berries onto DRBC plates.**

Possible ochratoxigenic species were isolated onto CZ agar plates to obtain pure cultures, for further identification of the individual species (Figure 43).

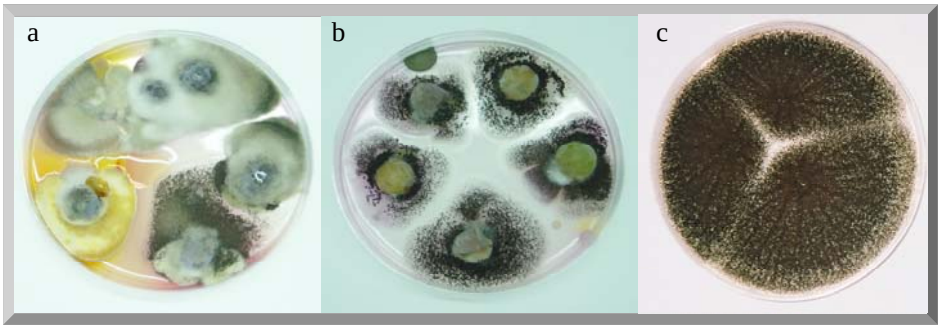


**Figure 43. Infestation of berries. a) Grape berries on DRBC infected by several fungi, after 7 days incubated at 25 ºC; b) Black aspergilli infecting grapes on DRBC; c) Colonies of *A. niger* aggregate growing on CZ.**

The first year of the study, a representative sample of these isolates was sent to an expert (Dr. Kozakiewicz, CABI Bioscience, UK, and Dr. Cabañes, UAB, Spain) for further authentication and verification and further screening using molecular techniques in order to unequivocally identify the OTA producing strains, particularly within the *A. niger* aggregate. Moreover, a workshop was organized in February 2002 in CABI Bioscience Mycological Institute, Egham, Surrey, UK, to help the partners of the project in black aspergilli identification.

### 7.5.5. Fungal screening for OTA production

The method used to extract OTA from fungal cultures in the studies presented in this section was based on Bragulat et al. (2001) methodology. It was a simple and clean screening method for detecting OTA production by fungi, based on HPLC detection and quantification of OTA in the extracts obtained from agar plugs cuts from pure Petri dish cultures.

After seven days of incubation of the possible ochratoxigenic mould at 25 ºC, three agar plugs (diameter 5-6 mm) were removed from the inner, middle and outer area of each colony. Plugs were weighted and introduced into 3-ml vials. Methanol (1 ml) was added, and the vials were shaken for 5 seconds (Autovortex SA6, Surrey, UK). After being left stationary for one hour, the extracts were shaken again, filtered (Millex$^{R}$ SLHV 013NK, Millipore, Bedford, MA, USA) into another vial and stored at 4 ºC until the analysis by HPLC with fluorescence detection (Figure 44).

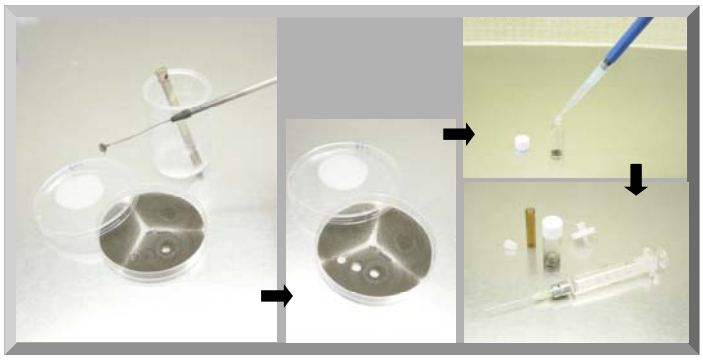

**Figure 44. Different steps in the extraction method of OTA from culture.**

## 7.6. INFLUENCE OF FARMING METHODS

### 7.6.1. Questionnaires

A model questionnaire was designed in order to study the possible factors which influence the prevalence of OTA producing fungi and the levels of OTA, at every stage of grape production, from the field to wine making. Questionnaires dealt with information of locality, climate, vineyard features, farming practices, harvest management, etc. (Appendix 1). Forms were filled annually with data of each of the vineyards sampled (n=40) and were sent to Dr. Paola Battilani (Faculty of Agriculture, Università Cattolica del Sacro Cuore, Piacenza, Italy). They included the information provided by the European countries involved in the study in a database, in order to compile a detailed picture of the different farming methods used across the Mediterranean basin considered as relevant to potential OTA formation.

## 7.7. INFLUENCE OF CLIMATE AND METEOROLOGICAL CONDITIONS.

### 7.7.1. Climate of Spain

Due to its complex orography and geographical situation, Spain has great climatic variability. The spatial differences of annual mean temperature values surpass 18 ºC on the Peninsula and the average annual accumulated precipitation ranges from barely 150 mm to over 2500 mm. It is also important to highlight the high interannual climatic variability and the noteworthy range of extreme daily values (Castro et al. 2005).

Peninsular Spain experiences mainly three climatic types: continental, maritime, and Mediterranean (www.ceit.es/Asignaturas/Ecologia/Hipertexto/03AtmHidr/113ClEsp.htm) (Figure 45).


**Figure 45. Main climatic regions in Spain** (www.ceit.es).

### 7.7.1.1. Temperature

- **Mean annual temperature**

There are latitudinal differences between northern and southern Spain in the mean annual temperatures, strengthened by the differences provided by the Atlantic ocean and the Mediterranean sea (Figure 45). Briefly, the values decrease in the coast-inland direction and increase from north to south, at equal altitude. Inland, values decrease from west to east.

- **Extreme temperatures**

It is well known that the 40 ºC threshold is surpassed every summer in some cities of Andalucía and less frequently, in Castilla-La Mancha, Extremadura, Múrcia, etc.

With regard to minimum absolute temperatures, on the coasts of the Peninsula and Islands, frost is infrequent or even non-existent. But the continentality and the altitudes of the inland Peninsula and of the mountain ranges on occasions permit very low minimums. Temperatures of below –10 ºC have been recorded in some points.

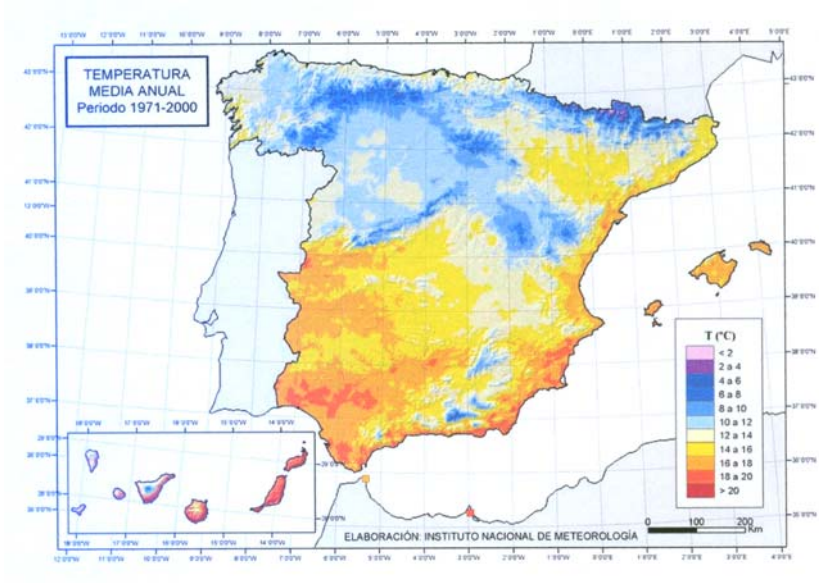


**Figure 46. Annual mean temperature (ºC)** (Spanish Meteorological Institute, 2003).

### 7.7.1.2. Rainfall

- **Mean annual rainfall**

The mean annual total rainfall has traditionally been used to distinguish three large zones in Spain: rainy, dry and semiarid Spain. The dividing line between the rainy and dry parts of Spain is usually established by the 800 mm isoyet and, in some cases, the 600 mm or intermediate one. The dividing line between dry Spain and semiarid Spain is established by the thresholds of 300 or 350 mm. The respresentation of three categories is not perfectly separated in space. Thus, the map of mean annual rainfall in Spain is a very complex one, with many enclaves with high or low pluviometry inserted into regions presenting the opposite sign (Figure 47). At joint scale, annual rainfall on Iberian Peninsula decreases from north to south and from west to east.

Other related parameters such as the existence of an interannual rainfall variability, seasonal regime and the rainfall intensity have to be present.

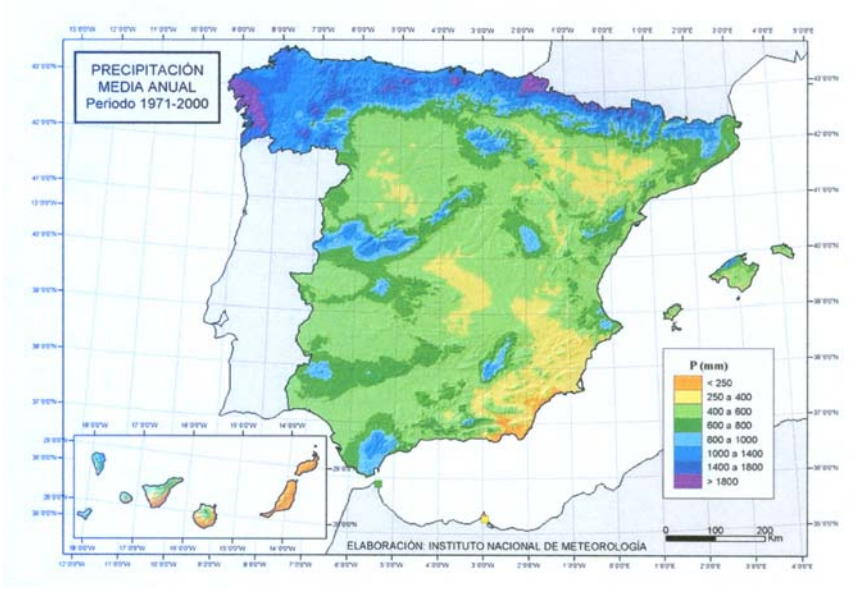


**Figure 47. Annual mean precipitation (mm)** (Spanish Meteorological Institute, 2003).

### 7.7.1.3. Other parameters

Solar radiation, insolation and cloudiness, hail, air humidity, atmospheric pressure and wind, are other parameters that could influence in the dissemination of spores and fungal contamination of the grapes at field. Data of some of these parameters may be collected and correlated to the occurrence of OTA in grapes.

## 7.8. OCCURRENCE OF OCHRATOXIGENIC FUNGI AND OTA IN SPANISH WINE GRAPES

### 7.8.1. Results of 2001

An extensive survey of filamentous fungi isolated from forty vineyards from four wine making regions of Spain and their capacity to produce OTA on CYA agar was carried out in 2001, in order to assess their potential for producing this toxin in grapes. Results are shown in the following paper:

Occurrence of OTA and toxigenic potential of fungal isolates from Spanish grapes. *Journal of the Science of Food and Agriculture* 84, 541-546 (2004).

Briefly, the fungal infection in berries increased with time, reaching a 100 % at harvest. A total of 386 isolates of *Aspergillus* section *Nigri* and 10 *Aspergillus ochraceus* were isolated and tested for their ability to produce OTA in CYA. Twenty-one strains produced OTA: 18 *Aspergillus* section *Nigri* and 3 *A. ochraceus*, the latest produced higher amounts than black aspergilli, with means of 10.76 μg $g^{-1}$ CYA and 1.42 μg $g^{-1}$ CYA, respectively. Despite this, black aspergilli are believed to be highly responsible for the OTA levels found in musts and wines, as it is more widespread in grapes.

Musts (n=40) produced from the grapes collected were analysed. 15% were found to contain OTA, concentrations ranging from 0.091 to 0.813 ng $ml^{-1}$ (detection limit: 0.07 ng $ml^{-1}$), but no correlation was found with the ochratoxigenic moulds isolated from grapes.

# Occurrence of ochratoxin A and toxigenic potential of fungal isolates from Spanish grapes

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**ABSTRACT**

This paper reports the results from an extensive survey of filamentous fungi isolated from wine-producing grapes and their capacity to produce ochratoxin A (OTA) on Czapek Yeast Autolysate agar (CYA), in order to assess their potential for producing this toxin in grapes. Grapes were sampled from four Spanish wine-producing regions during 2001. The fungal infection in berries increased with time, reaching a 100% at harvest.

A total of 386 isolates of *Aspergillus* section *Nigri* and 10 of *Aspergillus* section *Circumdati* were isolated and tested for their ability to produce OTA in CYA. 21 strains produced OTA (18 *Aspergillus* section *Nigri* and 3 *Aspergillus* section *Circumdati*). *Aspergillus* section *Circumdati* isolates produced higher amounts of OTA than *Aspergillus* section *Nigri* ones, with means of 10.76 μg $g^{-1}$ CYA and 1.42 μg $g^{-1}$ CYA, respectively. Despite this, black aspergilli are believed to be highly responsible for the OTA levels found in musts and wines, as it is more widespread in grapes.

Musts (n=40) produced from the grapes collected were analysed. 15 % were found to contain OTA, concentrations ranging from 0.091 to 0.813 ng $ml^{-1}$ (detection limit: 0.07 ng $ml^{-1}$), but no correlation was found with the ochratoxigenic moulds isolated from grapes.

**KEY WORDS:** *Aspergillus* section *Nigri; Aspergillus ochraceus*; ochratoxin A; grapes; must.

***Journal of the Science of Food and Agriculture* 84, 541-546 (2004)**

## INTRODUCTION

Mycotoxins are secondary metabolites produced by certain filamentous fungi. They can be produced in foods as a result of fungal growth. The increasing number of mycotoxin studies reflects the population high concern about their presence in commodities, as they are responsible for a wide range of toxic effects. Ochratoxin A (OTA) is an important nephrotoxic and nephrocarcinogenic mycotoxin and has been associated with Balkan Endemic Nephropathy and with the development of urinary tract tumours in humans.[1,2] Despite its toxicity, in the European Union it has only been regulated recently in a few food products, such as cereals and their based products (5 μg OTA $kg^{-1}$) and dry grapes (3 μg OTA $kg^{-1}$).[3] In Italy there is a regulation also for beer (200 ng OTA $l^{-1}$).[4] The European Union is considering to impose regulatory limits in more foodstuffs such as wine, grape juice, coffee and cocoa.

Ochratoxin A was discovered in 1965 as a secondary metabolite of *A. ochraceus* strains.[5] In the following years, several other *Aspergillus* and *Penicillium* species were reported as producers of this toxin, including various strains of species belonging to the section *Circumdati* (*A. ochraceus* group): *A. alliaceus*, *A. melleus*, *A. sulphureus*, *A. ostianus*, *A. petrakii*, *A. sclerotiorum*, *A. albertensis* and *A. auricomus*.[6-8] However, in the genus *Aspergillus*, the production of OTA has been recently related to species outside the section *Circumdati*. Thus, it has been clearly established as a metabolite of different species of the section *Nigri*, such as *A. niger*, *A. carbonarius*, *A. awamori* and *A. foetidus*.[9,10] As the taxonomy of the *Aspergillus* section *Nigri* is far from clear it is proposed to group its species according to morphological and cultural criteria,[11-15] or moreover to distinguish them by molecular studies.[16-20]

The black aspergilli are distributed world-wide, occurring on a great variety of substrates, and are widely used in food industry for the production of organic acids, enzymes, and other products.[21] *A. ochraceus* group and black aspergilli, such as *A. niger* and *A. carbonarius*, are the OTA-producing species most frequently found in warm and tropical regions of the world while, in temperate climates, OTA is produced by *P. verrucosum*, which is the only known ochratoxin-producing species in the genus *Penicillium*.[22]

Although certain commodities, such as cereals or pork and poultry meat, are known to be particularly susceptible to OTA contamination[2], there is an increasing incidence of detection of OTA in a large variety of foods, such as coffee,[23-30] beer[27-29] and milk.[31] OTA has also been recently reported in grapes, grape juices, musts and wine.[32-36]

The aims of this work were to screen for the presence of filamentous fungi, paying special attention to the above reported OTA-producing species in *Aspergillus* section *Nigri* and *Circumdati*, and to test these fungi *in vitro* for their OTA production capacity. Any correlation between the incidence of ochratoxigenic fungi and the presence of OTA in must was tested.

## EXPERIMENTAL

### Sampling design

Grapes were harvested from four important wine-producing regions in Spain, which belong to Costers del Segre (North-East), La Rioja (North), Penedés and Conca de Barberà (North-East) and Utiel-Requena (East) denominations of origin. Ten vineyards were chosen from each region. Both white (n=12) and red varieties (n=28) were sampled. The white varieties more frequently sampled were Chardonnay and Macabeo, and the red ones were Cabernet Sauvignon, Tempranillo and Garnacha. Some local varieties were also chosen. Sampling took place in four periods during the year 2001: (1) at setting (end of May-beginning of June), (2) 1 month later, (3) early veraison (beginning of August) and (4) just before harvest (mid-September). Ambient temperatures, rainfall and relative humidity during the time points of sample collections are shown in Table 1. In the first sampling period, ten vines were chosen approximately along the diagonals of each vineyard and labelled. The plants sampled changed in each sampling date: the plant following the one sampled in the previous date along the same row in the vineyard was chosen. One single bunch was collected from each plant at each sampling date. Bunches were put in paper bags and stored at 4ºC, until they were transferred to the laboratory where they were processed. Five berries were randomly taken from each bunch and direct plated onto Dichloran Rose Bengal Chloramphenicol medium (DRBC) in Petri dishes. All plates were incubated for 7 days at 25ºC. After this time, all *Aspergillus* section *Nigri* and *Circumdati* were isolated in Czapek Dox Agar (CZ) and incubated for 7 days at 25ºC for their classification and identification according to Dr. Kozakiewicz guidelines (CABI Bioscience, Egham, UK) (personal communication).

For each vineyard, musts were made by smashing and filtering the 10 bunches collected in the fourth sampling period. A total of 40 must samples were frozen (-18ºC) until analysis.

### Extraction, detection and quantification of OTA from culture

For the study of OTA production, isolates were inoculated on Czapek Yeast Autolysate agar (CYA) and incubated at 25ºC for 7 days.

Ochratoxin A was extracted by a variation of a simple method previously described.[37] Briefly, three agar plugs (diameter: 6 mm) were obtained from the inner, middle and outer area of each colony of potential ochratoxin-producers grown on CYA plates, and were introduced in a vial containing 900 µl of methanol. After 60 minutes, the extracts were shacken and filtered (Millex$^{R}$ SLHV 013NK, Millipore, Bedford, Massachusetts, U.S.A.) into another vial and stored at 4ºC until chromatographic analysis.

The production of OTA was detected and quantified by high-performance liquid chromatography (HPLC) with fluorescence detection ($\lambda_{exc}$ 330 nm; $\lambda_{em}$ 460 nm) using a $C_{18}$ column (Waters Spherisorb 5 μm, ODS2, 4.6x250 mm, Milford, Massachusetts, U.S.A.). The mobile phase (acetonitrile-water-acetic acid, 57:41:2) was pumped at 1.0 ml $min^{-1}$. The injection volume was 25 μl and the retention time was about 7 min. The detection limit of the analysis was 0.02 μg OTA $g^{-1}$ of CYA, based on a signal-to-noise ratio of 3:1. The ochratoxin standard was from *A. ochraceus* (Sigma-Aldrich, Steinheim, Germany). The standard solution was made in methanol (Merck, Darmstadt, Germany) and confirmed by using an UV spectrophotometer.

**Table 1. Ambient temperatures, rainfall and relative humidity during the time points of sample collections.**

| | | Temperature (ºC) | | | Rainfall (mm) | R.H. (%) |
|---|---|---|---|---|---|---|
| **Region** | **Month** | **$T_{min}$** | **$T_{mean}$** | **$T_{max}$** | | |
| Requena (Valencia) | May | 9.6 | 15.4 | 21.2 | 1.27 | 60 |
| | June | 14.5 | 22.9 | 31.3 | 0.18 | 64 |
| | July | 15.9 | 23.7 | 31.6 | 0.00 | 64 |
| | August | 17.6 | 25.0 | 32.1 | 0.10 | 68 |
| | September | 15.0 | 21.2 | 27.0 | 0.33 | 69 |
| Haro (La Rioja) | May | 8.9 | 15.4 | 21.8 | 0.51 | 57 |
| | June | 12.2 | 19.9 | 27.6 | 0.00 | 51 |
| | July | 14.5 | 20.8 | 27.2 | 1.32 | 55 |
| | August | 15.4 | 22.5 | 29.5 | 0.31 | 59 |
| | September | 11.2 | 17.0 | 22.8 | 1.99 | 65 |
| Lleida | May | 11.3 | 18.4 | 25.4 | 1.25 | 60 |
| | June | 15.2 | 23.5 | 31.7 | 0.26 | 48 |
| | July | 17.0 | 24.3 | 31.6 | 2.61 | 55 |
| | August | 18.6 | 26.0 | 33.4 | 0.14 | 53 |
| | September | 12.2 | 19.6 | 26.9 | 0.87 | 61 |
| Penedès* | May | 12.6 | 17.9 | 23.9 | 0.87 | 63 |
| | June | 16.2 | 22.3 | 28.3 | 0.16 | 62 |
| | July | 17.8 | 23.7 | 29.5 | 1.81 | 67 |
| | August | 19.9 | 25.6 | 31.2 | 0.05 | 68 |
| | September | 14.5 | 19.9 | 25.2 | 1.70 | 67 |

*Mean of the data from two meteorological stations located nearest to the sampled area.

### Extraction, detection and quantification of OTA from musts

The method for the determination of specific mycotic contaminants randomly occurring in wines from the *Office International de la Vigne et du Vin* was used.[38] Each sample (100 ml) was brought to a pH value of 7.4 by using NaOH (4M). Samples were passed through immunoaffinity columns (Ochraprep, Rhône Diagnostics Technologies LTD, Glasgow, Scotland) at a flow rate of 2-3 ml $min^{-1}$. Then, they were centrifuged (3830 g, 15 min) and filtered (Whatman num. 1). The columns were then washed with 20 ml of distilled water at a flow rate of 5 ml $min^{-1}$ and finally dried in an air stream (2 min). The desorption was carried out with 1.5 ml of methanol/acetic acid (98/2) solution that was slowly passed through the column. The eluate was then evaporated to dryness under a stream of nitrogen at 40ºC and dissolved in 2 ml of mobile phase (acetonitrile 48% - sodium acetate 4mM/acetic acid (19/1) 52%) prior to HPLC analysis. A sample (25 µl) was injected into the HPLC system (flow rate: 1.0 ml $min^{-1}$) equipped with a fluorescence detector ($\lambda_{exc}$ 230 nm; $\lambda_{em}$ 458 nm) and a $C_{18}$ column (Waters Spherisorb 5 µm, ODS2, 4.6x250 mm, Milford, Massachusetts, U.S.A.). The detection limit of the analysis was 0.07 ng OTA $ml^{-1}$ must, based on a signal-to-noise ratio of 3:1. The retention time of OTA under the conditions described was approximately 12 min.

### Statistical analyses of the data

Analysis of variance was made for percentage of *Aspergillus* section *Nigri* and *Circumdati* infection, for percentage of OTA-producing isolates, and for the amount of OTA detected in musts by using SAS program version 8.02 (SAS Institute, Inc., Cary, N.C., U.S.A.).

## RESULTS AND DISCUSSION

### Fungi isolated from grape samples

Figure 1 shows the percentage of infected berries of the total of 500 sampled in each region every sampling period, after 7 days of incubation at 25ºC on DRBC media. The fungal infection increased with time, reaching 100% of infection in the last sampling period in all regions.

Different genera were isolated from these grapes and the most frequent representatives were *Absidia*, *Alternaria*, *Aspergillus*, *Cladosporium*, *Epicoccum*, *Fusarium*, *Penicillium*, *Rhizopus* and *Ulocladium.* Among them, *Alternaria* was the most common genus in all samplings (80%). When we compare the vineyards studied no differences in distribution of these genera were detected. Other authors have also shown the wide range of fungi that infect grapes.[39-41]

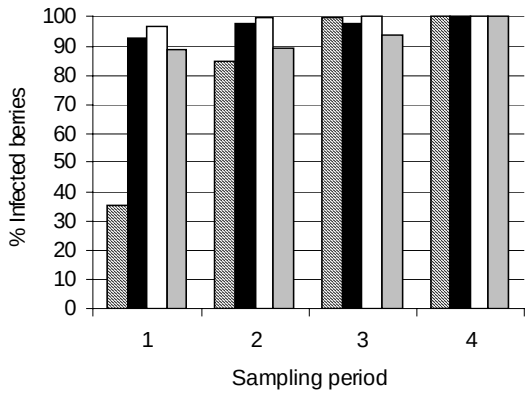


**Figure 1.** Percentage of infected berries found in each denomination of origin: La Rioja (▧); Utiel-Requena (■); Costers del Segre (□); Penedès-Conca de Barberà (▩), at setting (1); 1 month later (2); early veraison (3) and just before harvest (4).

The potential OTA-producing species were transferred to CZ plates in order to identify them. *Aspergillus* section *Nigri* and *Circumdati* isolates were found, but *P. verrucosum* was not detected. Serra *et al*[41] detected 192 isolates of *Penicillium* from Portuguese grapes, belonging to thirteen species and Sage *et al*[41] found that *Penicillium* (31%) and *Aspergillus* (10%) were the most often encountered genera out of 11 samples of grapes from Southern France. None of these two surveys found *P. verrucosum*. It is known that *P. verrucosum* appears more often in cool and temperate regions, such as in Northern Europe.[42] This could be the reason of the absence of this fungus in our samples. Thus the occurrence of ochratoxin in grapes from the regions sampled could be entirely attributed to *Aspergillus* species.

The percentage of infected berries which had *Aspergillus* section *Nigri* and *Circumdati* infection for each region are shown in Fig 2. At the first sampling date, few *Aspergillus* section *Nigri* and no *Aspergillus* section *Circumdati* were detected but, in the next sampling periods, the number of isolates of these species increased. However, that of the black aspergilli increase was higher than *A. ochraceus* group. The statistical analysis of the results showed a significant effect ($p<0.01$) of the sampled region and the sampling period in the number of *Aspergillus* section *Nigri* isolated from the grapes. The longer the grape was on the vine, the more black aspergilli appeared, with most being found in the fourth sampling period. In the case of the Costers del Segre denomination of origin, the number of black aspergilli isolates found in the third sampling date was significantly higher than in the other regions whereas, for the first, second and fourth sampling periods, no significant differences in the number of these strains were found between the different

regions. Looking at the meteorological data, the only difference found for Costers del Segre was as slightly higher temperature levels in the two months preceding harvest.

The statistical analysis of the number of *Aspergillus* section *Circumdati* isolated did not show any significant differences among regions or among sampling periods.

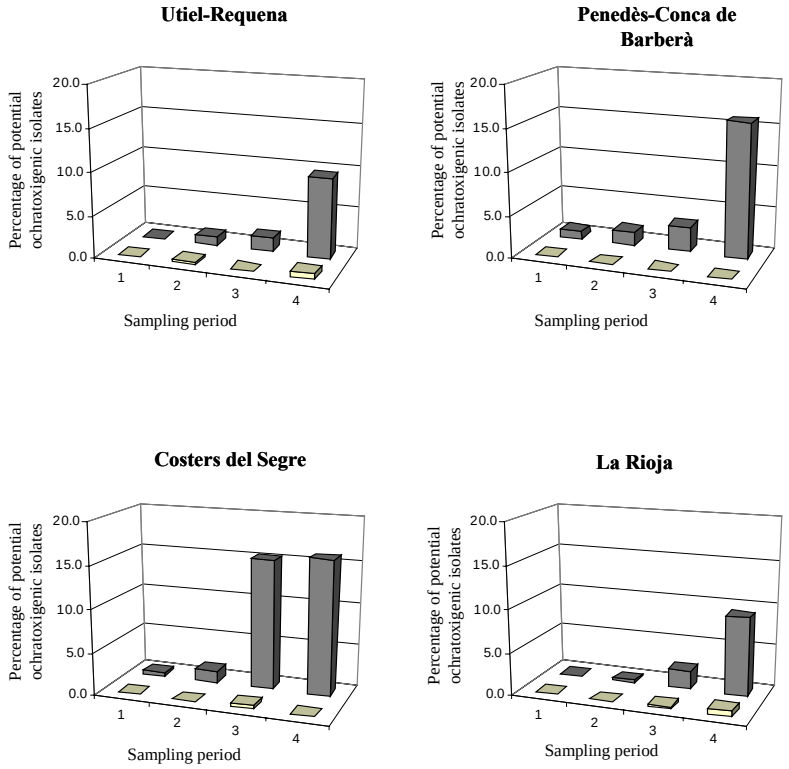


**Figure 2.** Percentage of infected berries by *Aspergillus* section *Nigri* ( ■ ) and section *Circumdati* (□) related to the total number of infected berries: at setting (1); 1 month later (2); early veraison (3) and just before harvest (4).

## Ochratoxigenic ability of *Aspergillus* section *Nigri* and *Circumdati* isolated from grapes

A total of 396 *Aspergillus* (386 *Aspergillus* section *Nigri* and 10 *Aspergillus* section *Circumdati*) were isolated and tested for their ability to produce ochratoxin A in CYA medium. Twenty-one isolates were shown to produce OTA [18 *Aspergillus* section *Nigri*

(4.6%) and 3 *Aspergillus* section *Circumdati* (30%)]. Less positive OTA *A. niger* strains were found by Serra *et al*[41] who detected only one out of 38 tested. Da Rocha Rosa *et al*,[40] found higher percentage of OTA-producing *A. niger* strains (16.6% and 30.18% from Argentinean and Brazilian grapes, respectively). Sage *et al*[41] showed that all *A. carbonarius* (n=14) tested *in vitro* on CYA and yeast extract sucrose produced OTA after 7 and 14 days of incubation.

When analysing the percentage of ochratoxigenic isolates, both sampling period and sampled region had significant effect ($p<0.01$). The number of ochratoxigenic isolates was significantly higher in the fourth sampling period for Costers del Segre than for the other regions sampled, where no statistical differences in that number were found among sampling periods. This dependence on the sampling period probably involves the environmental condition prevailing on the prevalence of OTA-producing strains. More studies on OTA occurrence in grapes from various geographical areas are needed in order to evaluate the different susceptibility to mycotoxin formation in relation to the specific growing area.

Table 2 shows the frequency of distribution of OTA concentrations from the ochratoxigenic isolates tested. They produced mainly low amounts of OTA (0-2.5 μg $g^{-1}$ CYA). The mean OTA production was 1.42 μg $g^{-1}$ for *Aspergillus* section *Nigri* and 10.76 μg $g^{-1}$ for *Aspergillus* section *Circumdati* when grown on CYA, with maximum concentrations of 2.82 μg $g^{-1}$ and 22.03 μg $g^{-1}$, respectively. The OTA production ranged from 0.5 to 87.5 μg $g^{-1}$ for the 14 *A. carbonarius* tested by Sage *et al*.[41] Although *A. ochraceus* group produced higher amounts of OTA than *Aspergillus* section *Nigri*, the high number of black aspergilli found in grapes suggested that they could be the main responsible for the frequent OTA levels found in grape juices and wines.

**Table 2. Frequency of distribution of OTA concentrations in CYA.**

| Concentration range (μg OTA $g^{-1}$ CYA) | Number of isolates | Mean (± SD) (μg OTA $g^{-1}$ CYA) |
|---|---|---|
| <DL – 2.5 | 17 | 1.23 ± 0.44 |
| 2.5 – 5 | 2 | 2.81 ± 0.02 |
| 5 – 10 | 1 | 9.27 |
| 10 – 15 | 0 | - |
| 15 – 20 | 0 | - |
| 20 – 25 | 1 | 22.03 |

DL, detection limit (μg OTA $g^{-1}$ CYA); SD, standard deviation.

**OTA detection in musts**

The analysis of the forty samples of handmade musts showed that six of them were OTA contaminated (15%) at low level. The most contaminated samples were two samples coming from Costers del Segre region (0.813 and 0.156 ng OTA $ml^{-1}$) and one sample from Utiel-Requena (0.293 ng $ml^{-1}$). Furthermore, three samples from La Rioja were found to be contaminated with OTA, at levels of 0.108 ng $ml^{-1}$, 0.071 ng $ml^{-1}$ and 0.091 ng $ml^{-1}$. In musts originating from Penedès and Conca de Barberà grapes, OTA was not detected above the detection limit (0.07 ng $ml^{-1}$).

No correlation between the incidence of ochratoxigenic fungi on grapes collected in the fourth sampling period and the level of OTA contamination in musts made with these grapes could be established, probably due to the limited number of musts containing OTA. However, Sage *et al*[41] have pointed to a possible correlation between the presence of ochratoxin-producing strains on grapes and the presence of OTA in musts in their study with 11 handmade musts. Despite this, most of the existing surveys deal with commercial musts, which commonly contain OTA.

More data on the occurrence of OTA in musts and ochratoxigenic moulds on grapes are required to establish a correlation.

## CONCLUSIONS

There is a significant grape infection by potential OTA producers at harvest. Although only a small number of them have been shown to be high producers, their implication in winemaking has to be taken into account, because OTA has been detected in musts derived from those grapes. It is important, therefore, to focus on the environmental conditions that may favour high OTA production in the field and in control strategies.

## ACKNOWLEDGEMENTS

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### 7.8.2. Results of 2002 and 2003

The same vineyards (n=40) as in 2001, were sampled at three different growth stages in 2002 and 2003. The aim was to study the fungi associated with grapes and their ability to produce OTA on synthetic media. Results are detailed in the following paper:

OTA-producing fungi in Spanish wine grapes and their relationship with meteorological conditions. *European Journal of Plant Pathology* (in press).

Among the total mycoflora of 2002 and 2003 samplings, 464 (7.7 %) and 648 (10.8 %) *Aspergillus* section *Nigri* (black aspergilli) strains were isolated, respectively, and were classified into three groups: isolates with uniseriate heads, *A. niger* aggregate and *A. carbonarius*. The latter presented the highest percentage of OTA-positive strains (82 % in 2002 and 76 % in 2003) and produced the highest levels of toxin (2.5-25 μg $g^{-1}$). The sampling year, sampling date, the region and their interactions presented significant differences in the number of black aspergilli isolated. Most black aspergilli were found in 2003 and at the sampling near harvest.

However, it is difficult to estimate growth and toxin production under field conditions in which temperatures fluctuate daily and seasonally. Thus, meteorological parameters of each region were analysed. A positive correlation between the number of black aspergilli in grapes and the temperature in the field was found. Grapes from 2003, the warmest year, and from Costers del Segre, the warmest region, were significantly the most contaminated. No significant correlation between black *Aspergillus* presence and other meteorological factors such as R.H. or rainfall could be established. Musts from all the vineyards were also analysed in both years, and no OTA was found in either year.

# Ochratoxin A-producing fungi in Spanish wine grapes and their relationship with meteorological conditions

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**ABSTRACT**

Forty vineyards from four wine making regions of Spain were sampled at three different growth stages in 2002 and 2003. The aim was to study the fungi associated with grapes and their ability to produce ochratoxin A (OTA) on synthetic media. Among the total mycoflora, 464 (7.7 %) and 648 (10.8 %) *Aspergillus* section *Nigri* (black aspergilli) strains were isolated in 2002 and 2003, respectively, and were classified into three groups: isolates with uniseriate heads, *A. niger* aggregate and *A. carbonarius*. The latter presented the highest percentage of OTA-positive strains (82 % in 2002 and 76 % in 2003) and produced the highest levels of toxin (2.5-25 μg $g^{-1}$). The sampling year, sampling date, the region and their interactions presented significant differences in the number of black aspergilli isolated. Most black aspergilli were found in 2003 and at harvest. A positive correlation between the number of black aspergilli found in grapes and the temperature in the field was found. Grapes from 2003, the warmest year, and from Costers del Segre, the warmest region, were significantly the most contaminated. No significant correlation between black aspergilli presence and other meteorological factors such as relative humidity or rainfall could be established. Musts from all the vineyards were also analysed in both years, although no OTA was found in either year.

**KEY WORDS**: *Aspergillus* section *Nigri*, *A. carbonarius*, musts, mycoflora, ochratoxin A, wine grapes.

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## INTRODUCTION

Ochratoxin A (OTA) is a toxic secondary metabolite naturally occurring in a wide range of foods both of vegetable and animal origin (van Egmond and Speijers, 1994). OTA has been reported in wine since 1996 (Zimmerli and Dick, 1996). Wine is estimated to be the second source of OTA in the diet after cereals in Europe, as it can represent up to 15 % of the total OTA intake (Codex Alimentarius Commission, 1999). OTA possesses teratogenic, nephrotoxic and immunotoxic properties and has been classified as a possible human carcinogen (Group 2B) (IARC, 1993).

OTA is produced by species in *Aspergillus* sections *Nigri* (black aspergilli) and *Circumdati*, commonly found in warm and tropical climates, with *Penicillium verrucosum* being the main source in temperate climates and more frequently associated with cereals (Pitt and Hocking, 1997). Recent studies have suggested that black aspergilli, essentially *A. carbonarius*, are the main species producing OTA in grapes (Abarca et al., 2003; Battilani et al., 2003; Bellí et al., 2004b; Cabañes, et al., 2002). With growing concern over European exposure to OTA, the European Union legislation authorities have recently set up a limit of 2.0 µg $l^{-1}$ wine, must or grape juice (European Commission, 2005).

Little information exists on mycoflora and potential OTA-producing fungi in Spanish wine grapes. This study focused on the identification of the common mycoflora in wine grapes from four important grape growing regions of Spain to study their progression during grape ripening in 2002 and 2003, with particular interest in ochratoxigenic species and their ability to produce OTA. An initial study was carried out by our team in 2001, and the results have been recently published (Bellí et al., 2004a). In the present study the objective was to correlate the fungal populations isolated over the three years with the meteorological conditions in the vineyards.

## MATERIAL AND METHODS

### Field sampling

Four wine-producing regions representing a cross section of five important Designations of Origin of Spain (La Rioja, Costers del Segre, Utiel-Requena, Penedés and Conca de Barberà) were chosen for the study. Ten fields were selected in each region (n=40) covering a range of foreign and regional grape varieties, both red and white. Samples were taken at three growth stages (1 month after setting, veraison and harvest time) in 2002 and 2003. Ten vines were chosen along the diagonals of each vineyard and a bunch of grapes was randomly collected from each vine. Bunches were collected in paper bags to reduce handling and prevent external contamination, and kept at 4 ºC until laboratory

analysis. Meteorological data of each sampled region was obtained from the Spanish National Institute of Meteorology database (INM, 2003).

**Mycoflora determination**

Five grapes were randomly chosen from each bunch and plated directly in Petri dishes containing Dichloran Rose Bengal Chloramphenicol medium (DRBC) (Pitt and Hocking, 1997) under sterile conditions. Plates were incubated for 7 days at 25 ºC and colonies of developing fungi were examined and classified into genera according to Pitt and Hocking (1997). Most of the potential OTA producers were isolated onto Czapek Dox agar (CZ) (Pitt and Hocking, 1997) for classification, and onto Czapek Yeast Extract agar (CYA) (Pitt and Hocking, 1997) for OTA production; both media were incubated at 25 ºC for 7 days. As morphological identification of black aspergilli is time-consuming and due to the high number of strains isolated in this study, they were classified according to the morphology of their spores and conidial heads into three groups: uniseriates, *A. niger* aggregate (biseriates excluding *A. carbonarius*) and *A. carbonarius*, as recommended by Dr. Z. Kozakiewicz (CABI Bioscience, UK) and Dr. J. Cabañes (Autonomous University of Barcelona, Spain).

**Screening of fungi for OTA production**

The method used was adapted from Bragulat et al. (2001). Three agar plugs, 6 mm in diameter, were extracted in 1 ml of methanol for 1 hour. The extracts were filtered (Millex$^{R}$ SLHV 013NK, Millipore, Bedford, Massachusetts, USA) before chromatographic analysis. A HPLC system with a fluorescence detector (Waters 474, Milford, Massachusetts, USA) ($\lambda_{exc}$ 330 nm; $\lambda_{em}$ 460 nm) and a C18 column (Waters spherisorb 5 μm, ODS2, 4.6 x 250 mm) were used. Mobile phase (acetonitrile-water-acetic acid, 57:41:2) was pumped at 1 ml min$^{-1}$. The ochratoxin standard was from *A. ochraceus* (Sigma-Aldrich, Steinheim, Germany). Recovery of added OTA to the media ranged from 80 to 100 %. The retention time was 7.1 min and the detection limit was 0.01 μg OTA g$^{-1}$ of CYA, based on a signal-to-noise ratio of 3:1.

**OTA in musts**

At the last sampling time of both years, the same ten bunches collected from each vineyard for the mycoflora study were crushed and the resulting musts (n=40) were analysed for OTA using the method of the Office International de la Vigne et du Vin (Bezzo et al., 2002). Briefly, 100 ml of each sample (pH 7.4 with NaOH 4M) were centrifuged (3830 g, 15 min) and filtered (Whatman n. 1). Afterwards, they were passed through an immunoaffinity column (Ochraprep, Rhône Diagnostics Technologies,

Glasgow, UK) at 2-3 ml $min^{-1}$. The column was then washed with 20 ml of distilled water (5 ml $min^{-1}$) and finally dried in an air stream (2 min). Desorption was carried out with 1.5 ml of methanol/acetic acid (98/2) solution. The eluate was evaporated to dryness at 40 ºC under a stream of nitrogen and redissolved in 2 ml of mobile phase (acetonitrile 48%-sodium acetate 4mM/acetic acid (19/1) 52%). About 25 µl of each final sample were injected into a HPLC system equipped with a fluorescence detector (Waters 474) ($\lambda_{exc}$ 230 nm; $\lambda_{em}$ 458 nm) and a $C_{18}$ column (Waters Spherisorb 5 µm, ODS2, 4.6 x 250 mm). The analysis was performed under isocratic conditions at a flow rate of 1 ml $min^{-1}$. Detection limit and retention time were 0.05 µg $l^{-1}$ and 11.5 min, respectively.

**Statistical analysis**

The percentages of infection of common mycoflora, black aspergilli species and OTA-producing isolates, were analysed by the General Linear Model Procedure of SAS (version 8.02, SAS Institute, Inc., Cary, N.C., U.S.A.) with Student-Newman-Keuls (SNK) test ($p$<0.05). The significance of the correlation between maximum, mean and minimum temperatures, relative humidity, rainfall, number of rainy days, number of black aspergilli isolates, number of OTA positive isolates, and number of uniseriates, *A. niger* aggregate and *A. carbonarius* isolates in 2001, 2002 and 2003, was assessed with the same program by using the Pearson coefficients at $p$<0.05.

**RESULTS**

The colonisation of grapes by fungi occurred rapidly in the field and increased from setting (75-85 %) to harvest (100 %) in all regions and in both years. The most common mycoflora isolated from grapes, in decreasing order, were: *Alternaria*, yeasts, *Aspergillus*, *Botrytis*, *Epicoccum*, *Cladosporium*, *Rhizopus*, *Penicillium*, *Fusarium*, *Mucor*, *Phoma*, *Trichoderma* and *Ulocladium*. No statistically significant differences were found between years or regions. Therefore, as an example, the fungi infecting grapes at each sampling date in 2003 in La Rioja region are shown in Figure 1. *Alternaria* was the highest component of the natural flora on the surface of fresh grapes, followed by yeasts. The number of *Aspergillus*, *Botrytis*, *Epicoccum*, *Rhizopus* and yeasts were statistically higher at harvest. The exception was *Alternaria*, which decreased from 95 % to 70 % in the later growth stages. The remaining genera were rarely isolated and did not follow any trend.

According to analysis of variance, all single factors: year, sampling date and region and their interactions, presented significant differences in the number of black aspergilli isolated (p<0.0001). A total of 464 (7.7 %) and 648 (10.8 %) black aspergilli were isolated in 2002 and 2003, respectively, distributed in the four regions sampled. The number of these moulds found at harvest was significantly higher than were found in the first or second sampling for the four regions (Figure 2). Grapes from Costers del Segre

were significantly the most contaminated every year, with approximately 300 strains isolated in 2003 and around 180 in 2002 at harvest, followed by those from Utiel-Requena with around 100 isolates in both years at harvest. However, no statistical differences were found between Penedés/Conca de Barberà and La Rioja regions.

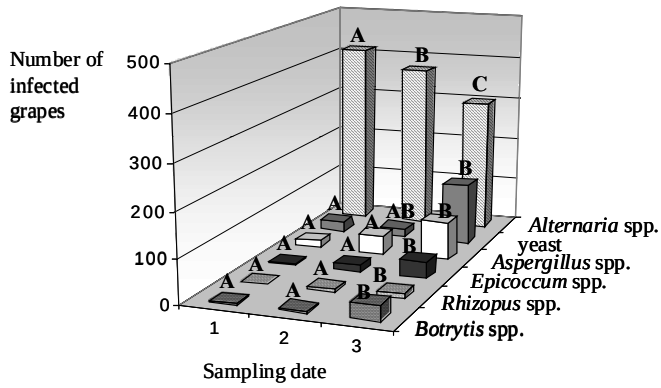


**Figure 1. Number of grapes infected by different fungi from a total of 500 grapes plated on DRBC, at each sampling date: (1) one month after setting, (2) veraison and (3) harvest, in 2003 in La Rioja region. Different letters over bars mean significant differences in the number of these fungi between sampling periods.**

Figure 3 shows the distribution of the black aspergilli isolates that were classified, with *A. niger* aggregate the most common species (75 % in 2002 and 53 % in 2003) followed by *A. carbonarius* (7 % in 2002 and 29 % in 2003). In contrast, the percentage of isolates with uniseriate heads was similar in both years (18 %). No isolates of *P. verrucosum* were detected, while few *A. ochraceus* isolates were found (19 in 2002 and 31 in 2003), most at harvest and representing approximately 15 % of strains which were OTA-producers.

In 2002, 7 % of the total number of black aspergilli strains isolated produced detectable levels of OTA in culture, whereas 25 % were detected in 2003. Among the black aspergilli groups, *A. carbonarius* presented the highest percentage of OTA-positive strains (82 % in 2002 and 76 % in 2003). A low percentage of *A. niger* aggregate isolates produced OTA (2 % in 2002 and 5 % in 2003) and no toxin was detected in any of the uniseriate strains in either year (Table 1). Furthermore, more than 95 % of the total number of positive isolates produced low amounts of OTA (<2.5 $\mu g\ g^{-1}$), although some isolates were found to produce OTA at higher levels (2.5 - 25 $\mu g\ g^{-1}$). The most toxigenic ones were *A. carbonarius* isolates in both years. However, subsequent analysis of musts detected no OTA. Fungi isolated from grapes were correlated to the meteorological data

in each sampling month for each region in 2002 and 2003 detailed in Table 2. A positive correlation between the number of black aspergilli isolated and the temperature in the field in the months preceding harvest was found (Table 3). High relative humidity (R.H.) also contributed to the infection of these fungi, mainly for the uniseriate group.

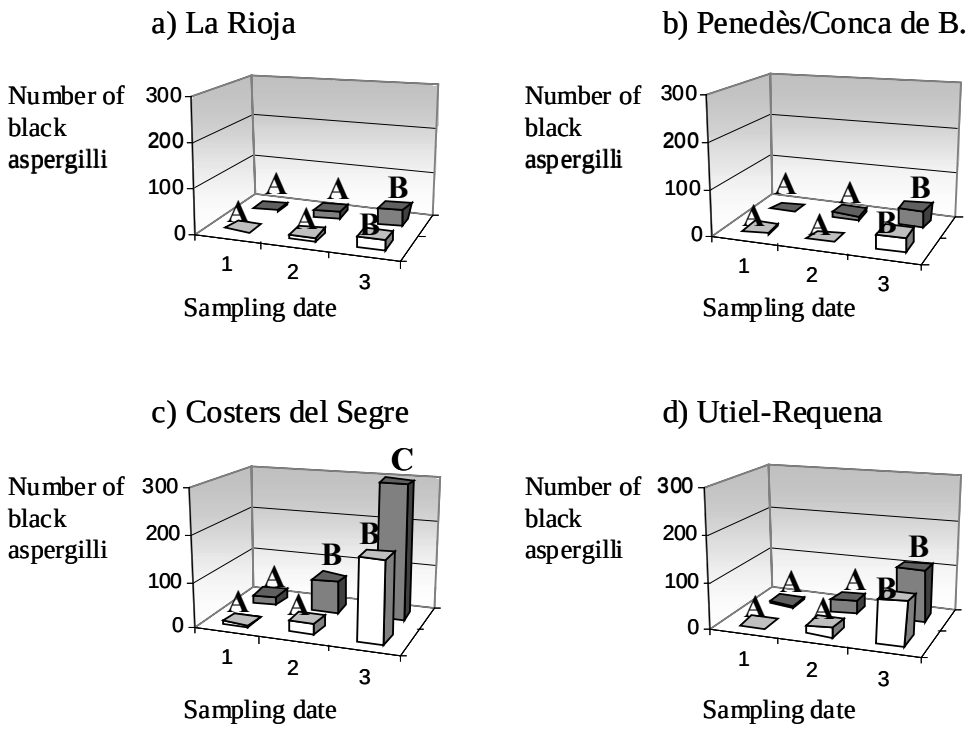


**Figure 2. Number of black aspergilli found on grapes plated on DRBC in □ 2002 and in ■ 2003 in four wine-making regions of Spain, at three sampling periods (2000 grapes per sampling period): (1) 1 month after setting, (2) veraison and (3) harvest. Different letters over bars mean significant differences in the number of these fungi between sampling periods.**

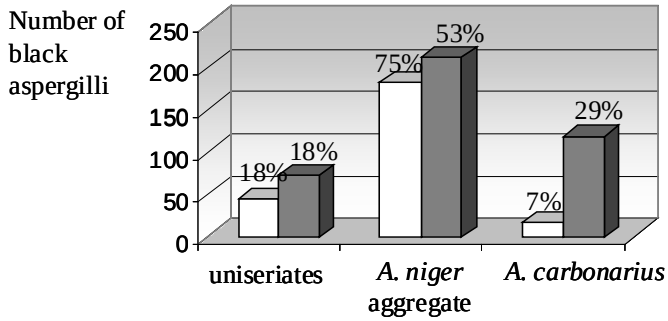


**Figure 3. Number of black aspergilli isolated from grapes in □ 2002 and in ■ 2003, classified into three groups and percentage of each group among all black aspergilli isolated.**

**Table 1. Percentage of black aspergilli isolates of each group found in each region at each sampling date in 2002 and 2003 among the total number of grapes analysed.**

| | | Uniseriate group | | *A. niger* aggregate | | *A. carbonarius* | |
|---|---|---|---|---|---|---|---|
| **S[a]** | **Region** | **2002** | **2003** | **2002** | **2003** | **2002** | **2003** |
| 1 | P/CB[b] | 0% (0/0) | 0% (0/0) | 0.01%(1/0) | 0.03%(2/0) | 0% (0/0) | 0% (0/0) |
| | UR | 0%(0/0) | 0% (0/0) | 0.10%(6/0) | 0.18%(11/0) | 0% (0/0) | 0% (0/0) |
| | R | 0.06% (4/0) | 0.06%(4/0) | 0.20%(12/0) | 0.46%(28/0) | 0% (0/0) | 0% (0/0) |
| | CS | 0% (0/0) | 0.20%(12/0) | 0.03%(2/0) | 0.10%(6/0) | 0% (0/0) | 0% (0/0) |
| 2 | P/CB | 0.25%(15/0) | 0.56%(34/0) | 0.16%(10/0) | 0.25%(15/1) | 0% (0/0) | 0.01%(1/1) |
| | UR | 0.40%(24/0) | 0.23%(14/0) | 1.18%(71/0) | 0.55%(33/2) | 0.01%(1/1) | 0% (0/0) |
| | R | 0% (0/0) | 0% (0/0) | 0% (0/0) | 0.06%(4/2) | 0% (0/0) | 0% (0/0) |
| | CS | 0% (0/0) | 0% (0/0) | 0.25%(15/0) | 0.26%(16/1) | 0% (0/0) | 0.10%(6/0) |
| 3 | P/CB | 0.01%(1/0) | 0% (0/0) | 0.88%(53/0) | 0.96%(58/5) | 0.01%(1/0) | 0% (0/0) |
| | UR | 0% (0/0) | 0% (0/0) | 0.05%(3/1) | 0.03%(2/-)[c] | 0.11%(7/6) | 0.11%(7/7) |
| | R | 0% (0/0) | 0.03%(2/0) | 0.03%(2/1) | 0.13%(8/-) | 0% (0/0) | 0% (0/0) |
| | CS | 0.01%(1/0) | 0.10%(6/0) | 0.10%(6/1) | 0.46%(28/-) | 0.13%(8/7) | 1.73%(104/82) |
| **TOTAL** | | **0.75%(45/0)** | **1.2%(72/0)** | **3.0%(181/3)** | **3.5%(211/11)** | **0.28%(17/14)** | **1.96%(118/90)** |

Numbers in brackets are the total number of black aspergilli isolated / the number of isolates producing OTA above the detection limit (0.01 μg $g^{-1}$ CYA).

[a]S, sampling (1, one month after setting; 2, veraison; 3, harvest);

[b]P/CB, Penedès/Conca-Barberà; UR, Utiel-Requena; R, La Rioja; CS, Costers del Segre;

[c](-) not tested for OTA.

**Table 2. Mean of 2001, 2002 and 2003 meteorological data of each region at each sampling period (1, June; 2, July; 3, August).**

| Region | Sampling | T max[a] (ºC) | T mean[b] (ºC) | T min[c] (ºC) | R.H. (%) | Rainfall (mm) | Rain (days) |
|---|---|---|---|---|---|---|---|
| Utiel-Requena | 1 | 30.3 | 22.9 | 15.5 | 62.1 | 10.2 | 1.3 |
| | 2 | 32.4 | 25.0 | 17.5 | 63.4 | 0.0 | 0.0 |
| | 3 | 32.3 | 25.3 | 18.2 | 65.5 | 21.7 | 2.0 |
| Rioja | 1 | 27.2 | 20.5 | 13.7 | 52.9 | 37.4 | 7.0 |
| | 2 | 27.6 | 20.9 | 14.3 | 52.5 | 23.8 | 5.0 |
| | 3 | 29.1 | 22.3 | 15.4 | 55.3 | 29.3 | 7.3 |
| Penedés/ Conca de Barberà | 1 | 29.2 | 22.6 | 16.5 | 62.2 | 20.8 | 4.3 |
| | 2 | 26.7 | 23.5 | 17.3 | 67.7 | 31.1 | 6.7 |
| | 3 | 30.7 | 24.0 | 18.2 | 68.9 | 21.8 | 4.7 |
| Costers del Segre | 1 | 30.9 | 23.1 | 15.8 | 58.2 | 11.5 | 3.0 |
| | 2 | 30.8 | 23.6 | 17.0 | 64.7 | 61.1 | 9.0 |
| | 3 | 32.4 | 25.1 | 18.2 | 62.6 | 20.5 | 4.7 |

(INM, 2003);
[a]T max: mean daily maximum temperature for each sampling stage;
[b]T mean: mean daily mean temperature for each sampling stage;
[c]T min: mean daily minimum temperature for each sampling stage;
R.H.: mean daily R.H. for each sampling stage.

**Table 3. Correlation between meteorological parameters with ochratoxigenic fungi isolated from grapes, by using the coefficients of Pearson.**

| | Black aspergilli | Black aspergilli OTA+ | Uniseriates | *A. niger* aggregate | *A. carbonarius* |
|---|---|---|---|---|---|
| T max | 0.40* | 0.26 | 0.23 | 0.44* | 0.25 |
| T mean | 0.46** | 0.28 | 0.21 | 0.51* | 0.26 |
| T min | 0.48** | 0.26 | 0.30 | 0.55** | 0.27 |
| R.H. | 0.20 | 0.09 | 0.36 | 0.14 | 0.10 |
| Rainfall | 0.10 | 0.17 | 0.06 | -0.02 | 0.15 |
| Num. rainy days | -0.01 | 0.01 | 0.22 | -0.22 | -0.02 |

*significant $p<0.05$; **significant $p<0.001$.

## DISCUSSION

The main moulds causing secondary rots of grapes are black aspergilli, *Alternaria*, *Rhizopus*, *Cladosporium* and *Penicillium*. These are generally associated with vine trash on soil, leaves, leaf buds and other residues in the field (MAPA, 1998). These fungi were the dominant genera isolated from grapes in the present survey as well as in another study in Argentina and Brazil by Da Rocha Rosa et al. (2002). They found that yeasts were a major component of the fungal population, and *Alternaria*, *Aspergillus* and *Botrytis* were frequently isolated. *Alternaria* and *Aspergillus* were also the most frequent moulds of the mycoflora of Argentinean grapes isolated by Magnoli et al. (2003). *Penicillium*, *Cladosporium* and *Botrytis* prevailed in Portuguese grapes (Abrunhosa et al., 2001). Da Rocha Rosa et al. (2002) suggested that the diversity of grape mycoflora depends on grape variety, degree of berry maturity, physical damage, viticulture practices and climatic conditions.

A positive correlation between the number of black aspergilli isolated from grapes and temperature was found (Table 3). This correlation was mainly due to *A. niger* aggregate. It is known that optimum temperatures for growth of *A. niger* aggregate *in vitro* are between 30-37 ºC; meanwhile the optimum for *A. carbonarius* and uniseriate strains are between 25-30 ºC (Bellí et al., 2004b; Mitchell et al., 2003). In addition, water activity ($a_w$) has been demonstrated to have an effect on *in vitro* growth of strains of black aspergilli, being the highest levels (0.98-0.995 $a_w$), similar to that of grapes, the optimum in most cases (Bellí et al., 2004a).

The highest number of black aspergilli were detected at harvest in the four regions and in both years. The same trend was found in the sampling carried out in 2001 (Bellí et al., 2004a), which suggests that late ripening marks a profound change in the ecological factors affecting fungal sporulation, dissemination of spores as well as microbial growth. External factors such as air movement, cultural practices and insect damage would disseminate spores to the surface of berries and start fungal infection. Moreover, grapes are more susceptible to fungal infection when approaching harvest as sugar content increases and the berry texture softens (MAPA, 1998). All of this, together with the increasing temperatures in the month preceding harvest, sometimes above 30 ºC (Table 2), could influence black aspergilli development. The general pattern of colonisation by fungal species of grapes, was not significantly different in 2001, 2002 and 2003; thus results can be considered representative of the situation in the sampled areas. However, more black aspergilli were isolated in 2003 than in the two previous years, probably because 2003 was an extremely hot year in Spain. High temperatures could also explain the higher number of black aspergilli found in Costers del Segre in 2002 and 2003.

Percentages of uniseriate isolates, *A. niger* aggregate and *A. carbonarius* (21, 60 and 19 %, respectively) found in a survey of Italian grapes in 1999-2000 (Battilani et al., 2003), were very similar to those found in the present study. High incidence of *A. niger*

aggregate was also found by Da Rocha Rosa et al. (2002) in a mycofloral survey of wine grapes from Argentina and Brazil. Less *A. carbonarius* were found, but 25 % of these were OTA producers (18-234 μg $g^{-1}$ on CYA). OTA was not detected in any of the must samples analysed, although in 2001, 15 % of the musts contained low amounts of OTA: five samples contained 0.091-0.293 ng $ml^{-1}$ and one 0.813 ng $ml^{-1}$ (Bellí et al., 2004b). Similar results for OTA in some years but not others has also emerged from an Italian study of OTA content in grapes, which concluded that temperature, rain and relative humidity are the main factors that influenced OTA production in grapes (Battilani and Pietri, 2002). Due to the absence of OTA in the musts analysed, no correlation between the incidence of OTA-producing strains in grapes and OTA in musts could be established from the present study. In contrast, Sage et al. (2002) found a strong correlation between these factors, as eight of eleven must samples were found to be contaminated with OTA (10-461 ng $l^{-1}$) and a significant number of *A. carbonarius* strains were previously isolated from grapes.

Ecophysiological studies with black aspergilli, and in particular *A. carbonarius*, are needed to determine the conditions that favour growth and toxin production. Moreover, it would be interesting to study the infection process of black aspergilli in grapes and the role grape skin damage, in order to determine preventive actions that minimise OTA content in grapes. Further investigations on the mechanisms of interactions and dominance of the fungi commonly isolated from grapes could be also developed.

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### 7.8.3. Review of 2001-03.

A review of the analysis of grapes during 2001, 2002 and 2003 was carried out. Data of other Spanish vineyards analysed by Cabañes et al. (Autonomous University of Barcelona, Spain) was included, in order to give a broader analysis of the Spanish ochratoxigenic mycobiota of grapes. Correlation between meteorological parameters and ochratoxigenic fungi was studied and revealed a significant positive correlation between black aspergilli infection and temperatures in the month preceding each sampling date. No significant correlation was found with either R.H. or rainfall. Biodiversity indexes were also calculated in this study. Black aspergilli species were the most abundant in grapes before harvest, and among them, *A. carbonarius* was the main OTA producer species and represented 78-100 % of the isolates tested. The results obtained clearly support the key role of *A. carbonarius* as the main source of OTA contamination in grapes. The complete review is shown in the following paper:

Mycobiota and OTA-producing fungi from Spanish wine grapes. *International Journal of Food Microbiology* (in press).

# Mycobiota and ochratoxin A producing fungi from Spanish wine grapes

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**ABSTRACT**

Grapes from three different regions with a long winemaking tradition in Spain were analysed at different growth stages in order to identify the ochratoxigenic mycobiota during three consecutive seasons. The correlation between meteorological parameters and ochratoxigenic fungi was studied and revealed a significant positive correlation between black aspergilli infection and temperatures in the month preceding each sampling date. No significant correlation was found with either relative humidity or rainfall. Biodiversity indexes were also calculated in this study. Black aspergilli species were the most abundant in grapes before harvest, and among them, *Aspergillus carbonarius* was the main OTA producer species and represented 78-100 % of the isolates tested. The results obtained clearly support the key role of *Aspergillus carbonarius* as the main source of OTA contamination in grapes.

**KEY WORDS**: mycobiota, ochratoxin A, *Aspergillus carbonarius*, black aspergilli, grapes.

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## INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin that has been shown to be nephrotoxic, immunosuppressive, teratogenic, carcinogenic and genotoxic (Creppy, 1999). It has been classified by the IARC as a possible human renal carcinogen (group 2B) and a recent study confirmed the hypothesis of the involvement of this mycotoxin in Balkan Endemic Nephropathy (BEN) aetiology (Vrabcheva *et al.,* 2004).

OTA exposure mainly occurs via the food chain with cereals and cereal products the most risky commodities. Maximum OTA levels in cereals, dried vine fruits and foods for infants and young children are regulated by the EU. Probably it will be extended to other commodities such as wine, grape juice, coffee, beer and cocoa (Anonymous, 2002, 2004).

Following cereals, wine is considered an important dietary source of OTA in Europe. Since the first report on OTA contamination in wine (Zimmerli and Dick, 1996) the occurrence of OTA in wine samples has been reported in various studies predominantly dealing with European wines.

OTA contamination of wine is due to fungal growth on grapes. Although *Penicillium verrucosum* and *Aspergillus ochraceus* are considered to be the main OTA producing species, they have not been reported as normal mycobiota of grapes. Recently, black aspergilli, mainly *A. carbonarius* and members of the *A. niger* aggregate have been reported as possible sources of OTA contamination in grapes (Da Rocha Rosa *et al*., 2002; Sage *et al.,* 2002; Battilani *et al*., 2003; Magnoli *et al.* 2003; Serra *et al.,* 2003; Bau *et al.,* 2004; Bellí *et al*., 2004a), wine (Cabañes *et al*., 2002) and dried vine fruits (Abarca *et al*. 2003; Heenan *et al*., 1998). The aim of this study was to identify the ochratoxigenic microbiota of grapes from vineyards located on three Spanish regions during three consecutive growing seasons and to correlate them with meteorological parameters in the vineyards.

## MATERIAL AND METHODS

### Sampling planning

Vineyards from three different regions with long winemaking tradition in Spain were chosen for the study. The regions are located as shown in Fig. 1. Overall, 10 vineyards were sampled in La Rioja region, 24 in Catalunya, and 11 in Valencia. The varieties cultivated in the different vineyards, including both red (Garnacha, Tempranillo, Bobal, Graciano, Cabernet Sauvignon, Merlot) and white (Macabeo, Moscatel, Chardonnay, Sauvignon blanc) represented common varieties in each area, some local ones as Bobal or Graciano and others which are more wide spread such as Cabernet Sauvignon.

Ten bunches were collected from the diagonals of each field at four growth stages: setting, one month after setting, early veraison and harvest, in 2001 and at the last three growth stages in 2002 and in 2003. Samples were sent to the laboratory as soon as they were collected and tested upon arrival.


**Figure 1. Location of the sampling areas in Spain.**

**Mycobiota identification**

Five asymptomatic berries were taken from each bunch at random and incubated at 25 ºC for 7 days, and plated on Dichloran Rose Bengal Chloramphenicol medium (Pitt and Hocking, 1997). After the incubation period, all fungal colonies were identified according to Pitt and Hocking (1997) criteria, and the percentage of grapes infected by each mould determined.

Black aspergilli colonies were picked and transferred to Czapek yeast extract agar (CYA) media (Pitt and Hocking, 1997), incubated at 25 ºC for 7 days for further testing for OTA production. During 2002 and 2003, they were also transferred to Czapek Dox agar (Pitt and Hocking, 1997) and allowed to grow at 25 ºC for 7 days to classify them into three main proposed groups (uniseriates, *A. niger* aggregate and *A. carbonarius*) (Abarca *et al.* 2004).

**Biodiversity Indexes**

(a) Simpson Index of diversity (SD) – a measurement that accounts for the richness and the percent of each subspecies from a biodiversity sample within a zone was used. The index assumes that the proportion of individuals in an area indicate their importance to diversity.

$SD = 1\text{-sum}(P_i^2)$

where $P_i$= abundance of a given species in a zone/total number of species

(b) Shannon-Wiener Index (H)– Similar to the Simpson's Index, this measurement takes into account subspecies richness and proportion of each subspecies within a zone.

$H = -\text{sum}(P_i \log[P_i])$

**OTA production**

All the isolates of black aspergilli and *A. ochraceus* were grown on CYA and tested for OTA production. OTA was determined following the methodology described by Bragulat *et al*. (2001). Three agar plugs, 6 mm in diameter, were extracted in 0.5 ml of methanol for one hour. The extracts were filtered (Millex$^R$ SLHV 013NK, Millipore, Bedford, Massachusetts, USA) before chromatographic analysis. A HPLC system with a fluorescence detector (Waters 474, Milford, Massachusetts, USA) ($\lambda_{exc}$ 330 nm; $\lambda_{em}$ 460 nm) and a C18 column (Waters spherisorb 5 μm, ODS2, 4.6 x 250 mm) were used. The mobile phase (acetonitrile-water-acetic acid, 57:41:2) was pumped at a rate of 1 ml $min^{-1}$. The ochratoxin standard was from *A. ochraceus* (Sigma-Aldrich, Steinheim, Germany). The retention time was 7.1 min and the detection limit was 0.01 μg OTA $g^{-1}$ of CYA, based on a signal-to-noise ratio of 3:1.

**Statistical analysis**

The variability of the percentages of infection of common mycobiota, *Aspergillus* section *Nigri* groups and OTA-producing isolates as a function of sampling growth stages, sampling years and regions, were analysed by the General Linear Model Procedure of SAS (version 8.02, SAS Institute, Inc., Cary, N.C., U.S.A.) with the Student-Newton-Keuls (SNK) test ($p<0.05$). The significance of the correlation between maximum, media and minimum temperatures, relative humidity, rainfall, number of rainy days, number of *A.* section *Nigri* isolates, number of OTA positive isolates, and number of uniseriates, *A. niger* aggregate and *A. carbonarius* isolates, was assessed with the same program by using the Pearson correlation coefficients at a $p<0.05$.

## RESULTS and DISCUSSION

### General fungal colonisation of grapes

Over the three years, there has been a high percentage of infected grapes right from the beginning of the field samplings (75-100%), with increasing percentages of infected berries from setting to harvest (100%). The most commonly isolated fungal genera were *Alternaria*, with a decreasing percentage from setting to harvest, yeasts and *Aspergillus*, with increasing percentages from setting to harvest, and *Cladosporium*, *Rhizopus*, and *Penicillium*. Isolates belonging to the following genera: *Arthrinium*, *Botrytis*, *Dreschlera*, *Epicoccum*, *Fusarium*, *Humicola*, *Phoma*, *Staphylocotrichum*, *Trichoderma*, and *Ulocladium*, were occasionally isolated from the grapes. Fungal population diversity increased from June to September (Table 1). This is an example from the results of the 2003 sampling, suggesting both a wider species richness (number of different species found) and a higher abundance in the sampled fields. No differences were found either in the overall fungal contamination or diversity among the different regions sampled.

**Table 1. Simpson and Shannon-Wiener Indices of biodiversity as a function of sampling dates and sampled areas in 2003.**

| Sampling | Region | Simpson Index | Shannon-Wiener Index | Simpson Index in black asp. | Shannon-Wiener Index in black asp. |
|---|---|---|---|---|---|
| One month after setting | La Rioja | 1.41 | 1 | 0.59 | - |
| | Catalunya | 1.82 | 1.59 | 0.88 | 0.38 |
| | Valencia | 1.47 | 1.56 | 0.59 | 0.41 |
| Early veraison | La Rioja | 1.67 | 1.48 | 0.84 | 0.33 |
| | Catalunya | 2.10 | 2.0 | 0.98 | 0.66 |
| | Valencia | 2.11 | 1.54 | 0.99 | 0.39 |
| Harvest | La Rioja | 3.95 | 1.87 | 1.54 | 0.56 |
| | Catalunya | 3.13 | 1.54 | 1.28 | 0.46 |
| | Valencia | 3.74 | 1.94 | 1.55 | 0.72 |

Previously little data was available on the mycobiota of grapes during ripening with nearly all dealing with fungi isolated at harvest time. *Alternaria*, *Aspergillus*, *Cladosporium* and *Penicillium* species have been reported as the predominant mycobiota in harvested grapes from Argentina and Brazil (Da Rocha Rosa *et al*., 2002; Magnoli *et al*., 2003), from France (Sage *et al*., 2002), and also during ripening of grapes from Spain

(Bau *et al.*2004; Bellí *et al.* 2004a). In harvested grapes from Portugal the most prevalent reported genera were *Botrytis*, *Cladosporium* and *Penicillium* (Abrunhosa *et al*., 2001).

**Potential OTA producers on grapes**

No *P. verrucosum* has been found in any of the years and regions sampled. Regarding *A. ochraceus* isolation, the mean percentage found over the three years was 0.16 % (Fig. 2), with no differences between years, regions or sampling dates. In contrast, more than 95 % of the total *Aspergillus* isolated belonged to section *Nigri*. This section infected 0.8 % of the berries in the first sampling, 2.7 % in the second, and 7.9 % at harvest (mean values for the three years). The significantly higher percentage of infection in 2003 was attributed to the extremely dry and hot weather conditions during this year (Table 2), while no significant differences were detected between years 2001 and 2002.

According to our results, black aspergilli have been recently reported as the predominant mycobiota of grapes at harvest time although they can be found on the surface of healthy grapes at all stages (Cabañes *et al.*, 2002; Da Rocha Rosa *et al*., 2002; Sage *et al.*, 2002; Battilani *et al*., 2003; Serra *et al*., 2003; Bau *et al.* 2004; Bellí *et al*., 2004a). Black spores provide protection from sunlight and UV light, providing a competitive advantage in warmer climates (Pitt and Hocking, 1997).

The correlation analysis between percentage of infection and meteorological variables (temperatures, relative humidity and rainfall) revealed a significant positive correlation between black aspergilli infection and maximum, minimum and mean temperatures in the month preceding each sampling date. No significant correlation was found with either relative humidity or rainfall (Table 3).

The reported optimal growth temperature for *A. niger* are 35-37 ºC (Panasenko, 1967; Leong *et al*., 2004) and 30-37 ºC (Bellí *et al.*, 2004b). For *A. carbonarius* the growth temperature range reported is 10-40 ºC (Pitt *et al.*, 2000), with the optimum between 25-35 ºC (Bellí *et al*., 2004b; Leong *et al*., 2004; Mitchell *et al*., 2004). Maximum OTA production for *A. niger* aggregate has been recently reported at 20-25ºC (Esteban *et al*., 2004) and for *A. cabonarius* at 15-20ºC (Bellí *et al*., 2004c; Esteban *et al*., 2004; Mitchell *et al*., 2004)

Looking into the berries infected by black aspergilli, it was observed that 0.4 % of the total berries was infected by uniseriates, with no differences among sampling dates or regions sampled (Fig. 3). *Aspergillus carbonarius* infected a reduced 0.3 %, except for the 2003 third sampling in the Catalunya region where 8.7 % of the berries were infected by this species. This high mean percentage was due to the high infection level in only some of the sampled fields. The region sampled in Catalunya was broader than the other two regions; therefore, this could be the reason for more dispersed results in terms of

black *Aspergilli* (*A. niger* aggregate and *A. carbonarius*, mainly). No *A. carbonarius* was isolated from La Rioja region in any of the samplings.

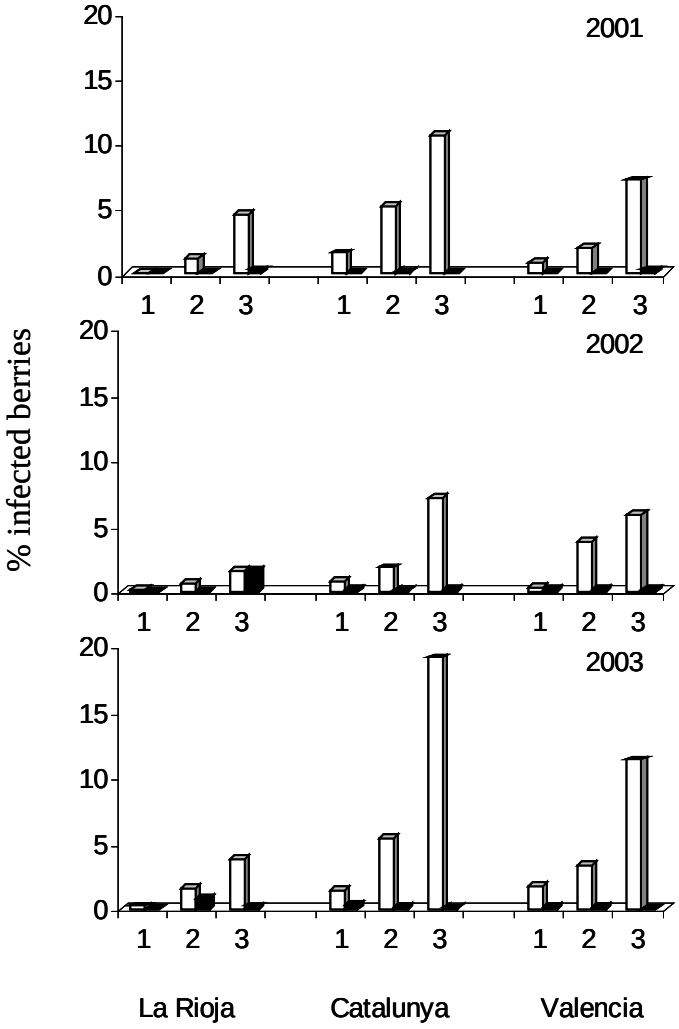


**Figure 2. Percentage of infected berries by black Aspergilli (□) and *A. ochraceus* (■) in the different years, regions and sampling dates (1, one month after setting; 2, veraison; 3, harvest).**

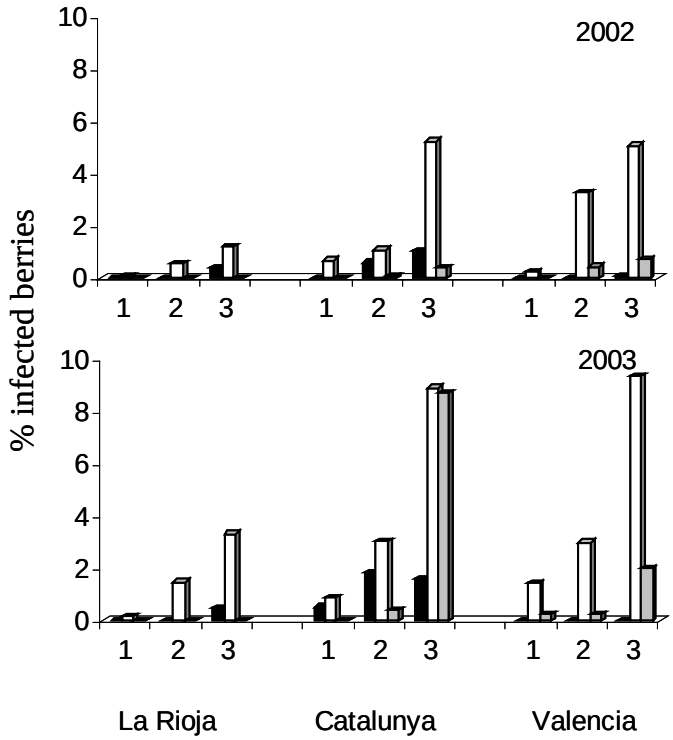


**Figure 3. Percentage of infected berries by *Aspergillus* section *Nigri*: uniseriates (■), *A. niger* aggregate (□) and *A. carbonarius* (▪) in the different years, regions and sampling dates (1, one month after setting; 2, veraison; 3, harvest).**

The total number of black aspergilli isolates, uniseriates, *A. carbonarius*, and *A. niger* aggregate accounted for 7-15%, 5-25% and 67-81%, respectively, depending mainly on the years, but not on sampling dates. *A. niger* aggregate, the most abundant group, was thus responsible for the general trends observed for black aspergilli. Firstly, a significant increase in *A. niger* aggregate was always observed from the first to the third sampling (mean isolation of 0.6, 2.1, and 5.5%, in the first, second and third sampling, respectively). Secondly, significant differences were found between year 2003 and the preceding two, due to the higher temperature levels registered. Finally, although not significant, there was a consistent trend in La Rioja region where lower number of *A. niger* aggregates were isolated. Regarding the diversity in black aspergilli populations for the different samplings and regions, in general there was an increasing diversity from setting to harvest mainly due to the increase in the number of isolates found in the *A. niger* aggregate group (see table 1).

**Table 2. Meteorological data[a] of each sampled region one month before each sampling (1, June; 2, July; 3, August) in 2001, 2002 and 2003.**

| | | 2001 | | | | | 2002 | | | | | 2003 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Region | Sampl. | Tmax[b] | Tmean[c] | Tmin[d] | RH[e] | Rain | Tmax | Tmean | Tmin | RH | Rain | Tmax | Tmean | Tmin | RH | Rain |
| Catalunya | 1 | 27.3 | 21.9 | 16.6 | 62 | 6.9 | 28.5 | 21.8 | 15.3 | 59 | 34.9 | 31.8 | 24.2 | 17.6 | 66 | 20.7 |
| | 2 | 28.6 | 23.5 | 17.0 | 67 | 66.8 | 29.2 | 22.6 | 16.1 | 68 | 20.7 | 31.8 | 24.4 | 17.7 | 68 | 5.9 |
| | 3 | 30.1 | 25.3 | 18.6 | 68 | 4.4 | 28.3 | 21.3 | 16.6 | 70 | 18.3 | 33.2 | 25.5 | 18.5 | 65 | 47 |
| La Rioja | 1 | 27.6 | 19.9 | 12.2 | 51 | 0.1 | 25.3 | 19.3 | 13.3 | 55 | 85.5 | 28.8 | 22.2 | 15.6 | 53 | 26.6 |
| | 2 | 27.2 | 20.8 | 14.5 | 55 | 40.9 | 26.3 | 19.8 | 13.2 | 55 | 24 | 29.3 | 22.2 | 15.1 | 47 | 6.6 |
| | 3 | 29.5 | 22.5 | 15.4 | 59 | 8.6 | 25.4 | 19.5 | 13.7 | 60 | 52.6 | 32.5 | 24.8 | 17.2 | 47 | 26.6 |
| Valencia | 1 | 31.3 | 22.9 | 14.5 | 64 | 5.5 | 28.5 | 21.8 | 15.1 | 65 | 19 | 31.0 | 23.9 | 16.8 | 57 | 6 |
| | 2 | 31.6 | 23.7 | 15.8 | 64 | 0.0 | 31.8 | 24.9 | 18.0 | 67 | 0 | 33.9 | 26.3 | 18.7 | 59 | 0 |
| | 3 | 32.1 | 25.0 | 17.6 | 68 | 3.0 | 30.6 | 24.2 | 17.8 | 73 | 32 | 34.2 | 26.7 | 19.2 | 56 | 30 |

[a] INM, Instituto Nacional de Meteorología, 2003; [b] maximum temperature (ºC); [c] mean temperature (ºC); [d] minimum temperature (ºC); [e] relative humidity (%)

**Table 3. Correlation between meteorological parameters and black aspergilli isolated from grapes, by using the coefficients of Pearson.**

| | ***Aspergillus* section *Nigri*** | ***Aspergillus* section *Nigri* OTA+** | **uniseriates** | ***A. niger* aggregate** | ***A. carbonarius*** |
|---|---|---|---|---|---|
| Maximum temperature | 0.36* | 0.30 | 0.25 | 0.40* | 0.25 |
| Mean temperature | 0.41** | 0.33 | 0.24 | 0.49* | 0.28 |
| Minimum temperature | 0.44** | 0.29 | 0.34 | 0.51** | 0.30 |
| Relative humidity | 0.15 | 0.10 | 0.40 | 0.10 | 0.17 |
| Rainfall | 0.09 | 0.23 | 0.12 | -0.01 | 0.19 |
| No rainy days | -0.02 | 0.03 | 0.26 | -0.11 | -0.03 |

*significant $p<0.05$; **significant $p<0.001$

**OTA-producing isolates from grapes**

The search for OTA producers in black aspergilli among the isolates from the berries led to the testing of 964 isolates for ochratoxigenic capacity in the years 2002-2003 (Table 4). The results were quite consistent, as no significant differences were found in the proportions of OTA positive isolates in the 3 groups, regardless of the sampling years and the regions. Thus, it could be concluded that uniseriates do not produce OTA, that a low percentage of isolates in the *A. niger* aggregate are ochratoxigenic (2-7%), and that most *A. carbonarius* produce OTA (78-100%). Isolates of *A. carbonarius* produced higher amounts of OTA than *A. niger* aggregate isolates (see table 4).

**Table 4. Ochratoxigenic isolates in *Aspergillus* section *Nigri* found in 2002 and 2003 samplings.**

| Year | Region | Number of isolates / % of OTA positive strains ( OTA range in μg/g) | | |
|---|---|---|---|---|
| | | Uniseriates | *A. niger* aggregate | *A. carbonarius* |
| 2002 | La Rioja | 4 / 0 | 14 / 7 (0.20 – 0.32) | - |
| | Catalunya | 17 / 0 | 162 / 2 (0.48 – 0.50) | 12 / 83 (4.30 – 61.0) |
| | Valencia | 24 / 0 | 107 /3 (0.21-0.43) | 13 / 92 (1.42 – 133.20) |
| | | | | |
| 2003 | La Rioja | 6 / 0 | 40 / 7 (0.79-0.81) | - |
| | Catalunya | 53 /0 | 283 / 3 (0.13-1.94) | 127 / 78 (0.05 – 477.3) |
| | Valencia | 14 / 0 | 65 / 5 (0.04-1.71) | 23 / 100 (0.25 – 47.7) |
| **Total** | | **118 / 0** | **671 / 4.5 (0.04-1.94)** | **175 / 87 (0.05-477.3)** |

The reported percentage of OTA-producing isolates of *A. ochraceus* are quite variable, ranging from 54.5% to values lower than 10% (Abarca *et al*. 2001; Pitt and Hocking, 1997). In this study 35% of the 65 isolates of *A. ochraceus* tested produced OTA. So this species is a relatively unimportant source of OTA in grapes.

The reported ability of *A. carbonarius* strains isolated from grapes ranged from 25 % to 100 % (Cabañes *et al*., 2002; Da Rocha Rosa *et al*., 2002; Sage *et al*., 2002; Battilani *et al*., 2003; Serra *et al*. 2003; Bau *et al.* 2004; Leong *et al*., 2004) while in the case of isolates belonging to *A. niger* aggregate this percentage was between 0 and 77% (Da Rocha Rosa et al., 2002; Battilani et al., 2003; Serra *et al*. 2003; Magnoli *et al*. 2003; Bau et al. 2004; Leong *et al*., 2004, Tjamos *et al.*, 2004). These results clearly show the role of *A. carbonarius* as the main source of OTA contamination in wine grapes.

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### 7.8.4. Results of 2004

To confirm the results obtained in 2001, 2002 and 2003, a sampling in 2004 was carried out. The fungi responsible for the presence of OTA in Spanish grapes and the mycoflora present in grapes before harvest were studied and compared with those of previous years.

Grapes from eight Spanish vineyards were screened for the presence of filamentous fungi, especially *Aspergillus* section *Nigri*, and tested for OTA production (Bragulat et al., 2001). Three Designations of Origin were sampled (Utiel Requena, Penedés/Conca de Barberà and Costers del Segre) before harvest ($1^{st}$-$15^{th}$ September 2004). The sampling protocol was the same as the one defined in previous years as well as the method to detect OTA *in vitro*.

Figure 48 shows the percentage of the main fungi isolated from grapes for each vineyard sampled in 2004. *Alternaria* was the most common genera isolated. High percentage of *Cladosporium*, yeasts and *Fusarium* were found in Utiel-Requena Designation of Origin, meanwhile *Botrytis*, *Penicillium*, *Rhizopus*, *A. flavus* and black *Aspergilli* were maximum in Penedés Designation of Origin. No significant differences in the number of *Epicoccum* and *Trichoderma* isolated were found among the regions.

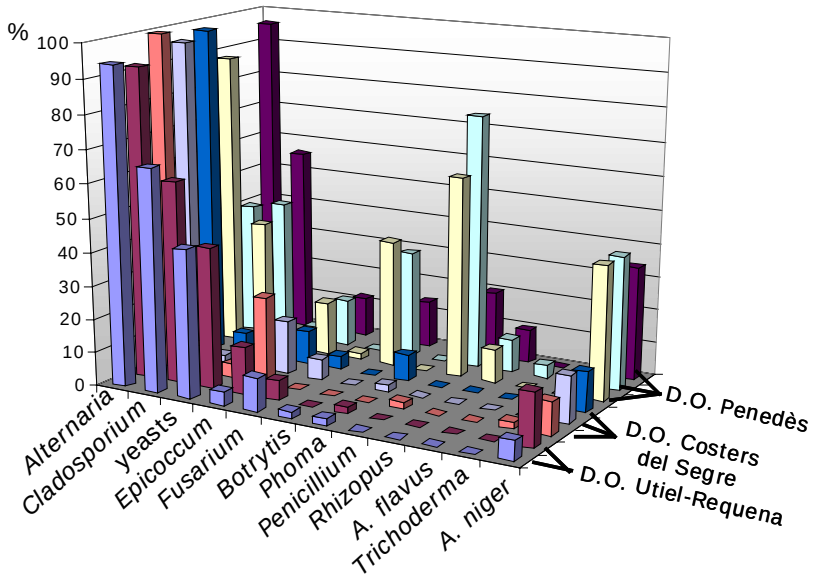


**Figure 48. Percentage of the main fungi isolated from grapes in each of the vineyards sampled in 2004.**

A total of 33 *Aspergillus* section *Nigri* were isolated and classified, distributed as table 33 shows. Twenty-four *A. niger* aggregate, 10 *A. carbonarius* and no uniseriates were detected. All *A. carbonarius* were OTA producers, whereas no OTA was detected from any of the *A. niger* aggregate isolates. The amounts of OTA detected, ranged from 0.02 to 10.20 µg $g^{-1}$ CYA. However, more than 80 % of the isolates produced less than 1 µg $g^{-1}$.

**Table 33. Number of *A. niger* aggregate and *A. carbonarius* and ochratoxigenic strains isolated in each of the vineyards sampled in 2004.**

| Origin | Vineyard | *A. niger* aggregate | *A. carbonarius* | Total | OTA + |
|---|---|---|---|---|---|
| Utiel-Requena D. O. | V1 | 2 | 0 | 2 | 0 |
| Utiel-Requena D. O. | V2 | 6 | 0 | 6 | 0 |
| Penedés D.O. | P1 | 4 | 3 | 7 | 3 |
| Penedés D.O. | P2 | 4 | 0 | 4 | 0 |
| Penedés D.O. | P3 | 2 | 1 | 3 | 1 |
| Costers del Segre D.O. | R1 | 3 | 3 | 5 | 3 |
| Costers del Segre D.O. | R2 | 2 | 2 | 4 | 2 |
| Costers del Segre D.O. | R3 | 1 | 1 | 2 | 1 |
| TOTAL: | - | 24 | 10 | 33 | 10 |

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