CHAPTER 8

ECOPHISIOLOGY OF OCHRATOXIGENIC MOULDS

8.1. FACTORS INFLUENCING GROWTH AND MYCOTOXIN PRODUCTION

Fungi have in the course of evolution diversified to exploit a wide variety of habitats. Different species hence require different conditions for optimal growth. Microbial metabolism is significantly influenced by the physical and chemical environment. Thus, toxin-producing fungi may invade food at pre-harvesting period, harvest-time, during post-harvest handling and in storage. According to the site where fungi infest food, toxinogenic fungi can be divided into three groups (Suttajit, 1989):

- **Field fungi**, includes species of plant pathogenic fungi, usually with high requirements of water, such as *Fusarium*, *Alternaria* and *Cladosporium*.

- **Storage fungi**, with lower requirements of humidity, are principally the genus *Aspergillus* and *Penicillium*.

- **Advanced deterioration fungi**, normally do not infest intact food, but easily attack damaged one and require high moisture content. Some examples are some other aspergilli species, *Chaetomium*, *Scopulariopsis*, *Rhizopus*, *Mucor* and *Absidia*.

Mycotoxigenic fungi grow better under certain environmental conditions. However, the presence of these fungi does not mean the mycotoxin synthesis, as production of secondary metabolites is not essential to the synthesizing organism. In fact, the conditions in which these moulds produce their mycotoxins are very specific and independent of those required for fungal growth. Conversely, the visible absence of mould does not mean that no mycotoxins are present since these may remain in the product long after the producing fungus has disappeared. It is not possible to entirely prevent the formation of mycotoxins, but eliminating the conditions necessary for fungal growth helps prevent formation of the toxin.

The main factors that influence growth and production of *Aspergillus* toxins include temperature, pH and moisture. But apart from environmental factors, chemical and biological factors clearly play a role (Figure 49). Under some circumstances these effects are additive. Under others, the implication is that synergistic interactions lead to a combined effect of greater magnitude than the sum of constraints applied individually. This has been described by Leistner and Rödel (1976) as the '**hurdle concept'**. Moreover, hurdles are frequently combined to minimise the impact of processing on the quality and to improve the safety of ready-to-eat foods.

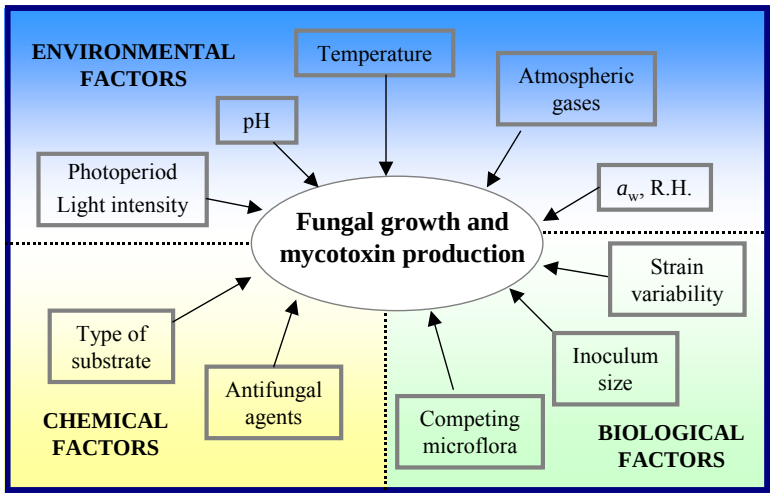

Figure 49. Principal factors influencing fungal growth and mycotoxin production (modified from EC, 1994).

8.1.1. Environmental factors:

The large and diverse group of microscopic foodborne yeasts and moulds includes several hundred species. The ability of these organisms to attack many foods is due in large part to their relatively versatile, environmental requirements. There are several major parameters governing fungal invasion, growth and production of mycotoxins:

8.1.1.1. Temperature

Temperature is an important environmental factor affecting growth and mycotoxin production by moulds. Fungi are capable of surviving under the full range of temperatures normally experienced in environments in which they live. The temperature range usually reported for fungal growth is broad (10-35°C), with a few species capable of growth below or above this range.

Fungi can be divided according to their tolerance to temperature in psychrotolerant, psychrophilic, mesophilic, thermotolerant and thermophilic fungi.

In general, the optimum temperature for mycotoxin production is below the optimum for growth. The production of different mycotoxins by the same specie is also related to the temperature level.

Consequently, temperature can be considered one environmental way to control or prevent mycotoxin production in foods, mainly in storage steps, as sometimes temperature is not easy to control, particularly in agricultural commodities.

8.1.1.2. Water availability

Living organisms consist largely of water. Hence if an organism is to grow has to take up water from the environment. Whether water enters or leaves a cell depends on the difference between the water potential of the cell and that of the surrounding medium, water moving from a region of high to one of lower water potential (Carlile and Watkinson, 1996). In microbiology, three measures of the water availability have been used: water potential (ψ), water activity (a_w), and relative humidity (R.H.).

Water potential is the sum of numerous components, of which the most important are osmotic, matric and turgor potential, and is measured in units of pressure.

Water activity is a fundamental property of aqueous solutions, and by definition is the ratio of the vapour pressure of the water in the substrate (p) to that of pure water at the same temperature (p_o):

$$a_w = \frac{p}{p_0}$$

Water activity ranges from zero (water absent) to 1.0 (pure water). For an ideal solution a_w is independent of temperature, and in actual practice, the a_w of a given solution varies only slightly with temperature within the range of temperature permitting microbial growth.

The relationship between water potential and water activity is given by the next equation, where the value of k depends on temperature and is, for example, 1.37 at 25 ºC and 1.35 at 20 ºC:

$$\psi \text{ (Mpa)} = k \ln a_w$$

Not only is the availability of water in the surrounding liquid phase of importance to fungi, but the water content of the adjacent gas phase. The water content of the atmosphere is expressed in terms of **relative humidity**, the ratio of the water vapour pressure of the gas phase being considered, to that of a saturated atmosphere at the same temperature. It is hence the same ratio as water activity but expressed as a percentage.

In most of the studies presented in this thesis, a_w was used to describe the status of the water in solution or substrate in preference to R.H., which applies more strictly to the surrounding atmosphere. Under equilibrium conditions the two terms are interchangeable.

A required a_w in the environment of a fungus may be obtained either by fixing the water content or the solute concentration in the culture substrate or by keeping the substrate in equilibrium with an atmosphere of controlled R.H.

Moisture requirements of foodborne moulds are relatively low; most species grow at a 0.85 a_w or less, although yeasts generally require a higher water activity. 0.60 a_w is considered the limit for cell growth, but spores of *Aspergillus* and *Penicillium* for example, are able to survive at lower a_w for several years (Carlile and Watkinson, 1996).

Moisture control is the best and most economical means to control the environment to prevent mould growth and mycotoxin production.

8.1.1.3. Hydrogen ion concentration (pH)

In general, there is a lack of information on the effect of pH on fungal growth parameters, in spite of a considerable literature on growth in relation to the initial pH of media. This data is of limited value, since fungal metabolism alters pH during their evolution.

Hydrogen ion concentration in a medium could affect growth either indirectly by its effect on the availability of nutrients or directly by action on the cell surfaces. The acid/alkaline requirement for growth of all yeasts and moulds is quite broad, ranging from pH 3 to above pH 8, with optimum around pH 5, if nutrient requirements are satisfied. In general, *Aspergillus* species are more tolerant to alkaline pH while *Penicillium* species appear to be more tolerant to acidic pH (Wheeler et al., 1991).

It is seen that in situations near neutral pH, fungi must compete with bacteria for niches, and at higher a_w values most fungi are not competitive in mixed culture. However, where a_w is below 0.90, fungi become dominant irrespective of pH. In specialised niches where bacteria do not appear to have a role as pathogens, specific *Fusarium* and *Penicillium* species are dominant even at neutral pH and high a_w (Wheeler et al., 1991).

8.1.1.4. Light

There are some reports that illumination will increase or more commonly reduce the rate at which fungi spread across an agar surface. Such effects are sometimes due to the photochemical destruction of components of the medium but in other instances a direct

effect on metabolism seems likely. The biosynthesis of pigments, mainly carotenoids, as consequence of light action has been demonstrated.

8.1.1.5. Availability of oxygen

Organisms can obtain energy by oxidative (respiratory) metabolism or by fermentation. The implications for oxygen requirements of the occurrence of respiration, fermentation or both in a fungus divided them in obligate aerobes, facultative anaerobes and obligate anaerobes (Carlile and Watkinson, 1996):

Food spoilage moulds, like almost all other filamentous fungi and yeasts, have an absolute requirement for oxygen. However, many species appear to be efficient oxygen scavengers, so that the total amount of oxygen available, rather than the oxygen tension, determines growth. The concentration of oxygen dissolved in the substrate has a much greater influence on fungal growth than atmospheric oxygen tension (Pitt and Hockings, 1997). The most oxygen demanding moulds will colonise the surface of the food, while the less exigents could be found inside the food.

Although probably not economically feasible, one sure way to prevent mycotoxin contamination of cereals and other food, is to store them under anaerobic conditions, e.g. CO_2 or nitrogen. For instance, this could be done in large airtight silos. The moulds would not grow, but this type of environment control is sometimes understandably unrealistic.

8.1.2. Biological factors

8.1.2.1. Competing mycoflora

The simultaneous presence of different microorganisms, as bacteria or other fungi, could disturb fungal growth and the production of mycotoxins. Therefore, several microorganisms have been reported as biological pest control agents. It is seen that *Trichoderma harzianum* produces a lytic enzyme, chitinase, which has an antifungal activity against a wide range of fungal strains, among them, *A. niger*. (Nampoothiri et al., 2004, Valero et al., 2005). However, black aspergilli have been reported as dominant genera among most of the common mycoflora in grapes, such as *Alternaria*, *Cladosporium*, *Eurotium*, *Candida* and some *Penicillium* (Valero et al., 2005). A study of Bae et al. (2004) showed a clear inhibition of the growth of *A. carbonarius* and other fungi by *Bacillus thuringiensis*. This is a biological insecticide that has been used in agricultural practices for over 50 years. The commercially available product consists of a mixture of endospores and parasporal proteins which are toxic to insects, in the case of vines, to apple moth for example. The study of Bae et al. (2004) suggests that apart of the

current use of *B. thuringiensis* in viticulture to control insects, it could be of great interest to control spoilage and mycotoxigenic fungi. Although *B. thuringiensis* is a prevalent organism on wine grapes, it is not able to grow in grape juice or wine, and therefore, does not adversely impact on wine quality or safety.

8.1.2.2. Strain variability

As already emphasized, the production of mycotoxins is influenced by both the genotype of the organism and the physicochemical environment in which it is growing. The production of any particular mycotoxin depends on the strain and not only on the species. However, OTA biosynthesis of ochratoxigenic *Aspergillus* spp. is determined more by environmental conditions than by the inherent ability of the organism to produce OTA.

If an isolate does not produce OTA under given conditions, this does not justify any conclusion about its general ability to produce OTA. Also, any grouping into "OTA producers" and "non-producers" based on such data will be misleading. In contrast, OTA production in the genus *Penicillium* appears to be stable and evenly distributed (Mühlencoert et al., 2004).

8.1.2.3. Inoculum

The source of inoculum for most of the fungi affecting vegetal products is likely the soil. Many studies have been done dealing with cereals, finding extensive colonisation of crop residues and soils, in productions highly contaminated with mycotoxins (McGee et al, 1996). They found significantly higher populations in summer months of the year, and mycotoxigenic fungi were recovered more frequently from crop residues in continuous culture than in culture rotation plots. It is known that the survival structure of most fungi in the soil may be in debris or in soil, as mycelium or as conidia, and depending on the fungi, as sclerotia.

8.1.2.4. Insects and other vectors

Physical damage of food due to insects and pests is a disturbing problem mainly in tropical regions, particularly as food contaminants in the field, more than in the storage. But insects and other arthropods could function as vectors. Many different insects have the capability of promoting infection of various crops with mycotoxigenic fungi. A successful vector must naturally encounter the fungal agent and carry it to the crop/site in a viable condition. This transport may be directly to the plant or in close enough proximity for the fungus to arrive at the plant site by other means, such as other insects, water or air (Dowd, 1998).

Both insects and mites are one of the vectors able to introduce fungal spores inside the foods by the lesions that they produce in them. Insects may contribute to the infection of agricultural commodities in different ways: they may transport primary inoculum, move inoculum throughout the commodity, disseminate spores within the food and/or facilitate colonization and infection by injuring the foodstuff (Payne, 1998). The timing of insect injury also contributes to the levels of mycotoxin found. Consequently, an infestation of insects predisposes the food to the attack of different fungi and the resultant production of mycotoxins (Farrar and Davis, 1991).

Water and wind are other vectors that favour the dissemination of fungal spores among agricultural food commodities. Airborne conidia of a wide range of fungi are present each year in the fields, although differing greatly across years. Airborne conidia levels were higher in irrigated fields than in non-irrigated ones, in a study of aflatoxin contamination carried out by Jones et al. (1981).

8.1.3. Chemical factors

8.1.3.1. Nutritional factors

All forms of life, including moulds, require exogenous materials to build into biomass. As heterotrophs, the moulds require organic compounds for both the synthesis of biomass (anabolic metabolism) and to produce the energy to drive these reactions (catabolic metabolism). These aspects of metabolism are frequently referred to as primary metabolism (Smith and Moss, 1985).

Fungi can use a number of different **carbon sources** to fill their carbon needs for the synthesis of carbohydrates, lipids, nucleic acids and proteins. Oxidation of sugars, alcohols, proteins, lipids, and polysaccharides provides them with a source of energy. Differences in their ability to utilise different carbon sources, such as simple sugars, sugar acids, and sugar alcohols, are used, along with morphology, to differentiate the various yeasts. Fungi require a source of **nitrogen** for synthesis of amino acids for proteins, purines and pyrimidines for nucleic acids, glucosamine for chitin, and various vitamins. Depending on the fungus, nitrogen may be obtained in the form of nitrate, nitrite, ammonium or organic nitrogen as no fungus can fix nitrogen. Most fungi use nitrate, which is reduced first to nitrite and then to ammonia. Therefore, availability and type of nutritional factors such as carbon source and nitrogen source can also affect both mycotoxin production and morphological differentiation.

Other major nutrients for fungi are sulphur, phosphorus, magnesium and potassium, which can be supplied to most fungi as salts. Trace elements like iron, copper, manganese, zinc and molybdenum are required by nearly all fungi as cofactors for enzymes. But in high amounts, some trace elements can become toxic for some fungi.

OTA production by *A. ochraceus* strains varied with the different concentrations of yeast extract (0-4%) and sucrose (0-4%) in a laboratory medium (Atalla and El-Din, 1993). Engel (1976) also tested different carbohydrate sources as lactose, maltose, fructose, glucose, saccharose, etc., and found the highest yields of OTA by *A. ochraceus* in maltose-containing medium. Bee pollen added to the medium has been demonstrated that stimulates OTA production by *A. ochraceus* (Medina et al., 2004).

Moreover, consistency of food exerts considerable influence over the kind of spoilage to which a food is susceptible. Filamentous fungi are more obvious spoilage in solid substrates where they have ready access to oxygen.

8.1.3.2. Antifungal agents

A wide range of antifungal agents are used in combating biodeterioration and in preventing or treating fungal diseases of plants. In these contexts, they are commonly referred to as **fungicides**. Others are used for treating disease in animals and man, and are simply referred to as **antifungal agents.** Antimicrobial agents produced by means of a microbial fermentation, called **antibiotics**, by the plant on which the mould is growing, or added as biocides during crop management, are other factors interacting with the growth and metabolism of a mould. Antifungal agents differ widely in their chemical nature and in their properties and mode of action (Carlile and Watkinson, 1996).

The effect of pesticides is interesting as they are largely used to control several diseases in plants. The correct use of fungicides to diminish fungal mycoflora could lead to a diminution in the amount of mycotoxins produced. But certain number of studies showed that the use of sub-lethal concentration could favour the production of the toxins (Moss and Frank, 1987). It is also possible that the pesticide decreases the synthesis of the mycotoxins without affecting the fungal growth (Draughton and Ayres, 1978, 1982).

8.2. METHODS FOR MEASURING FUNGAL GROWTH

Growth may be defined as the orderly increase in cell components leading to an increase in biomass (Prosser, 1995). The growth form of filamentous fungi is complex; extension of individual hyphae is localized at the tip, whereas biomass synthesis supporting that growth may take place throughout the mycelium. The growth of a fungus can be measured in various ways such as increase in colony diameter, increase in dry weight, rate of production of different type of metabolites, etc.

The mechanisms involved in the control and regulation of mycelial growth are better studied on solid medium than in submerged cultures, as fungi are adapted to growth on solid substrates.

8.2.1. Direct methods

Some of the direct methods to determine fungal growth are stated afterwards:

8.2.1.1. Growth of hyphal extension

Measure of hyphal extension rate is measured microscopically on solid medium as an increase in length, e.g. measure of the increase in radii of circular colonies, daily or every other fixed period of time, after inoculating the mould in an agar plate and incubate it at the appropriate conditions. This is probably the most common technique for estimation of growth of filamentous fungi on solid media. A growth rate function can be derived by plotting colony diameter against time and measuring the slope of the straight part of the line. Analysis is now greatly facilitated by the increased availability of image analysis systems which enable automated measurement of hyphal lengths and subsequent kinetic analysis of data (Wiebe and Trinci, 1990; Gray and Morris, 1992).

8.2.1.2. Measure of fungal biomass

Moulds are usually grown on the surface of a cellophane membrane, overlaying the agar, from which the biomass can be washer or otherwise removed for the determination of the dry weight. This measure is also possible in liquid cultures. In both solid and liquid media, separation of biomass from the growth medium is slow, tedious and requires relatively large amounts of biomass for accuracy. Turbidimetric techniques are less reliable due to the heterogeneous nature of liquid cultures of filamentous fungi.

8.2.2. Indirect methods

8.2.2.1. Ergosterol

Fungal plasma membranes are similar to mammalian plasma membranes, differing in having the nonpolar sterol ergosterol, rather than cholesterol, as the principal sterol. The plasma membrane regulates the passage of materials into and out of the cell by being selectively permeable. Membrane sterols provide structure, modulation of membrane fluidity, and possibly control of some physiologic events. Fungal growth and biomass could therefore be estimated by measuring this specific component of fungi. Quantifying ergosterol production in foods has proved more difficult. Since now, ergosterol content has been mainly assayed in cereal samples. The determination of ergosterol is also valuable in correlating metabolites such as aflatoxins and OTA (Gourama and Bullerman, 1995; Saxena et al. 2001).

8.2.2.2. Impedimetry and conductimetry

Metabolites produced by growth of microorganisms in liquid media alter the medium's impedance and conductance. The use of changes in these properties has been used to estimate fungal growth. A major problem of these techniques involves the selection of suitable media, but when the method is set up, this method results rapid and effective.

8.2.2.3. Adenosine triphosphate (ATP)

Another measure of microbial biomass is the measure of the bioluminescence emitted by the molecules of fungal ATP. However, living plant cells contain also high levels of ATP and fungi are often very difficult to separate from food materials.

8.2.2.4. Pectinesterase

The fundament of this technique is that gas liquid chromatography is used to determine the amount of methanol released from pectin by the fungal enzyme pectinesterase. This is considered a rapid method for detecting viable spores of spoilage fungi, but it needs some improvements before practical application.

8.2.2.5. Fungal volatiles

It consists in measuring the effects of fungi on foods, rather than fungi *per se*. Fungi produce chemical volatiles during growth and particular chemicals can be detected and therefore measure fungal growth in an indirect way.

Several commercial gas sensor array instruments are now available on the market covering a variety of chemical sensor principles, system design and data analysis techniques. A series of different detection principles can be used in chemical gas sensors: heat generation, conductivity, electrical polarization, electrochemical activity, optical properties, dielectric properties and magnetic properties. In principle, the results obtained from a gas-sensor array represent qualitative and quantitative information of the composition of the headspace gas mixture of a sample. The technique should therefore have a great potential in a number of applications related to food. Numerous **electronic nose** studies related to food already have been published, but the electronic nose technology applied on food must be regarded as being in its early stage. A goal of this technology is to explore the use of an electronic nose for rapid detection of food spoilers and pathogens via development of a standard curve of some potential volatile compounds that can be used to develop some specific aroma-labeled substrates.

8.2.2.6. Immunological techniques

Fungal cell wall proteins produce antigens, which can be detected by immunological methods. Some antigens are derived from components common to a wide range of fungi, and hence are indicative of general fungal growth, while others are genus or even species specific.

8.2.2.7. Molecular methods

They are based on nucleic acid sequences that are specific to the target fungi. The most known method is called **nucleic acid hybridisation** and it involves the selection, cloning and chemical labelling of sequences specific to the target organism. These are then used as probes to detect RNA or DNA of the pathogen in extracts of the substrate. DNA may be specific at almost any taxonomic level.

In some instances the detection and identification of the causal agent(s) may be secondary to other consideration. For example, it may be more important to quantify the amount of pathogen present rather than just determine its identity. Several approaches have been taken to develop diagnostic assays, and are divided into immunological and DNA-based systems, this last generally being **polymerase chain reaction** (PCR). In contrast to hybridisation, PCR-based assays for detecting mycotoxins in fungi have been widespread in the last years. PCR is an extremely sensitive technique and involves the enzymatic amplification of a target DNA sequence by a thermostable DNA polymerase.

8.3. METHODS FOR THE ANALYSIS OF MYCOTOXINS

The different stages involved in the analytical process of mycotoxins detection are sampling, extraction, clean-up, separation, detection and confirmation, and their have been detailed previously for OTA analysis in wine (see 6.3.4.1.). Moreover, the particular methodology used for OTA analysis from fungal cultures has been explained in section 7.5.5. However, a short description of the general steps involved in the analysis of mycotoxins in general is provided afterwards:

Mycotoxins are rarely uniformly distributed throughout natural products. They are generally found in high concentrations at the sites where the toxigenic fungi have invaded the product. When analysing large quantities or lots of agricultural products, care must be exercised to achieve a situation where the final samples for analysis can be representative of the whole lot.

Extraction can be the most problematic and the one in which losses or failure to extract the most difficult to quantify, as mycotoxins normally occur in extremely low

concentrations. It is generally accepted that spiking of the matrix with the analyte of interest is a required process for method validation (Shephard, 2001). Although many interfering compounds may be partially removed during the extraction sequence, further clean-up of the extract is normally necessary.

The traditional clean-up systems generally involved either solvent portioning and/or open column chromatography on silica adsorbent. The development of solid phase extraction (SPE) cartridges containing packing with various surface chemistries allowed more rapid and efficient clean-up process. However, the introduction of the immunoaffinity columns (IAC) in which specific antibodies are bound to a solid matrix, has allowed an even more specific clean-up process (Scott and Trucksess, 1997).

Classical analytical separation methods for mycotoxins include TLC, HPLC, gas chromatography (GC) and MS. Mass spectrometry offers the ideal confirmatory technique via the detection of molecular ions at specific chromatographic retention times and via the generation of a compound specific fragmentation pattern. In recent years, most of these techniques have been coupled with immunoaffinity techniques to simplify extraction and improve mycotoxin recovery and measurement from foodstuffs (Patel et al., 2004). They are based on specific monoclonal and polyclonal antibodies produced against the toxin. Commercial immunological techniques are divided into IAC and ELISA. There are commercially available ELISA kits, as well as IAC, for many mycotoxins such as aflatoxins (general or specifics), OTA, deoxynivalenol, fumonisins, T-2 toxin, zearalenone, etc.

A number of technologies in separation science are emerging. Biosensors, capillary electrophoresis with fluorescence detection, molecular imprint polymers, automation and microarrays are emerging technologies for mycotoxin analysis that are not yet widely available commercially, but will likely in the future play an increasing role in mycotoxin analysis.

8.4. ECOPHYSIOLOGICAL PARAMETERS INFLUENCING OCHRATOXIGENIC FUNGI

8.4.1. Effects on *A. ochraceus* and *P. verrucosum*

Preventing the presence of mycotoxins may concern the detection of contaminated stocks early in order to redirect these harvests to uses others than foodstuffs. However, the loss in value of the contaminated harvest inevitably results in a loss of income for the farmer. Strategies for the prevention of the infections are thus clearly useful, both to public health and the economy of farming operations and therefore to producing countries. To facilitate

the prevention approach, a good understanding of the ecological factors which favour infection and the production of mycotoxins is an essential condition.

Current scientific literature on the influence of environmental factors on OTA production has been focused mainly on *P. verrucosum* (Table 34) and *A. ochraceus* (Table 35). Ecophysiological experiments on *A. ochraceus* are reported from the early seventies (Schindler and Nesheim, 1970; Sansing et al. 1973). Studies on *P. verrucosum* dated from the same time, but with the name of *P. viridicatum* (Northolt et al. 1979). Environmental conditions affecting ochratoxigenic groups of fungi have been recently compiled (Sanchis, 2004). *A. niger* was extensively used in the half of the 20th century in investigations on fungal nutrition and physiology. Steinberg (1956) studied its growth. Bonner (1948) studied the effect of temperature and humidity on this fungus. Later, Rai et al. (1967) used *A. niger* in experiments on the effects of light, temperature, pH, oxygen and R.H, but in all these studies black aspergilli species were considered as *A. niger*. Much work on black aspergilli was needed since 1996, when Zimmerli and Dick highlighted the importance of this species in grapes and wine.

Data on the ecophysiological characteristics strongly depends on the substrate where the fungi are growing. Substrates used in the studies showed in the next two tables were diverse, such as barley grain (Ramakrishna et al., 1993; Pardo et al., 2004; Ramos et al., 2005), barley extract agar (Pardo et al., 2005d), bread analogue (Arroyo et al., 2005), grape-juice analogue (Pardo et al., 2005a), green coffee (Suárez-Quiroz et al., 2004; Pardo et al., 2005b, 2005c), melon seed (Pitt and Christian, 1968), etc.

8.4.2. Effects on black aspergilli

The effect of several ecophysiological factors was studied on both black aspergilli growth and OTA production. The study intervals for each factor were fixed in line with the conditions encountered during grape cultivar. A summary of the ecophysiological parameters studied in each experiment is shown in Table 36.

Table 34. Main ecophysiological characteristics of *P. verrucosum*.

	Growth	OTA	Reference
Range T (ºC)	n.d. n.d. 0-31 10-30	7-31 4-? n.d. n.d.	Northolt et al. (1979) ICMSF (1996) Pitt and Hocking (1997) Pardo et al. (2005d)
Optimum T (ºC)	n.d. 20 20 20	24 n.d. 20 n.d.	Northolt et al. (1979) Ramakrishna et al. (1993) Pitt and Hocking (1997); ICMSF (1996) Pardo et al. (2005d)
Range a_w	0.79-? 0.80-? 0.85-0.99	n.d. 0.86-? n.d.	Northolt et al. (1979) Pitt and Hocking (1997); ICMSF (1996) Pardo et al. (2005d)
optimum a_w	n.d. 0.90-0.95 0.97 0.95-0.99	0.95-0.99 <0.97 0.93-0.95 n.d.	Northolt et al. (1979) Ramakrishna et al. (1993) Arroyo et al. (2005) Pardo et al. (2005d)
Range pH	4.5-6.0	4.5-6.0	Arroyo et al. (2005)
Optimum pH	6.0	6.0	Arroyo et al. (2005)
Range time (days)	-	?-35	Arroyo et al. (2005)
Optimum time (days)	-	28-35	Arroyo et al. (2005)
Germination time (hours)	12-24 –favourable conditions 18 h		Northolt et al. (1979) Ramakrishna et al. (1993)

n.d. not determined

Table 35. Main ecophysiological characteristics of *A. ochraceus*.

	Growth	OTA	Reference
Range T (ºC)	10-45 n.d. 8-37 10-37 10-40 10-30 10-30	15-30 12-37 12-37 12-37 10- n.d. n.d. 20-30	Sansing et al. (1973) Northolt et al. (1979) ICMSF (1996); Pitt and Hocking (1997) Ramos et al. (1998) Suárez-Quiroz et al. (2004) Pardo et al. (2005a) Pardo et al. (2005b; 2005c)
Optimum T (ºC)	20-25 n.d. 24-31 25-30 25-30 30 20-30	25-30 31-37 31 25-30 35 n.d. 20-30	Sansing et al. (1973) Northolt et al. (1979) ICMSF (1996); Pitt and Hocking (1997) Ramos et al. (1998) Suárez-Quiroz et al. (2004) Pardo et al. (2005a) Pardo et al. (2004; 2005b; 2005c)
Range a_w	0.77-? 0.79-? n.d. 0.79-? 0.80-0.99 0.94-0.99 0.85-0.99	0.85-? 0.83-? 0.80-? n.d. 0.90-0.99 n.d. 0.85-0.99	Pitt and Christian (1968) Northolt et al. (1979) Adebajo et al. (1994) Pitt and Hocking (1997) Suárez-Quiroz et al. (2004) Pardo et al. (2005a) Pardo et al. (2004; 2005b; 2005c)
optimum a_w	0.95-0.99 n.d. 0.96-0.98 0.95 0.99 0.95-0.99	n.d. 0.99 0.98 0.95 n.d. 0.99	Pitt and Christian (1968) Northolt et al. (1979) Ramos et al. (1998) Suárez-Quiroz et al. (2004) Pardo et al. (2004; 2005a) Pardo et al. (2005b; 2005c)
Range pH	3-10	n.d.	Wheeler et al. (1991)
Optimum pH	3.5-4	n.d.	Wheeler et al. (1991)
Range time (days)	-	4-14	Sansing et al. (1973)
Optimum time (days)	6-10 (20-25ºC) n.d.	10-12 (25ºC) 21 (25-30ºC)	Sansing et al. (1973) Ramos et al. (1998)
Germination time (days)	0.5-2 –favourable conditions		Northolt et al. (1979)

n.d. not determined

Table 36. Summary of the ecophysiological studies of black aspergilli presented in this thesis.

A. carb (origin)	***A. niger* aggr. (origin)**	**Uni (origin)**	**Medium**	**Solute**	**a_w**	**T (ºC)**	**photo-period**	**fungi-cides**	**Growth (up to x days)**	**OTA (extraction time)**	**Sectior**
2 (S)	2 (S)	2 (S)	SNM	Glycerol Glucose	0.90, 0.93, 0.95, 0.98, 0.995	25	-	-	✓ (60 d)	-	8.4.2.1. A
6 (S, F, I, P)	8 (S, F, I, P)	6 (S, I, P)	SNM	Glycerol	0.90, 0.93, 0.95, 0.98, 0.995	25	-	-	✓ (60 d)	-	8.4.2.1 A
4 (S, F, I, P)	3 (S, I, P)	3 (S, I, P)	SNM	Glycerol	0.90, 0.93, 0.95, 0.98, 0.995	10, 15, 20, 30, 37	-	-	✓ (60 d)	-	8.4.2.1. A
8 (S, F, I, P)	-	-	SNM	Glycerol	0.90, 0.93, 0.95, 0.99	15, 20, 30, 35, 37	-	-	✓ (30 d)	✓ (7 d)	8.4.2.1. B
4 (S, F, I, P)	-	-	grapes damaged/ undamaged	Glycerol	80, 90, 100 % R.H.	20, 30	-	-	✓ % of infection	✓ (7 d)	8.4.2.1. C
2 (S, I)	2 (S, P)	-	SNM	Glycerol	0.90, 0.93, 0.95, 0.98, 0.995	25	-	-	-	✓ (5, 10, 15, 20 d)	8.4.2.2 D
4 (S, T)	-	-	SNM	Glycerol	0.96	7, 15, 20, 25, 30, 35, 42	-	-	✓ (10 d)	✓ (2, 4, 6, 8, 10 d)	8.4.2.2. E
3 (S, I, F)	-	-	SNM	Glycerol	0.99	20, 28	✓ light/	-	✓ (3, 5, 10 d)	✓ (3, 5, 10 d)	8.4.2.3 F

							dark				
3 (I, F, S)	-	-	SNM	Glycerol	0.99	20, 30	-	✓ n=26	✓ (3, 5, 10 d)	✓ (7 d)	8.4.2.4. G
2 (F, S)	-	-	SNM	Glycerol	0.99	20, 30	-	✓ n=13	MIC (30 d)	-	8.4.2.4. G
2 (F, S)	-	-	grapes	Glycerol	100 % R.H.	20, 30	-	✓ n=6	✓ % of infection (7 d)	✓ (7 d)	8.4.2.4. G
field	field	field	grapes	-	field	field	field	✓ n=2	✓	✓	8.4.2.4 H

S, Spain; I, Italy; P, Portugal; F, France; T, Tunisia;

A: Bellí et al. (2004). Influence of water activity and temperature on growth of isolates of *Aspergillus* section *Nigri* obtained from grapes. *Int. J. Food Microbiol.* 96, 19-27.

B: Bellí et al. (2005). *Aspergillus carbonarius* growth and ochratoxin A production on a synthetic grape medium in relation to environmental factors. *J. Appl. Microbiol.* 98, 839-844.

C: Bellí et al. (2005). Skin damage, high temperature and relative humidity as detrimental factors for *Aspergillus carbonarius* infection and ochratoxin A production in grapes. *Food Control* (submitted).

D: Bellí et al. (2004). Incubation time and water activity effects on ochratoxin A production by *Aspergillus* section *Nigri* strains isolated from grapes. *Lett. Appl. Microbiol.* 38, 72-77.

E: Marín et al. (2005). Kinetics of ochratoxin A production and accumulation by *Aspergillus carbonarius* on synthetic grape medium at different temperature levels. *J. Food Sci.* (submitted).

F: Bellí et al. (2005). Effect of photoperiod and day-night temperatures simulating field conditions on growth and ochratoxin A production of *Aspergillus carbonarius* strains isolated from grapes. *Food Microbiol.* (in press).

G: Bellí et al. (2005). Effect of chemical treatments on ochratoxigenic fungi and common mycobiota of grapes (*Vitis vinifera*). *J. Food Prot.* (submitted).

H: Bellí et al. (2005). Impact of fungicides on *Aspergillus carbonarius* growth and ochratoxin A production on synthetic grape-like medium and on grapes. *Food Add. Cont.* (submitted).

8.4.2.1. Effect of water activity and temperature

The purpose of the following experiments was to study the effect of environmental factors such as a_w, temperature, and their interaction on the growth rate and OTA production of black aspergilli on SNM and natural grapes.

- **Influence of water activity and temperature on growth of isolates of *Aspergillus* section *Nigri* obtained from grapes**

The effects of a_w and temperature on growth of *Aspergillus* section *Nigri* isolated from wine grapes were investigated on SNM medium. Temperatures in the range of 10-37 ºC were tested. Optimum temperatures for growth were between 30 and 37 ºC. Water activity levels ranging from 0.90 to 0.995 were tested. Optimum a_w for growth was 0.98 in most cases. Statistical differences were found among the groups tested (*A. carbonarius*, *A. niger* aggregate and *A.* section *Nigri* uniseriates). Growth rates models for the factors assayed have been obtained, and the results are shown in the following paper.

Influence of water activity and temperature on growth of isolates of *Aspergillus* section *Nigri* obtained from grapes. *International Journal of Food Microbiology* 96, 19-27 (2004).

- ***Aspergillus carbonarius* growth and OTA production on a synthetic grape medium in relation to environmental factors**

The effects of a_w (0.90-0.99 a_w), temperature (15-37 ºC) and their interaction on growth and OTA production by eight isolates of *A. carbona*rius were investigated on SNM medium. Growth data were modelled by an MLR regression and response surface models were obtained. *A. carbonarius* grew much faster at 30 ºC than at the other temperature levels tested and its growth rate increased with increasing a_w, maximum growth rate being between 0.95 and 0.99 a_w. In general, isolates grew faster at 35-37 ºC than at 20 ºC, although no significant differences were found between these temperatures. OTA accumulation was also favoured by high a_w levels, and although it was observed in the whole range of temperatures, maximum amounts were detected at 20 ºC. No OTA was found at the most unfavourable growth conditions. Optimum a_w level for growth seems to correspond with optimum for OTA production, meanwhile the most propitious temperature for the toxin production was below the best one for growth. Prediction of *A. carbonarius* growth would allow estimating their presence and therefore, the OTA production, as it was found that conditions for the toxin production were more limited than those permitting growth. The study is shown in the following paper:

Aspergillus carbonarius growth and OTA production on a synthetic grape medium in relation to environmental factors. *Journal of Applied Microbiology* 98, 839-844 (2005).

- **Water relations of germination, growth and OTA production by European isolates of *Aspergillus carbonarius* from wine and table grapes.**

This study has reviewed the effect of temperature and water availability on mycelial growth and OTA production by *A. carbonarius* isolates from several countries, and has added the effect of the pH. It also examined the effect of these parameters on spores germination and germ tube extension. Germination was found very rapid (< 24 h) at 0.90-0.99 a_w and 25-35 ^oC. This was also reflected in measurements of germ tube extention. Growth of two isolates from each country showed some differences in relation to steady state a_w levels. Only a few isolates could grow at 0.88 a_w, and none at 0.85 a_w. Surface response curves were developed to show the optimum and marginal conditions for growth of representative isolates of *A. carbonarius*. Growth was influenced by pH x a_w conditions. Growth was better at pH 4.5 and 7 than pH 2.8. In contrast to growth, OTA production was found to be optimum at 15-20 ºC over the range 0.99-0.95 a_w.

Water relations of germination, growth and OTA production by European isolates of *Aspergillus carbonarius* from wine and table grapes. *International Journal of Food Microbiology* (submitted).

- **Skin damage, high temperature and relative humidity as detrimental factors for *Aspergillus carbonarius* infection and OTA production in grapes**

This work describes the impact of skin damage on visible *A. carbonarius* colonization and OTA production in grapes at different temperatures and R.H. Four ochratoxigenic *A. carbonarius* strains isolated from wine grapes from four different European countries were used. Artificially damaged and undamaged table grapes were surface-disinfected and inoculated. Grapes were stored at three levels of R.H. (80, 90 and 100 %) and at two levels of temperature (20 and 30 ºC). After seven days, the infection percentage of *A. carbonarius* was recorded and OTA accumulation in berries was analysed. Damaged grapes were more commonly infected and colonies were more developed than in undamaged ones (Figure 50); consequently more OTA was detected in the first ones. Temperature and R.H. had significant influence on both infection and toxin content. The amount of OTA detected at 30 ºC was higher than at 20 ºC in most of the treatments. The highest R.H. (100 %) led to maximum amounts of OTA while no significant differences were found between 90 % and 80 % in the OTA content. Any opening in the grape

skin facilitate the penetration and development of the mould. Infection and therefore OTA production in grapes must be specially marked in hot and humid years. The results confirmed the effect of temperature and R.H. and indicate that damage of the berries contributes to infection and OTA production. The implementation of preventive measures in order to minimise berries damages in the field by controlling pathogenic fungi and insects during grape growing and removing visibly damaged grapes at harvest may significantly reduce OTA contamination in grapes. The study is shown in:

Skin damage, high temperature and relative humidity as detrimental factors for *Aspergillus carbonarius* infection and OTA production in grapes. *Food Control* (submitted).

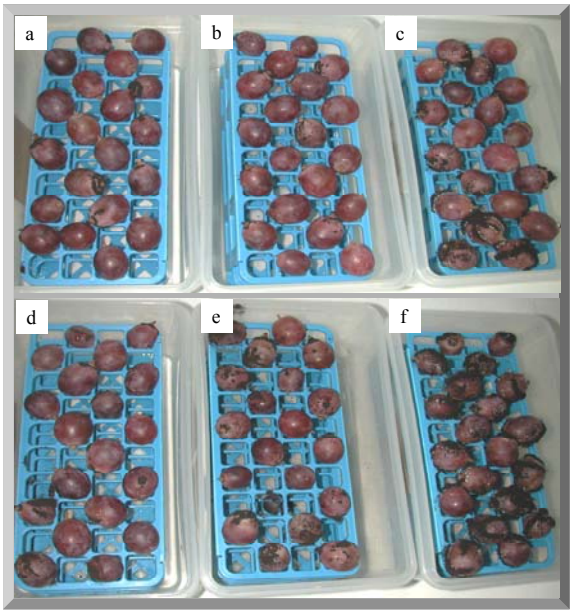

Figure 50. Damage (d, e, f) and undamaged (a, b, c) grapes after seven days of incubation at 30 ºC and at different R.H: 80 % (a, d), 90 % (b, e) and 100 % (c, f) R.H.

Influence of water activity and temperature on growth of isolates of *Aspergillus* section *Nigri* obtained from grapes

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ABSTRACT

The effects of water activity (a_w) and temperature on growth of *Aspergillus* section *Nigri* isolated from wine grapes were investigated on an agar medium with composition similar to that of grapes. Temperatures in the range of 10-37 ºC were tested. Optimum temperatures for growth were between 30 and 37 ºC. Water activity levels ranging from 0.90 to 0.995 were tested. Optimum a_w for growth was 0.98 in most cases. Statistical differences were found among the groups tested (*A. carbonarius*, *A. niger* aggregate and *A.* section *Nigri* uniseriates). Growth rates models for the factors assayed have been obtained.

KEY WORDS: Water activity, temperature, solute, *Aspergillus* section *Nigri*, growth, grapes.

1. INTRODUCTION

Ochratoxins are secondary metabolites produced by moulds belonging to several species of the genera *Aspergillus* and *Penicillium*. The most extensively studied compound of this group, ochratoxin A (OTA), has been shown to be a nephrotoxic, immumosuppressive, teratogenic and carcinogenic agent (JEFCA, 1991).

The presence of OTA has been reported in a number of plant products and occasionally in body fluids and kidneys of animals and humans (Xiao et al., 1996; Solti et al., 1997; Burdaspal and Legarda, 2000). First reported in wines in 1996 (Zimmerli and Dick, 1996), OTA has since been found in other grape beverages. Fungi belonging to *Aspergillus* section *Nigri* are the main OTA producers in these products. In addition, recent studies have shown that the three major black species, *A. carbonarius*, *A. niger* aggregate, and *A.* section *Nigri* uniseriates, are all very common in grapes at harvest (Battilani et al., 2003, Bellí et al., 2004).

The most used measure of the availability of water to microorganisms is water activity (a_w), the ratio of the vapour pressure of the water in the substrate to that of pure water at the same temperature and pressure. Other environmental factors such as temperature, pH, nutritional factors, etc. also affect mycelial growth and mycotoxin production by moulds. Temperature and a_w are the main factors influencing germination, growth and sporulation of spoilage fungi (Magan and Lacey, 1984).

The objectives of this study were to determine *in vitro* the effect of a_w and temperature on mycelial growth of several isolates of *Aspergillus* section *Nigri* isolated from grapes, on a synthetic nutrient medium similar to grape composition.

2. MATERIAL AND METHODS

2.1. Fungi

All the fungi used in this study were isolated from European wine grapes during the year 2001. Isolates were classified as *A. carbonarius* (W120, 93cr4, A0204, 36br4, 01UAs219, 01UAs294, A0933, CA332, Mu644), *A. niger* aggregate (93RJ4, 11V4, 01UAs203, 01UAs127, A1099, A1109, C432, Mu246), and *Aspergillus* section *Nigri* uniseriates (73r3, W118, 01UAs128, A0704, A0212).

Isolates were obtained from (1) the Faculty of Agriculture, Università Cattolica del Sacro Cuore, Piacenza, Italy; (2) the Institut National Polytechnique de Toulouse, Ecole Nationale Supérieure Agronomique de Toulouse, France; (3) the Departamento Engenharia Biologica, Universidade do Minho, Braga, Portugal; Departament de Sanitat i d'Anatomia Animals, Facultat de Veterinària, Univ.

Autònoma de Barcelona, Spain; (4) the Department Tecnologia d'Aliments, Escola Tècnica Superior d'Enginyeria Agrària, Universitat de Lleida, Spain, where samples of each isolate are held in their culture collection.

2.2. Growth medium

A synthetic nutrient medium (SNM) with composition similar to that of grapes between veraison and ripeness (modified from Delfini, 1982) was used. It had the following composition: D (+) glucose, 70 g; D (-) fructose, 30 g; L (+) tartaric acid, 7 g; L (-) malic acid, 10 g; $(NH_4)_2SO_4$, 0.67 g; $(NH_4)_2HPO_4$, 0.67 g; KH_2PO_4, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.75 g; NaCl, 0.15 g; $CaCl_2$, 0.15 g; $CuCl_2$, 0.0015 g; $FeSO_4 \cdot 7H_2O$, 0.021 g; $ZnSO_4$, 0.0075 g; (+) catechin, 0.05 g, distilled water, 1 L; pH adjusted to 4.2 with KOH (2N); agar, 20 g.

2.3. Water activity and solute type

Water activity was determined with a water activity meter (AquaLab, Decagon CX-2, Pullman, Washington, U.S.A.). Amounts of solute (glycerol or glucose) necessary to adjust the SNM growth medium to 0.90, 0.93, 0.95, 0.98 and 0.995 a_w were calculated and added (Table 1). These amounts were determined by interpolation in two experimental curves, one for each solute, obtained by plotting different concentrations of solutes versus resulting a_w. Additional, control plates were prepared and measured at the end of each experiment in order to detect any significant deviation.

Table 1. Amounts (g) of solute (glycerol or glucose) necessary to make up 100 ml of medium (SNM) at the required a_w.

a_w	glycerol	glucose
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

2.4. Inoculation and incubation

Fungi were grown on Czapek Yeast Autolysate Agar (CYA) for 7 days at 25 ºC to obtain heavily sporulating cultures. Spore suspensions were obtained by harvesting spores of each isolate from these cultures and suspending them in sterile distilled water containing 0.005 % of a wetting agent (Tween 80, Probus, Barcelona, Spain). The final concentration of the spores was assessed by using a Thoma chamber, and was adjusted to 10^6 spores ml^{-1}. SNM agar plates (20 ml) were needle inoculated centrally with each spore suspension. Plates with the same a_w were sealed with parafilm, distributed in polyethylene bags (20 plates/bag) and finally incubated at the required temperature (10, 15, 20, 25, 30 and 37 ºC).

2.5. Measurement of growth

Mycelial growth rates were determined by daily measurement of two right-angled diameters of the colonies. Measurements were carried out for a maximum of 60 days. Linear regression of colony radius against time (days) was used to obtain the growth rates (mm day^{-1}) under each set of growth conditions. Lag phase for growth was defined as the time (days) to reach 5 mm of diameter.

2.6. Experiments

The study was divided into three parts as two preliminary trials were carried out before the main experiment.

2.6.1. Effect of different solutes (glycerol and glucose) on the growth rates of six *A.* section *Nigri* isolates, at 25 ºC on SNM

A preliminary trial was carried out comparing growth rates of two isolates of *A. carbonarius* (W120, 93cr4), two isolates of *A. niger* aggregate (W119, 11V4) and two isolates of *A.* section *Nigri* uniseriates (73r3, W118), at different a_w (0.90, 0.93, 0.95, 0.98 and 0.995), on SNM at 25 ºC. The media were modified to achieve the correct a_w by using either glycerol or glucose in order to study the effect of these solutes on the mould growth and to decide which would be more suitable for further studies.

A full-factorial design with three replicates was applied. The factors assayed were solute (glycerol and glucose) and isolates (n=6) as qualitative factors, and a_w levels (n=5) as a quantitative factor. The response recorded was colony diameter.

2.6.2. Effect of a_w on growth rates of 20 *A.* section *Nigri* isolates at 25 ºC on SNM

Subsequent detailed experiments on the influence of a_w on growth of a larger number of isolates (six *A. carbonarius*, eight *A. niger* aggregate and six uniseriates), were carried out at 25 ºC on unmodified medium (0.995 a_w) and media modified with glycerol to 0.98, 0.95, 0.93 and 0.90 a_w. The objective was to verify the results of the first trial on a_w influence on these species growth in order to establish interspecific differences.

A full-factorial design with three replicates was applied. The factors assayed were isolate (n=20) as a qualitative factor, and a_w levels (n=5) as a quantitative factor. The response recorded was colony diameter.

2.6.3. Temperature and a_w influence on the growth of 10 black *Aspergillus* isolates on SNM

The combined effect of temperature and a_w on the growth of ten black Aspergilli isolates (four *A. carbonarius*, three *A. niger* aggregate and three uniseriates) on SNM was studied. This medium was modified in the range of a_w 0.90-0.995 by the addition of glycerol. Plates were incubated at five different temperatures (10, 15, 20, 30, 37ºC).

A full-factorial design with three replicates was applied. The factors assayed were isolate (n=20) as a qualitative factor, and a_w levels (n=5) and temperature (n=5) as quantitative factors. The response recorded was colony diameter.

2.7. Statistical treatment of the results

Colony diameters (mm) were statistically analysed (SAS Institute, version 8.2, Inc., Cary, N.C., U.S.A), so that effects of single factors (isolate, solute, a_w and temperature), and their interactions could be assessed at statistically significant differences (p<0.05). Growth rates were modelled by polynomial multiple lineal regression (MLR) with six coefficients (b_0, b_1, b_2, b_{12}, b_{11} and b_{22}): growth rate= $b_0 + b_1 a_w + b_2 T + b_{12} a_w T + b_{11} a_w^2 + b_{22} T^2$. The resulting response surface models (RSM) were obtained with the Unscrambler® software, version 7.6 (CAMO ASA, Oslo, Norway), including the significant factors, interactions and quadratic terms.

3. RESULTS AND DISCUSSION

3.1. Effect of glycerol and glucose on the growth rates of six *A.* section *Nigri* isolates, at 25 ºC on SNM

No significant differences between glycerol and glucose were found, although the growth was slightly faster and the lag phase for growth shorter with glucose, as sugars are the preferred carbon source for *A. niger* (Hatzinikolaou and Macris, 1995). However, glycerol was chosen for subsequent experiments as the dissolution of high amounts of glucose was more time-consuming than working with glycerol.

Significant differences were found between isolates, a_w and their interaction. The differences in the isolates were due to differences among the different groups, as observed with further statistical analysis. Isolates belonging to the same group had similar growth rates and lag phases for growth under all the conditions tested, however, there were differences among the different groups: *A. carbonarius* growth rates were significantly lower than those of *A. niger* aggregate and uniseriate isolates; moreover, lag phases for growth of *A. carbonarius* and uniseriates were higher than those of *A. niger* aggregate. Maximum growth rates and the shortest lag phases for growth were observed for all fungi at high a_w (0.95-0.995) (Fig. 1).

3.2. Effect of a_w on growth rates of 20 *A.* section *Nigri* isolates at 25 ºC on SNM

The isolates tested showed different growth rates and responses to a_w and the significant differences found were mainly attributed to the group to which the isolate belonged (*A. carbonarius*, *A. niger* aggregate and uniseriates). Analysis of variance revealed that water relation profiles were similar for all the isolates of *A. niger* aggregate, being the ones which presented the fastest growth rates, higher than 5 mm day^{-1} except at the lowest a_w. All *A. carbonarius* isolates tested presented similar growth rates, below 5 mm day^{-1}, except for the isolate 01UAS294 which reached 7.8 mm day^{-1} at 0.98 and 0.995 a_w. Uniseriate isolates also showed the same behaviour, with the maximum growth rates at 0.98 a_w, except for isolate A0704, which presented the lowest growth rates values of this group.

Water activity effect was statistically significant for *A. carbonarius* and uniseriate isolates; *A. carbonarius* grew faster at higher a_w (0.98-0.995), whereas similar growth rates were detected at 0.93 and 0.95 a_w, and the lowest growth rates were at 0.90 a_w (Fig. 2). Growth of uniseriates was significantly lower at 0.90 a_w but similar at the other a_w levels tested. No differences on the growth rates of *A. niger* aggregate at the different a_w levels were detected, although the optimum a_w level for growth was between 0.98-0.995 a_w. As no statistical differences were found among the isolates tested into each group (except for the two cases mentioned

above), figure 2 shows the influence of a_w on the growth rates of two isolates of each group. The country of origin of each isolate did not have any significant influence on their growth rates.

This second trial confirmed that at 25ºC on SNM, all *A.* section *Nigri* grew better at high levels of a_w, with an optimum between 0.95-0.995 and also confirmed the differences on the growing patterns of the different groups in the section *Nigri*.

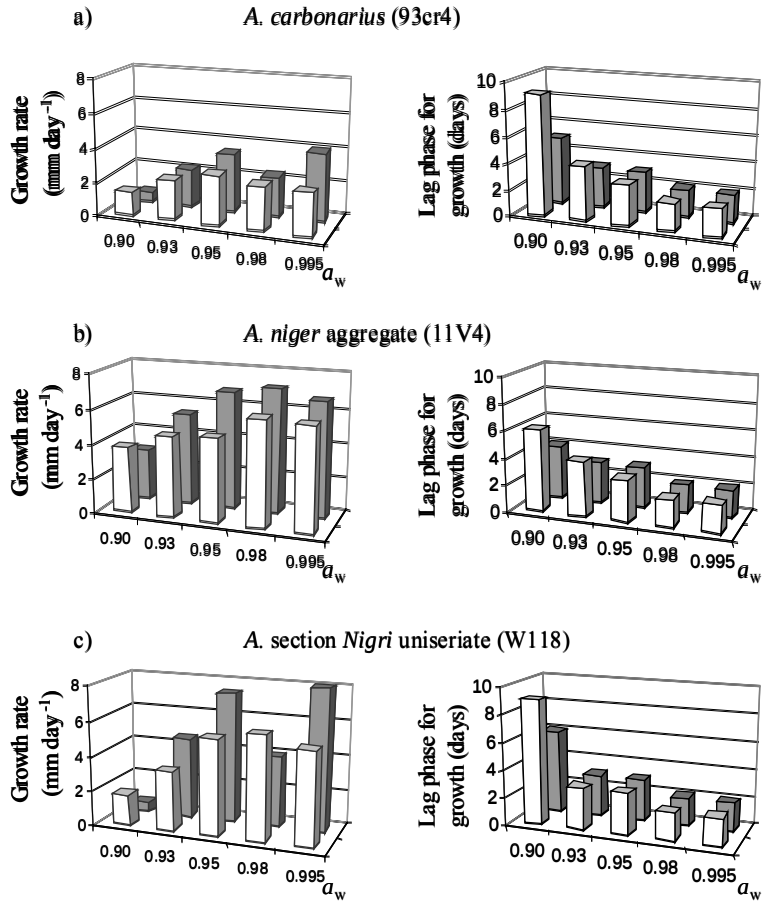

Figure 1. Effect of a_w on growth rate (mm day^{-1}) and lag phase (days until the colony diameter reached 5 mm) of three black *Aspergilli* isolates at 25ºC on SNM. Solutes were glycerol (□) and glucose (■).

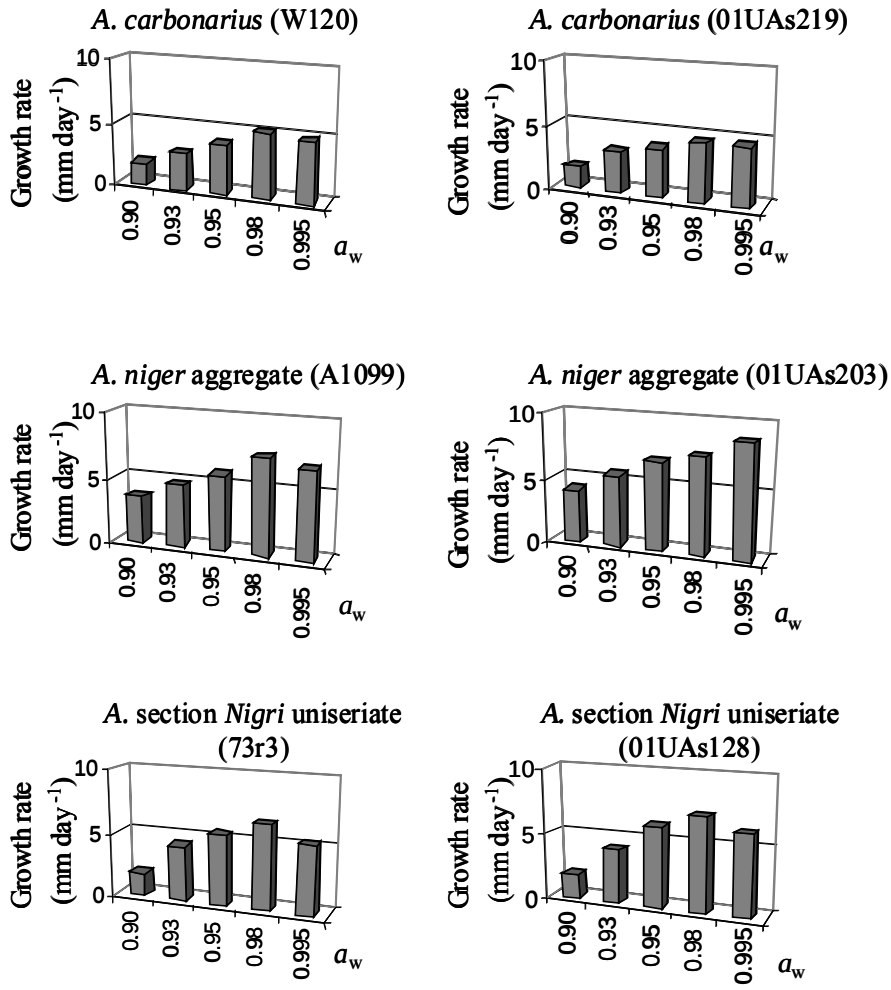

Figure 2. Effect of a_w on growth rate (mm day^{-1}) of six black Aspergilli isolates at 25ºC on SNM.

3.3. Temperature and a_w influence on the growth of 10 black *Aspergillus* isolates on SNM

Lag phases for growth were noticeably influenced by both temperature and a_w. Generally, the time required to reach the linear phase increased with decreasing temperatures. Water activity influenced lag phases for growth in the same way as at 25 ºC in the previous sections.

There were statistical differences between the growth of the different isolates tested, due again to the group to which they belonged (*A. carbonarius*, *A. niger* aggregate and uniseriate). Growth was influenced by both factors assayed (a_w and temperature) and their interaction. Table 2 shows growth rates and statistical significance of each isolate under all a_w at the different temperature levels tested. The growth of *A. niger* aggregate was higher than the growth of the other two groups, except at low temperatures (10-20 ºC), when the growth rates were very similar for the isolates of different groups. At 30 and 37 ºC, *A. carbonarius* growth rates were below 5 mm day^{-1}. Again, the isolate 01UAs294 grew faster than the other isolates of *A. carbonarius* tested, but this isolate has been shown to present some molecular differences in comparison with the others (Dr. J. Cabañes, Animal Health and Anatomy Department, Autonomous University of Barcelona, Spain, personal communication). Furthermore, there were differences on the growth of the isolates belonging to the other two groups of black Aspergilli, although these differences were not attributable to the country of origin.

In all cases, the minimum growth rates were at 10 ºC, increasing slightly at 15 ºC and again at 20 ºC. Similar results for *A. carbonarius* were found by Mitchell et al. (2003), who reported no isolate growing at 10 ºC whereas at 15 ºC growth was only possible at higher a_w levels. In our study, at the highest temperatures (30 and 37 ºC), all the isolates showed higher growth rates than at the remaining temperatures with 30 ºC being the optimum for *A. carbonarius* and uniseriate isolates, although the latter had highest growth rates at 37 ºC than at 30 ºC when a_w level was the lowest (0.90). Similarly, other authors found optimum temperature for *A. carbonarius* between 25 ºC and 35 ºC (Leong et al., 1999; Mitchell et al., 2003). In the present study, all *A. niger* aggregate isolates growth rates at 30 ºC and 37 ºC were not significantly different regardless of a_w. The effect of environmental factors on the growth of one isolate of *A. niger* was studied by Vats et al. (2002) and found that the isolate grow best at 30 ºC but contrary to us, found a sharp decline at 37 ºC and no growth at 45 ºC and above.

For all the isolates and temperatures assayed, growth rates increased with a_w reaching the optimum at 0.98 a_w and decreasing slightly at 0.995 a_w. at 10 ºC, the growth of all the isolates was negligible when a_w levels were low (0.90 and 0.93 a_w), increasing slightly at 0.95, 0.98 and 0.995 a_w. All the isolates were more tolerant of low a_w at temperatures close to the optimum.

Models of growth rates versus a_w and temperatures were obtained (Fig. 3). Negative values must be interpreted as null values. Table 3 presents the model coefficients estimated by multiple linear regression for main effects, as well as interaction and quadratic terms.

Table 2. Growth rates (mm day^{-1}) of ten *A.* section *Nigri* isolates at different a_w and temperatures on SNM.

		Growth rates (mm day^{-1})					
Isolate	**a_w**	**10º C**	**15º C**	**20º C**	**25 ºC**	**30º C**	**37º C**
A. carbonarius (36br4)	0.90	0^{a}	0.13^{a}	0.83^{a}	1.77^{a}	1.94^{a}	0.54^{a}
	0.93	0^{a}	0.68^{b}	1.88^{b}	2.92^{b}	3.74^{a}	2.97^{b}
	0.95	0.28^{b}	1.14^{c}	2.35^{c}	3.92^{b}	4.22^{b}	3.32^{c}
	0.98	0.43^{c}	1.51^{c}	2.46^{c}	5.12^{b}	5.38^{b}	4.51^{c}
	0.995	0.31^{b}	1.05^{c}	1.3^{bc}	4.77^{b}	4.02^{ab}	3.2^{c}
A. carbonarius (A0933)	0.90	0^{a}	0.09^{a}	0.45^{a}	1.34^{a}	1.01^{a}	0.44^{a}
	0.93	0^{a}	0.69^{b}	1.61^{b}	2.06^{ab}	3.4^{b}	1.92^{b}
	0.95	0.21^{b}	1.01^{c}	2.17^{c}	2.94^{b}	3.65^{b}	2.04^{b}
	0.98	0.36^{c}	1.26^{c}	2.57^{c}	2.74^{b}	3.45^{b}	2.62^{b}
	0.995	0.16^{a}	0.92^{c}	2.07^{c}	3.36^{b}	3.76^{b}	2.24^{b}
A. carbonarius (Mu644)	0.90	0^{a}	0.51^{a}	1.46^{a}	1.55^{a}	3.47^{a}	4.06^{a}
	0.93	0^{a}	0.82^{b}	1.77^{b}	2.84^{b}	4.58^{a}	7.18^{a}
	0.95	0.39^{b}	0.99^{c}	2.31^{c}	3.05^{b}	5.15^{a}	7.3^{a}
	0.98	0.62^{c}	1.28^{d}	2.62^{c}	2.97^{b}	4.54^{a}	5.13^{a}
	0.995	0.4^{d}	0.89^{d}	1.85^{c}	2.90^{b}	4.51^{a}	4.68^{a}
A. carbonarius (01UAs294)	0.90	0^{a}	0.07^{a}	0.71^{a}	3.15^{a}	4^{a}	2.18^{a}
	0.93	0^{a}	0.33^{a}	2.14^{b}	5.18^{a}	5.85^{a}	5.15^{b}
	0.95	0^{a}	0.74^{b}	2.61^{c}	5.87^{a}	7.57^{a}	5.79^{b}
	0.98	0.14^{b}	1.43^{c}	2.73^{bc}	7.57^{a}	9.11^{a}	6.35^{b}
	0.995	0.09^{ab}	1.00^{c}	2.36^{c}	7.81^{a}	6.97^{a}	3.07^{ab}
A. niger aggregate (11V4)	0.90	0^{a}	0.64^{a}	1.62^{a}	4.16^{a}	4.62^{a}	4.89^{a}
	0.93	0^{a}	1.06^{b}	2.57^{bc}	6.34^{a}	7.04^{a}	7.23^{a}
	0.95	0.18^{a}	1.35^{c}	3.35^{b}	7.06^{a}	8.29^{a}	9.17^{a}
	0.98	0.39^{b}	1.38^{c}	3.16^{b}	8.64^{a}	9.34^{a}	8.9^{a}
	0.995	0.47^{b}	1.14^{c}	2.49^{c}	9.53^{a}	5.24^{a}	7.54^{a}
A. niger aggregate (A1109)	0.90	0^{a}	0.69^{a}	1.77^{a}	3.08^{a}	4^{a}	3.81^{a}
	0.93	0.32^{a}	1.13^{b}	2.5^{b}	3.96^{a}	4.9^{a}	7.07^{b}
	0.95	0.45^{b}	1.41^{c}	2.79^{c}	4.27^{a}	5.1^{a}	7.83^{b}
	0.98	0.61^{c}	1.5^{c}	3.15^{c}	4.73^{a}	6.14^{ab}	6.29^{b}
	0.995	0.45^{b}	1.32^{c}	2.76^{bc}	4.63^{a}	5.56^{b}	5.09^{b}
A. niger aggregate	0.90	0^{a}	0.52^{a}	1.81^{a}	4.00^{a}	4.26^{a}	3.92^{a}

(01UAs127)	0.93	0^{a}	0.91^{b}	2.55^{b}	5.43^{a}	6.42^{a}	7.05^{a}
	0.95	0.22^{a}	1.49^{c}	3.16^{b}	6.28^{a}	7.27^{a}	7.16^{a}
	0.98	0.43^{b}	1.51^{c}	3.2^{b}	7.36^{a}	7.84^{a}	9.03^{a}
	0.995	0.12^{a}	1.23^{c}	2.51^{ab}	7.40^{a}	6.97^{a}	5.98^{a}
A. section *Nigri* uniseriate (W118)	0.90	0^{a}	0.44^{a}	1.59^{a}	1.77^{a}	2.64^{a}	3.74^{a}
	0.93	0^{a}	0.55^{a}	1.59^{a}	3.88^{b}	2.9^{b}	1.79^{a}
	0.95	0.46^{b}	0.92^{b}	2.22^{b}	6.01^{b}	5.87^{b}	2.31^{a}
	0.98	0.78^{c}	1.47^{c}	3.71^{b}	6.81^{b}	7.56^{b}	3.54^{a}
	0.995	0.06^{ab}	0.8^{b}	3.59^{b}	5.46^{b}	7.6^{b}	6^{a}
A. section *Nigri* uniseriate (A0212)	0.90	0^{a}	0.02^{a}	0.13^{a}	3.57^{a}	0.77^{a}	3.01^{a}
	0.93	0^{a}	0.23^{b}	0.94^{b}	3.46^{b}	3.98^{b}	0.51^{ab}
	0.95	0.06^{b}	0.29^{b}	1^{b}	6.37^{b}	6.99^{b}	0.89^{ab}
	0.98	0.23^{c}	0.8^{c}	2.72^{c}	8.67^{b}	5.52^{b}	5.45^{c}
	0.995	0.17^{c}	1.55^{d}	2.14^{d}	5.62^{b}	4.9^{b}	1.33^{b}
*A.*section *Nigri* uniseriate (01UAs128)	0.90	0^{a}	0^{a}	0.4^{a}	1.83^{a}	1.64^{a}	2.93^{a}
	0.93	0^{a}	0.35^{b}	1.64^{b}	4.10^{b}	2.93^{b}	2.38^{a}
	0.95	0^{a}	0.75^{c}	2.37^{c}	6.15^{b}	5.23^{c}	2.17^{a}
	0.98	0.14^{b}	1.37^{d}	3.25^{c}	7.19^{b}	7.01^{bc}	5.99^{a}
	0.995	0.11^{b}	1.36^{d}	1.72^{c}	6.17^{b}	6.99^{c}	3.6^{a}

For each isolate, data in the same column followed by different letters are significantly different in LSMEANS test.

Results obtained on culture media cannot easily be extrapolated to natural systems as the reactions in the field can be modified by the ecosystem (Magan and Lacey, 1984). However, these preliminary results can provide an indication of the growing patterns of these species as affected by environmental conditions, and may be a first step for further experimental design at field conditions. Furthermore, studies directly on grapes are in progress.

Table 3. Model coefficients obtained by MLR for effects of a_w and temperature on growth rates (mm day^{-1}) on SNM.

	Regression coefficients		
Factors	***A. carbonarius*[a]**	***A. niger* aggregate[b]**	**uniseriates[c]**
Intercept (b_0)	-11.316**	-11.926**	-21.258**
a_w (b_1)	11.290**	10.674**	21.465**
Temperature (b_2)	0.172**	0.276**	0.172**
a_w x T (b_{12})	0.195^{ns}	0.268*	0.321*
a_w^2 (b_{11})	-0.505**	-0.656**	$3.587 \cdot 10^{-2}$ ^{ns}
T^2 (b_{22})	-0.535^{ns}	-0.310*	-0.859^{ns}
R^2	0.693	0.907	0.659

^{ns} Not significant; R^2 = percentage of variation explained by the model;
Values of the interaction and square effects for:
[a] *A. carbonarius* : T* a_w = 0.1010 (T-22.4) x 29.1636 (a_w – 0.951); $(T)^2$ = (0.1010 (T-22.4))^2; $(a_w)^2$ = (29.1636 (a_w – 0.951))^2.
[b] *A. niger* aggregate : T* a_w = 0.1008 (T-22.4) x 29.1145 (a_w – 0.951); $(T)^2$ = (0.1008 (T-22.4))^2; $(a_w)^2$ = (29.1145 (a_w – 0.951))^2.
[a] Uniseriates : T* a_w = 0.1008 (T-22.4) x 29.1145 (a_w – 0.951); $(T)^2$ = (0.1008 (T-22.4))^2; $(a_w)^2$ = (29.1145 (a_w – 0.951))^2.
* significant p<0.05; ** significant p<0.01.

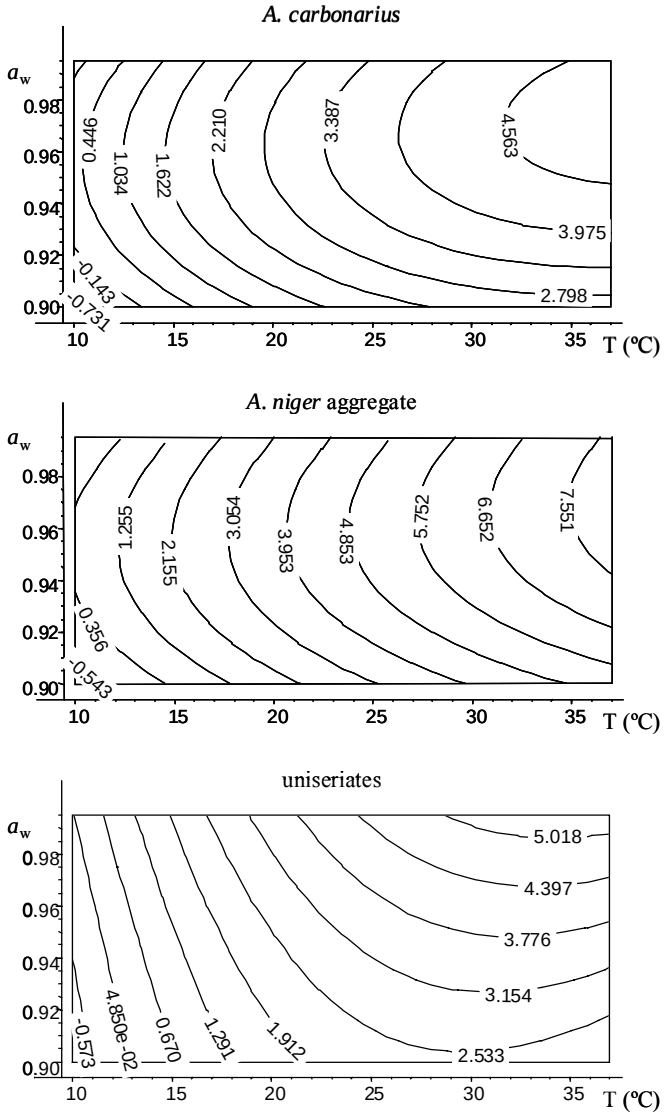

Figure 3. Response surface contour plots showing the effect of a_w and temperature on growth rates (mm day^{-1}) of *Aspergillus* section *Nigri*: a) *A. carbonarius* b) *A. niger* aggregate and c) uniseriates, on SNM.

4. CONCLUSIONS

A. section *Nigri* may grow at all the temperatures tested in this study, ranging from 10 to 37 ºC, with an optimum between 30 and 37 ºC, with the latter conditions being frequent in the field close to harvest time. However, at 10 ºC the growth of all the isolates was negligible when a_w levels were low (0.90 and 0.93 a_w). The optimum a_w for black Aspergilli to grow, seems to be at 0.98, similar to the a_w of grapes in the field. Thus field conditions are likely to be conducive to optimum growth of these species. Furthermore, there were statistical differences between the growth of the different isolates tested, mainly due to the group to which the isolate belonged (*A. carbonarius*, *A. niger* aggregate and uniseriate), with the *A. niger* aggregate presenting the fastest growth rates in the three trials and *A. carbonarius* the lowest.

A better knowledge of the growth condition for OTA-producing fungi is critical for an understanding of the ecological niche occupied by these black *Aspergillus* spp. Moreover, further studies should be conducted to improve understanding of the ecology and to study the effect of the variables that most likely would influence toxin production by the main ochratoxigenic moulds in grapes. Based on these studies, remedial measures to prevent their establishment could be implemented.

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Aspergillus carbonarius growth and ochratoxin A production on a synthetic grape medium in relation to environmental factors

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SUMMARY

Aims:
The effects of water activity (0.90-0.99 a_w), temperature (15-37 ºC) and their interaction on growth and ochratoxin A (OTA) production by eight isolates of *Aspergillus carbonarius* were investigated on synthetic nutrient medium (SNM) with composition similar to grapes.
Methods and Results:
Growth data were modelled by an multiple linear regression and response surface models were obtained. *Aspergillus carbonarius* grew much faster at 30 ºC than at the other temperature levels tested and its growth rate increased with increasing a_w, maximum growth rate being between 0.95 and 0.99 a_w. In general, isolates grew faster at 35-37 ºC than at 20 ºC, although no significant differences were found between these temperatures. OTA accumulation was also favoured by high a_w levels, and although it was observed in the whole range of temperatures, maximum amounts were detected at 20 ºC. No OTA was found at the most unfavourable growth conditions.
Conclusions:
Optimum a_w level for growth seems to correspond with optimum for OTA production, meanwhile the most propitious temperature for the toxin production was below the best one for growth.
Significance and Impact of Study:
Prediction of *A. carbonarius* growth would allow estimating their presence and therefore, the OTA production, as it was found that conditions for the toxin production were more limited than those permitting growth.

KEY WORDS: *A. carbonarius*, grapes, growth, ochratoxin A, temperature, water activity.

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INTRODUCTION

Ochratoxin A (OTA) is an isocumarin derivative of the secondary metabolism of different filamentous fungi. It is a genotoxic carcinogen for animals and humans. Sources of this mycotoxin can be vegetal materials (cereals, coffee, beer, wine and fruit juices) as well as food products based on animal tissues (Varga et al. 2001). Moreover, the occurrence of OTA in grapes, raisins, wine and other derivatives has gained increasing attention in the last few years.

Fungi belonging to the genera *Aspergillus* and *Penicillium* are described as the main OTA producers. It has been observed that in grapes, *Aspergillus* section *Nigri* (black aspergilli) are dominant in the field before harvest and some of its members have been reported to be potential OTA producing fungi in grapes (Da Rocha Rosa et al. 2002; Sage et al. 2002; Battilani et al. 2003; Magnoli et al. 2003; Bellí et al. 2004a). In particular *A. carbonarius* seems to be confirmed as the target pathogen, especially because a high percentage (75-100%) of its isolates are able to produce the toxin (Cabañes et al. 2002; Bellí et al. 2005).

Growth of ochratoxigenic species such as *A. ochraceus* and OTA formation, has been shown to be affected by temperature, water activity (a_w) and other factors in different substrates such as cereals (Lee and Magan 2000; Pardo et al. 2004). However, *A. carbonarius* isolates have been rarely studied, as they were recently described as OTA producers. Preliminary studies with a relatively low number of isolates showed that a_w and temperature play an important role in the development and OTA production by these fungi (Mitchell et al. 2003; Bellí et al. 2004b,c).

In this study, eight isolates of *A. carbonarius* isolated from wine grapes were grown on synthetic media, with composition similar to grapes, at different combinations of a_w and temperature to determine the marginal and optimum conditions for mycelial growth and OTA production. A suitable model for prediction of *A. carbonarius* growth as a function of a_w and temperature was also obtained.

MATERIALS AND METHODS

Isolates

Eight OTA-producing isolates of *A. carbonarius* obtained from wine-grapes from four European countries: Italy (W9, W198), France (W37, W38), Portugal (W89, W104) and Spain (W120, W128) were used in this study. Isolates were supplied by the Faculty of Agriculture, Università Cattolica del Sacro Cuore, Piacenza, Italy; the Institut National Polytechnique de Toulouse, École Nationale Supérieure Agronomique de Toulouse, France; the Departamento Engenharia Biologica, Universidade do Minho, Braga, Portugal; the Departament de Sanitat i d'Anatomia Animals, Facultat de Veterinària,

Univ. Autònoma de Barcelona, Spain and the Departament de Tecnologia d'Aliments, Escola Tècnica Superior d'Enginyeria Agrària, Universitat de Lleida, Spain, where samples of each isolate are held in their culture collection.

Medium and water activity modification

Studies were carried out *in vitro* using synthetic nutrient medium (SNM) composed of D (+) glucose, 70 g; D (-) fructose, 30 g; L (+) tartaric acid, 7 g; L (-) malic acid, 10 g; $(NH_4)_2SO_4$, 0.67 g; $(NH_4)_2HPO_4$, 0.67 g; KH_2PO_4, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.75 g; NaCl, 0.15 g; $CaCl_2$, 0.15 g; $CuCl_2$, 0.0015 g; $FeSO_4 \cdot 7H_2O$, 0.021 g; $ZnSO_4$, 0.0075 g; (+) catechin, 0.05 g; agar, 20 g; water until 1000 ml; pH 4.2 reached with KOH (2N) (modified from Delfini, 1982). This medium simulates grape composition at veraison and its a_w was 0.99. SNM was modified to 0.90, 0.93 and 0.95 a_w by adding different amounts of glycerol (Bellí et al. 2004c). Determinations were done by using a water activity meter (AquaLab CX-2, Decagon, Pullman, Washington, U.S.A.). Autoclaved media (20 ml) were poured into 9 cm diameter sterile plastic Petri dishes and used immediately.

Inoculation, incubation and mycelium measurement

Spore suspensions (10^6 spores ml^{-1}) from each fungal isolate grown on SNM were prepared and used to needle-inoculate the centre of the SNM Petri plates. Plates were incubated at different temperatures (15, 20, 30, 35 and 37 ºC) in sealed polyethylene bags in order to maintain a constant relative humidity (R.H.) level. Diameters of the growing colonies were measured daily with the aid of a binocular magnifier, for up to 30 days. Treatments were repeated three times.

OTA analysis

Additional SNM plates were prepared as in the growth experiment and inoculated. OTA was extracted by a variation of Bragulat et al. (2001) method. Three plugs (diameter: 6 mm) were removed from the inner, middle and outer area of the colony, after seven days of incubation at different combinations of temperature (15, 20, 30, 35 and 37 ºC) and a_w (0.90, 0.93, 0.95, 0.99) levels. Plugs were introduced in a vial containing 1 ml of methanol. After 60 minutes, the extracts were shaken and filtered (Millex^R SLHV 013NK, Millipore, Bedford, Massachusetts, U.S.A.) into another vial and stored at 4 ºC until the analysis by high performance liquid chromatography (HPLC) with fluorescence detection (Waters 474, Milford, Massachusetts, U.S.A.) (λ_{exc} 330 nm; λ_{em} 460 nm). Acetonitrile-water-acetic acid (57:41:2) (1.0 ml min^{-1}) was the mobile phase and a C_{18} column (Waters Spherisorb 5 µm, ODS2, 4.6x250 mm) was used. The injection volume was 25 µl and the retention time was 7.1 minutes. The detection limit was 0.02 µg OTA

g^{-1} of SNM, based on a signal-to-noise ratio of 3:1. The ochratoxin A standard was from *A. ochraceus* (Sigma-Aldrich, Steinheim, Germany). The standard solution was made in methanol and concentration confirmed by using an UV spectrophotometer. Treatments were performed in triplicate.

Statistical treatment of the results

The regression lines of colony diameters against days after inoculation were calculated for each a_w and temperature combination with Microsoft Excel program (version 2002; Microsoft, Munich, Germany) and were used to obtain the growth rate under each treatment conditions.

Temperature and a_w effects on mycelial growth and OTA production were statistically analysed with SAS software (version 8.02, SAS Institute, Inc., Cary, N.C., U.S.A.) by analysis of variance followed by either LSMEAN or Student-Newman-Keuls (SNK) multiple-range tests. Statistical significance was judged at $P<0.0001$.

Polynomial multiple linear regressions (MLR) and the resulting response surface models (RSM) were obtained for prediction of *A. carbonarius* growth rates, with the Unscrambler software (CAMO ASA, Oslo, Norway, version 7.6), including the significant factors, interactions and quadratic terms.

RESULTS

Temperature and a_w influence on mycelial growth

Analysis of variance revealed that the factors a_w and temperature and their interaction, had a significant influence on mycelial growth of the eight *A. carbonarius* isolates tested ($P<0.0001$). No significant differences among the isolates tested were observed (data not shown).

The combined effects of a_w and temperature on growth rates (mm day^{-1}) are shown in Table 1. Optimum temperature for growth was 30 ºC for all a_w levels tested, except for isolate W9 at 0.99 a_w, W37 at 0.90 and 0.99 a_w and W120 at 0.93 a_w, where growth was faster at 35 ºC than at 30 ºC, and W128 and W198 at 0.99 a_w, where maximum growth was detected at 20 ºC.

Growth rates presented a peak at 30 ºC at 0.95 a_w, and at 0.99 a_w in the case of W38, W89 and W120 isolates, being superior of 10 mm day^{-1} for this last one.

Table 1. Growth rates (mm day^{-1}) of eight *A. carbonarius* isolates at different a_w and temperatures on SNM ± standard error.

		Growth rates (mm day^{-1})				
Isolate	**a_w**	**15 ºC**	**20 ºC**	**30 ºC**	**35 ºC**	**37 ºC**
W9	0.90	N.G.	0.84 ± 0.09	2.86 ± 0.08	2.00 ± 0.06	1.49 ± 0.06
	0.93	0.65 ± 0.07	3.03 ± 0.09	5.37 ± 0.19	3.92 ± 0.17	3.42 ± 0.21
	0.95	2.09 ± 0.03	4.26 ± 0.06	8.76 ± 0.22	5.79 ± 0.12	4.45 ± 0.16
	0.99	2.86 ± 0.09	5.61 ± 0.20	7.35 ± 0.16	7.69 ± 0.27	5.69 ± 0.21
W37	0.90	N.G.	0.94 ± 0.07	1.73 ± 0.31	2.21 ± 0.05	1.53 ± 0.09
	0.93	0.34 ± 0.09	2.05 ± 0.12	5.05 ± 0.17	3.18 ± 0.42	3.42 ± 0.09
	0.95	1.52 ± 0.03	4.03 ± 0.08	7.75 ± 0.19	5.68 ± 0.16	5.24 ± 0.15
	0.99	2.76 ± 0.10	3.63 ± 0.16	6.08 ± 0.26	6.74 ± 0.41	5.87 ± 0.31
W38	0.90	N.G.	0.71 ± 0.04	1.47 ± 0.12	1.27 ± 0.14	0.91 ± 0.09
	0.93	0.42 ± 0.07	2.53 ± 0.08	4.96 ± 0.24	3.74 ± 0.07	3.24 ± 0.16
	0.95	1.81 ± 0.04	4.11 ± 0.04	7.02 ± 0.31	5.99 ± 0.22	4.81 ± 0.18
	0.99	2.62 ± 0.09	5.94 ± 0.16	7.93 ± 0.24	7.78 ± 0.31	5.84 ± 0.17
W89	0.90	N.G.	0.78 ± 0.10	1.94 ± 0.13	1.16 ± 0.07	0.93 ± 0.09
	0.93	0.25 ± 0.06	2.36 ± 0.07	3.78 ± 0.23	3.11 ± 0.13	3.44 ± 0.15
	0.95	1.86 ± 0.04	4.15 ± 0.07	6.75 ± 0.36	4.75 ± 0.15	4.70 ± 0.16
	0.99	2.86 ± 0.10	5.00 ± 0.25	6.83 ± 0.13	6.42 ± 0.34	4.32 ± 0.11
W104	0.90	N.G.	0.94 ± 0.05	2.63 ± 0.10	1.34 ± 0.12	1.32 ± 0.08
	0.93	0.69 ± 0.08	2.49 ± 0.11	4.99 ± 0.19	3.74 ± 0.27	3.53 ± 0.17
	0.95	2.01 ± 0.02	4.32 ± 0.06	7.65 ± 0.14	4.66 ± 0.15	4.84 ± 0.22
	0.99	2.50 ± 0.10	5.63 ± 0.12	7.44 ± 0.37	5.55 ± 0.64	5.11 ± 0.13
W120	0.90	N.G.	1.02 ± 0.11	2.47 ± 0.10	2.43 ± 0.15	2.21 ± 0.13
	0.93	0.77 ± 0.06	2.98 ± 0.07	4.51 ± 0.09	4.84 ± 0.10	4.31 ± 0.09
	0.95	2.13 ± 0.05	5.20 ± 0.07	7.75 ± 0.16	5.96 ± 0.23	4.49 ± 0.12
	0.99	2.90 ± 0.15	7.02 ± 0.24	10.1 ± 0.41	6.05 ± 0.25	5.72 ± 0.20
W128	0.90	N.G.	0.60 ± 0.06	1.88 ± 0.10	1.20 ± 0.08	0.82 ± 0.04
	0.93	0.50 ± 0.07	1.91 ± 0.07	5.38 ± 0.19	2.59 ± 0.12	1.94 ± 0.21
	0.95	1.53 ± 0.03	3.90 ± 0.05	8.29 ± 0.10	4.36 ± 0.36	3.39 ± 0.12
	0.99	3.37 ± 0.12	7.42 ± 0.33	6.54 ± 0.15	5.83 ± 0.16	4.84 ± 0.16
W198	0.90	N.G.	0.63 ± 0.10	1.92 ± 0.17	1.11 ± 0.12	1.36 ± 0.13
	0.93	1.12 ± 0.03	3.17 ± 0.04	5.90 ± 0.23	3.67 ± 0.17	3.87 ± 0.04
	0.95	2.32 ± 0.02	4.45 ± 0.11	8.01 ± 0.20	5.49 ± 0.19	4.44 ± 0.08
	0.99	2.91 ± 0.11	7.13 ± 0.29	6.80 ± 0.19	5.69 ± 0.24	4.82 ± 0.10

N.G. no growth

No significant differences were found between 20, 35 and 37 ºC, although in general, isolates grew faster at 35 and 37 ºC than at 20 ºC.

Growth at 0.95 and 0.99 a_w was significantly higher than at 0.93 a_w, and this one significantly higher than at 0.90 a_w, where little growth was detected in few of the isolates. Minimum growth rates (<4 mm day^{-1}) were obtained at 15 ºC regardless of a_w level and isolates tested. No growth was observed at this temperature at 0.90 a_w, and it was very low (<1.5 mm day^{-1}) at 0.93 a_w.

The proposed model that allows predicting growth rates of *A. carbonarius* is shown in Fig. 1. It is suitable for any *A. carbonarius* isolate, as no significant differences were found between the eight isolates. The percentage of variance explained by the model (R^2 coefficient) varied between 0.86 and 0.96.

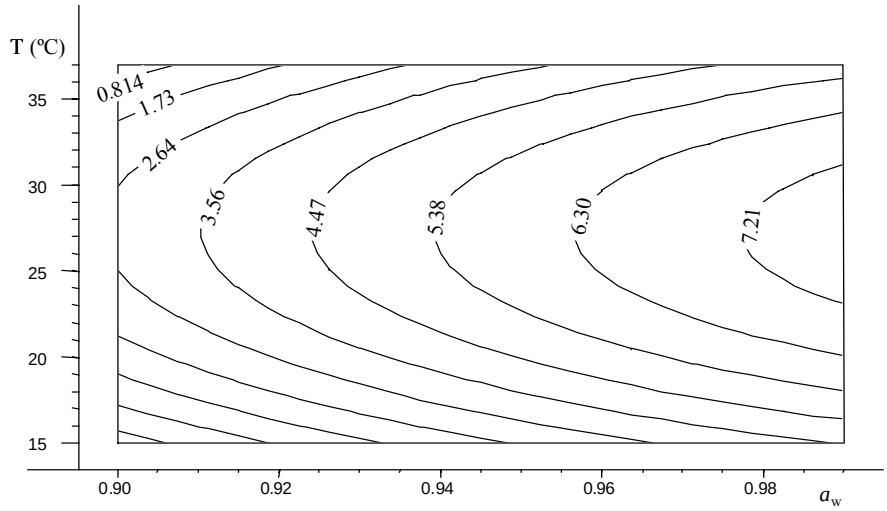

Figure 1. Response surface contour plot showing the effect of a_w and temperature on the growth rates (mm day^{-1}) of *A. carbonarius* (W128) isolate, on SNM (R^2=0.86).

Temperature and a_w influence on OTA production

All single factors (a_w, temperature and isolate) and the interaction isolate*temperature had a significant effect on OTA production (Table 2).

Maximum amounts of OTA were detected at 0.95 a_w for W120 and at 0.99 a_w for the other isolates tested (Table 3). OTA production decreased with decreasing a_w, although

no significant differences were found between 0.95 and 0.93 a_w and between 0.93 and 0.90 a_w. No OTA was detected at 0.90 a_w and only two isolates (W37 and W120) produced low amounts at 0.93 a_w at 20 ºC.

OTA was detected under the whole range of temperatures tested, although at 15 ºC only three isolates (W37, W38 and W120) produced OTA at 0.99 a_w. At 35 and 37 ºC, OTA was only detected at the highest a_w level tested. Temperature was statistically significant only for the higher OTA producing isolates (W38 and W120), where a peak production of OTA occurred at 20 ºC followed by 15 ºC. Significantly less OTA was found at 30, 35 and 37 ºC, with no significant differences among them. For the other isolates tested and in general, 20 ºC seems also the optimum temperature for producing OTA after 7 days of incubation, although no significant differences were observed between temperatures.

The highest mean OTA concentrations of 9.13 μg g^{-1} SNM at 0.98 a_w and 20 ºC, and of 20.5 μg g^{-1} SNM at 0.95 a_w and 20 ºC were detected for W38 and W120 isolates, respectively. The lowest OTA producers were the Italian (W9, W198) and the Portuguese (W89, W104) isolates, producing less than 2 μg g^{-1}, meanwhile the amount of OTA produced by W37 and W128 isolates was up to 5 μg g^{-1}.

Table 2. Analysis of variance of the effects of a_w and temperature on OTA production by eight isolates of *A. carbonarius* after 7 days growing on Synthetic Nutrient Medium (SNM).

Factors	Mean Square	F-value
a_w	46.34	7.53*
isolate	144.48	23.47*
T	88.63	14.40*
a_w*T	6.57	1.07^{ns}
isolate*T	42.42	6.89*
a_w*isolate	4.49	0.73^{ns}
a_w*isolate*T	6.71	1.09^{ns}

* Significant (P<0.0001); ^{ns} Not significant.

Table 3. Mean ochratoxin A concentrations (μg g^{-1}) produced by eight isolates of *A. carbonarius* incubated at different a_w and temperatures on Synthetic Nutrient Medium (SNM) for 7 days.

Temperature		15 ºC	20 ºC	30 ºC	35 ºC	37 ºC
Isolate	a_w	(μg g^{-1})[a]	(μg g^{-1})	(μg g^{-1})	(μg g^{-1})	(μg g^{-1})
W9	0.90	N.G.	N.G.	< d.l.	< d.l.	< d.l.
	0.93	N.G.	< d.l.	< d.l.	< d.l.	< d.l.
	0.95	N.G.	0.19 ± 0.16	0.12 ± 0.21	< d.l.	< d.l.
	0.99	< d.l.	0.57 ± 0.50	0.18 ± 0.31	< d.l.	< d.l.
W37	0.90	N.G.	N.G.	< d.l.	< d.l.	< d.l.
	0.93	N.G.	0.22 ± 0.38	< d.l.	< d.l.	< d.l.
	0.95	N.G.	0.25 ± 0.43	< d.l.	< d.l.	< d.l.
	0.99	2.28 ± 3.95	4.95 ± 7.32	1.47 ± 1.78	0.47 ± 0.81	< d.l.
W38	0.90	N.G.	N.G.	< d.l.	< d.l.	< d.l.
	0.93	N.G.	< d.l.	< d.l.	< d.l.	< d.l.
	0.95	N.G.	6.09 ± 6.34	0.10 ± 0.19	< d.l.	< d.l.
	0.99	3.66 ± 4.17	9.13 ± 2.14	1.88 ± 0.95	2.03 ± 1.92	3.11 ± 1.81
W89	0.90	N.G.	N.G.	< d.l.	< d.l.	< d.l.
	0.93	N.G.	< d.l.	< d.l.	< d.l.	< d.l.
	0.95	N.G.	0.71 ± 0.76	< d.l.	< d.l.	< d.l.
	0.99	< d.l.	0.21 ± 0.13	0.77 ± 0.38	1.52 ± 2.64	1.09 ± 1.55
W104	0.90	N.G.	N.G.	< d.l.	< d.l.	< d.l.
	0.93	N.G.	< d.l.	< d.l.	< d.l.	< d.l.
	0.95	N.G.	< d.l.	0.03 ± 0.06	< d.l.	< d.l.
	0.99	< d.l.	0.91 ± 1.13	0.37 ± 0.37	< d.l.	0.96 ± 1.66
W120	0.90	N.G.	N.G.	< d.l.	< d.l.	< d.l.
	0.93	N.G.	14.00 ± 19.99	0.03 ± 0.03	< d.l.	< d.l.
	0.95	N.G.	20.49 ± 7.07	1.70 ± 0.08	< d.l.	< d.l.
	0.99	14.25 ± 4.25	10.60 ± 8.51	10.36 ± 6.42	1.75 ± 1.53	2.60 ± 0.94
W128	0.90	N.G.	N.G.	< d.l.	< d.l.	< d.l.
	0.93	N.G.	< d.l.	< d.l.	< d.l.	< d.l.
	0.95	N.G.	2.21 ± 1.95	< d.l.	< d.l.	< d.l.
	0.99	< d.l.	2.55 ± 0.73	1.84 ± 2.29	1.79 ± 3.10	1.17 ± 1.41

W198	0.90	N.G.	N.G.	< d.l.	< d.l.	< d.l.
	0.93	N.G.	< d.l.	< d.l.	< d.l.	< d.l.
	0.95	N.G.	0.08 ± 0.13	< d.l.	< d.l.	< d.l.
	0.99	< d.l.	< d.l.	1.69 ± 1.46	1.21 ± 1.78	< d.l.

[a]Mean value of three replicates; N.G. No growth; d.l. detection limit (0.02 μg OTA g^{-1} SNM).

DISCUSSION

The present study has focused on *A. carbonarius*, the main fungal species responsible for OTA accumulation in grapes, and puts the emphasis on the effect of ecological factors on the toxin production as a measure to predict further contamination in the field.

Aspergillus carbonarius growth was found to occur optimally at 30 ºC. This temperature was previously reported as the temperature that allows maximum growth rates for four *A. carbonarius* isolates (Bellí et al. 2004b). Similarly, they found that *A. carbonarius* grew faster at higher a_w (0.98-0.995), whereas no significant differences in growth rates were detected at 0.93 and 0.95 a_w. Other studies suggested that the optimum temperature for *A. carbonarius* growth was between 25-30 ºC (Leong et al. 2004) or between 25-35 ºC, depending on the isolate, but most of the cases growing faster at 35 ºC than at 25 ºC (Mitchell et al. 2003). Contrary to us, they found a different behaviour of the isolates tested in terms of a_w, with optimum varying from 0.93 to 0.987 a_w. However, in most of the cases, optimum was 0.98 a_w, similarly to our results. The greatest tolerance to low a_w in all the isolates studied was found at temperatures close to the optimum ones.

The range of temperatures allowing *A. carbonarius* growth proposed in other studies is similar to the one showed in this one (15-37 ºC). In general, growth at temperatures below 15 ºC is not common and it is only possible at the highest a_w levels (Mitchell et al. 2003; Bellí et al. 2004b). *A. carbonarius* is sensitive to higher temperatures compared with the *A. niger* aggregate (Bellí et al. 2004b), showing much reduced growth at 42 ºC (Leong et al. 2004).

Provided that a_w of grapes is 0.95-0.99 and mean temperature levels in the vineyards from June to September may vary between 20 and 26 ºC, and approximately 70 days with maximum temperature higher or equal to 30 ºC (INM, 2003), approximately 60 % of the time in the period preceding harvest (four months), would give suitable conditions for *A. carbonarius* growth and therefore for OTA production.

In this study, OTA was analysed after an incubation period of 7 days, as it is usually enough to allow OTA detection, although some *A. carbonarius* isolates required more days to reach maximum production in YES and CYA (Bragulat et al. 2001). Five days resulted in the maximum OTA accumulation for *A. carbonarius* growing on SNM,

dropping off after 10 days, but no information was given between these two incubation periods (Bellí et al. 2004c).

Optimum temperature for OTA production was observed at 20 ºC for most of the isolates. Mitchell et al. (2003) found that OTA production by *A. carbonarius* was greater at 25 ºC than at 20 ºC. Similarly to us, they reported that more toxin was produced at higher a_w levels.

High R.H. and temperature levels in the field during the months preceding harvest could also favour OTA production in grapes. Moreover, other factors such as the situation of the vineyards, meteorological extreme conditions like hailstone or the fluctuation of the environmental factors between day and night, could also influence positively or negatively in the final OTA content in wine grapes, although their effect is not known yet.

Testing isolates of different countries of origin may have explained the different adaptation of the isolates to different climatic conditions. However, no significant conclusions have been drawn about this aspect from the results, as all isolates behaved similarly in terms of growth and in OTA production at the a_w and temperature conditions tested, although the minimum amounts of OTA were detected in the Italian and Portuguese isolates.

Predicting the growth of *A. carbonarius* could be important to estimate the production of OTA by these species, as the conditions for OTA formation are more restricted than those for growth, as reported before for other mycotoxins (Marín et al. 1999).Therefore, if growth is restricted, OTA will not be present.

Finally, further investigations are required to determine the ability of *A. carbonarius* to produce OTA when growing on natural substrates as grapes, instead of on SNM, to observe if they behave similarly or the penetration of the grapes skin pose a hurdle to them in an approach to better understand growth conditions and OTA production by these fungi.

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Water relations of germination, growth and ochratoxin A production by *Aspergillus carbonarius* isolates from wine and table grapes from the northern Mediterranean basin

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ABSTRACT

This study has examined the effect of temperature, water availability, temperature x pH interactions on germination, germ tube extension, mycelial growth and ochratoxin production by isolates of *Aspergillus carbonarius* obtained from 6 different countries (Portugal, Spain, France, Italy, Greece and Israel). This has shown that germination is very rapid (< 24 h) at 0.90-0.99 a_w and 25-35^{o}C. This was also reflected in measurements of germ tube extension. Growth of two isolates from each country showed some differences in relation to steady state a_w levels. Only a few isolates could grow at 0.88 a_w, and none at 0.85 a_w. Surface response curves were developed to show the optimum and marginal conditions for growth of representative isolates of *A.carbonarius*. Growth was influenced by pH x a_w conditions, better at pH 4.5 and 7 than pH 2.8. In contrast to growth, OTA production was found to be optimum at 15-20^{o}C over the range 0.99-0.95 a_w. The pH effects on OTA was different from that obtained for growth with higher amounts of OTA produced at pH 2.8 and 7 than pH 4.5. This study suggests that microclimatic conditions need to be carefully monitored close to harvest as this could determine the OTA production during post-harvest processing.

KEY WORDS: germination, germ tube extension, growth, ochratoxin, *Aspergillus carbonarius.*

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INTRODUCTION

Aspergillus section *Nigri* is commonly found in soil where it is able to saprophytically colonise and effectively survive on crop debris producing large numbers of spores which can become air-borne. The spores of species in this group of spoilage fungi can subsequently contaminate ripening crops in Mediterranean, tropical and subtropical regions. In the mid-1990s it was discovered that within this group *A.carbonarius* was responsible for the production of the class 2b carcinogenic mycotoxin ochratoxin A (OTA; Abarca et al., 1994; Cabanes et al., 2002). Subsequently, *A.carbonarius* and OTA were detected in grapes, wine and other grape products (Zimmerli and Dick, 1996; MacDonald et al., 1999; Majerus et al., 2000; Bragulat et al., 2001; Battilani and Pietri, 2002; Stefanaki et al., 2003).

The presence of OTA as a contaminant of grapes, grape products and wine is dependent on environmental conditions at some stage being conducive to and facilitating germination, germ tube extension, establishment and mycelial colonisation to occur. The most important factors governing these components of the life cycle of micro-organisms are water availability, temperature and their interaction with the nutrient status of the food matrix. For processing of grapes, pH is also important. There have been some studies of the effect of water and temperature on germination and growth of isolates of the *A.niger* group, prior to knowledge of the existence of *A.carbonarius*. For example, Ayerst (1969) and Marin et al. (1998) examined *A.niger* group isolates from cereal grain. More recently, Parra et al. (2004) and Para and Magan (2004) studied growth and sporulation capacity of *A.niger* wild-type and genetically-modified strains and modelled the effect of interacting water activity (a_w) x temperature conditions on growth. However, only a few studies have examined the effect of these abiotic variables on growth and OTA production by the *A.niger* Section nigri group (Belli et al., 2004a; Belli et al., 2004b; Mitchell et al., 2003; 2004).

The objectives of this study were to examine the effect of water activity, temperature and pH on germination, germ tube extension, growth and OTA production by a range of *A.carbonarius* isolates from different parts of Europe to identify the similarity and differences in environmental conditions at which a low and high risk exists for spore germination, mycelial colonisation and most importantly, OTA contamination of grapes and grape products. A series of experiments were carried out in vitro on an artificial grape medium representative of mid-veraison.

MATERIALS AND METHODS

Fungal isolates

The range of isolates used in this study came from all over Europe and are detailed in Table 1. As these same isolates have been used in other studies reference numbers are also given for cross-referencing with other studies. The identities of these isolates were all confirmed by Dr. Z. Lawrence (CABI BioSciences, Egham, Surrey, U.K.).

Table 1. List of isolates examined from six different isolates of *Aspergillus carbonarius*, two from each country.

Isolate Number (Country code)	Origin, Code used
MPVA A 1102	Italy, Ita1
MPVP A 933	Italy, Ita2
01UAs219	Portugal, Port1
01UAs263	Portugal, Port2
W120	Spain, Esp1
W128	Spain, Esp2
SA 636	France, Fra1
Mu 141	France, Fra2
G 458	Greece, Gre1
G 444	Greece, Gre2
1-4-1-9-10.8	Israel, Isr1
1-4-1-9-7.7	Israel, Isr2

Media, water activity modification and inoculation

Studies were conducted *in vitro* on a synthetic grape juice medium (SGM) representative of grape composition at mid-veraison. This consisted of D(+) glucose 70g, D(-) fructose 30g, L(-) tartaric acid 7g, L(-) malic acid 10g, $(NH_4)_2HPO_4$ 0.67g KH_2PO_4 0.67g, $MgSO_4$ 7 H_2O 1.5g NaCl 0.15g $CaCl_2$ 0.15g $CuCl_2$ 0.0015g, $FeSO_4$ 7 H_2O 0.021g, $ZnSO_4$ 7 H_2O 0.0075g, (+) Catechin hydrate 0.05g, agar 25g in a litre of medium. This was adjusted with 2M KOH to pH 4.0 – 4.2. All chemicals were obtained from Sigma/Aldrich.

All experiments were carried out over the range of 0.99-0.85 a_w by the addition of either glucose or glycerol (Sigma). The unmodified medium was 0.987 a_w and this was used as

the control treatment. The temperatures used were 10, 15, 20, 25, 30, 35, 37 and 40°C. The a_w of all cooled treatment media were checked using a Aqualab® WP4 (Decagon Devices, Inc Washington USA) connected to a PC using Hyperterminal software (Higraeve Inc Michigan USA) and found to be within 0.005 a_w of the treatment values. pH was modified to 2.6, 4.0, 7.0 by the addition of HCl or KOH (Sigma) and final values were found to be within 0.1 of the target values.

Four types of measurement were made: (i) germination, (ii) germ tube extension, (iii) growth and (iv) OTA production. For germination studies a spore suspension (10^5 spores ml^{-1}) was made in water solutions containing 0.01% tween 80 (Merck) at each a_w treatment. A 0.2 ml aliquot was spread-plate over the surface of the SGM treatment Petri plates. At least three replicates of each treatment were incubated at 15, 20, 25 and 30°C. Agar discs (1.5 cm diameter) were aseptically removed periodically, two per replicate, and stained with trypan blue and covered with a coverslip and kept at 4°C until examination with a microscope. This enabled temporal data to be obtained on germination rates. The germ tube lengths of up to 25 single spores were measured randomly. The spores were considered to have germinated if the germ tube was equal to or longer than the spore diameter (Magan, 1988).

For growth studies all treatments and replicates were inoculated centrally with a small loop of spore suspension (5 µl) from a 10^5 spores ml^{-1} stock solution obtained from 10 day old SGM culture of the same a_w. All studies were carried out with a minimum of three replicates per treatment and repeated at least twice. Replicates of the same treatment were stored in controlled environment chambers of the same a_w/temperature to maintain treatment conditions for a maximum of 56 days.
Temporal mycelial extension rates were measured daily in two directions at right angles to each other until the Petri plates were fully colonised. The radial extension rates were plotted against time and the growth rates calculated using linear regression (mm day^{-1}) at each different a_w and temperature level, for each replicate and treatment. All experiments were carried out with at least three replicates per treatment and repeated.

Quantification of Ochratoxin A production

A series of replicates (at least three per experiment) were destructively sampled for OTA quantification for each treatment condition. Six agar plugs were removed from each replicate plate using a cork borer (4.5mm diameter) across the radius of each colony every 5 days for 20 days. Where necessary samples were frozen and subsequently analysed for OTA content. The method used was adapted from Bragulat *et al.* (2001). The plugs were placed into a 3 ml microtube (Eppendorf, UK) and weighed. 1ml of HPLC grade methanol (Fischer, UK) was added and the samples shaken and incubated at room temperature for 60 minutes. The extracts were filtered (Millex® HV 13mm, Millipore)

directly into amber HPLC vials (Jaytee Biosciences LTD, UK) and stored at 4°C until HPLC analysis was performed. This method gave an extraction efficiency of about 90%.

The HPLC system used consisted of a Millipore Water 600E system controller, a Millipore 712 WISP autosampler and a Millipore Waters 470 scanning fluorescence detector (Millipore Corporation Massachusetts USA)(excitation 330 nm, emission 460 nm). The samples were separated using a C18 Luna Spherisorb ODS2 column (150 x 4.6mm, 5µm) (Phenomenex), with a guard column of the same material. Run time for samples was 12 minutes with OTA detection after 5.75 minutes. The flow rate of the mobile phase (acetonitrile:water:acetic acid; 57:41:2) was 1 ml min^{-1}. The detection limit was <0.01 µg OTA g^{-1} SGM, based on a signal to noise ratio of 3:1. Analysis of the results was carried out on a computer with Kroma systems 2000 software (Bio-tek Instruments, Milan, Italy). In this paper data on OTA production after 10 days is presented although information on other times was also gathered.

Statistical analyses of the data

The data was statistically analysed in two ways. The effect of a_w x temperature was examined with ANOVA using Statistica (Statsoft, Tulsa, U.S.A.). Differences in growth rates for isolates from each European region were also compared in relation to the medium modification with glucose/glycerol, and differences between strains. Using a polynomial multiple linear regression (MRL) the surface response curves were obtained using the above programme.

RESULTS

Effect of water and temperature on germination and germ tube extension

Temperature, aw and time affected both germination and germ tube extension of conidia. Generally germination and initial establishment was very rapid at 25-30°C, especially under freely available water conditions. However, at 15°C germination was significantly slower with about 50% after 36 hrs. Furthermore, germ tube extension was just over 100 µm after 72 hrs, while at all other temperatures tested the germ tubes had reached about 300 µm in 24 hrs.

Germination was rapid at between 0.99-0.90 a_w with almost 100% of spores successfully germinating within 24 hrs. At 0.88 a_w, there was a lag of about 18 hrs before germination occurred, with almost all spores germinating after about 60 hrs. The germ tube extension reflected this. Only in the drier conditions (e.g. 0.88 a_w) were germ tube lengths < 30 µm

after about 60 hrs. At 0.85 a_w there was no germination in the time frame of these experiments. Results were similar for isolates from both Italy and Portugal.

Effect of water, temperature and pH interactions on growth of isolates from different parts of Europe

Optimum radial extension (approx. 10 mm day^{-1}) was at 30-35°C at 0.99-0.93 a_w. Figure 1 compares the growth rate of two isolates each from 6 different countries at different steady state a_w levels at 25°C. This shows that there are some intraspecific differences between isolates from a single country, and between isolates from different countries. Under drier conditions (0.88 a_w) only a few isolates grew, with more than 50% unable to grow over the 56 day incubation period.

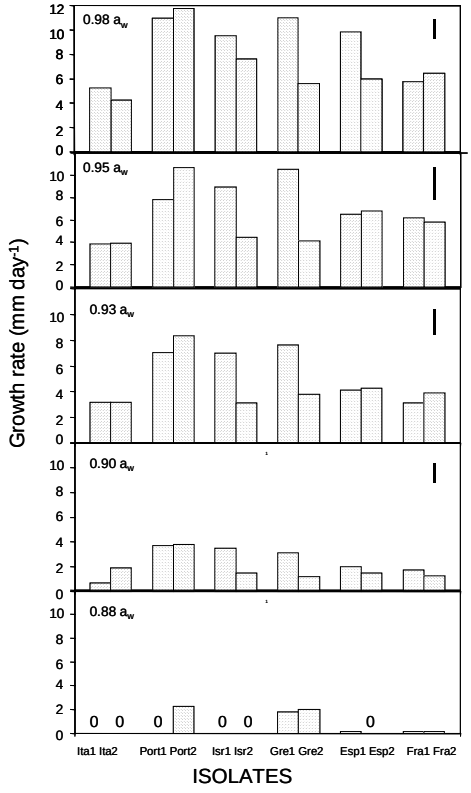

Figure 1. Comparison of growth of two isolates each of *A.carbonarius* from 6 different countries at different steady state water activity conditions at 25°C. Bars indicate LSD (P=0.05). See Table 1 for key to fungi.

Data for individual isolates was subjected to statistical analysis to produce two dimensional profiles of temperature x a_w for growth. Figure 2 shows an example of this for two isolates. This confirms the optimum conditions of a_w and temperature for growth are in the range 25-35^oC, at 0.98-0.92 a_w.

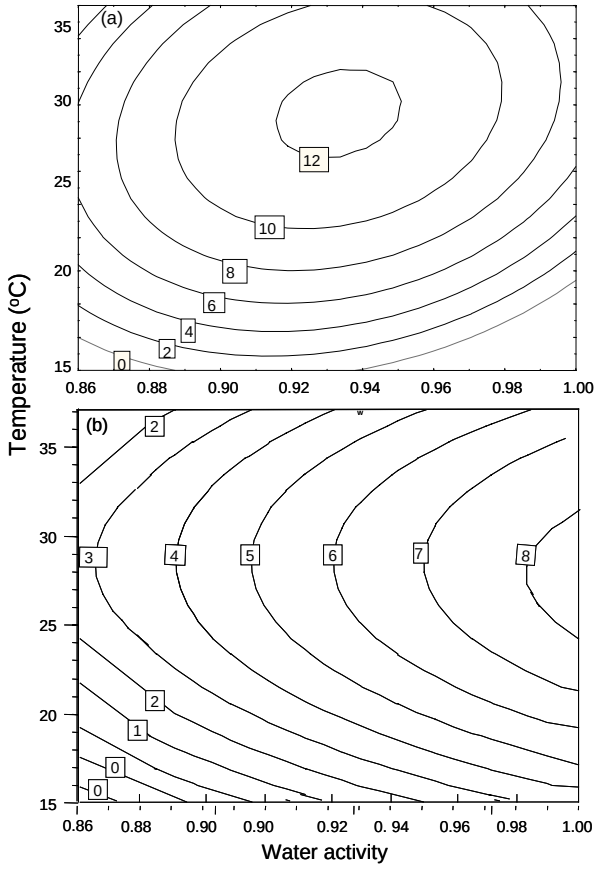

Figure 2. Surface response curves for two isolates from (a) Portugal; Port1 and (b) Greece; Gre1 of *Aspergillus carbonarius*. Numbers on the isopleths represent growth rates (mm day^{-1}).

The effect of three pH levels x a_w on growth of an isolate of *A.carbonarius* (isolate Ita1) at different temperatures showed that it was better at pH 4 than 7 or 2.6, regardless of the a_w level used. Growth was inhibited at pH 2.6 and 7.0 at 0.90 a_w, while at pH 4 growth was inhibited at 0.88 a_w.

Effect of environmental factors on ochratoxin production

Figure 3 shows the effect of temperature on OTA production at different steady-state a_w levels by a single isolate and also compares OTA production by three other isolates in relation to temperature at one steady state a_w level. The amounts of OTA produced by different isolates varied markedly. Some produced < 1 µg g^{-1} medium, while others produced up to 10 µg g^{-1} medium. Overall, this showed that the optimum temperature for OTA production is in the range 15-20°C with a marked decrease at > 25°C. The data at different temperatures and a_w levels were used to produce surface response profiles of conditions for OTA production. This shows the optimum, marginal and maximum conditions for OTA production by two strains of *A.carbonarius* from different countries (Figure 4). This confirms that 0.95-0.98 a_w and 15-20°C were optimum for OTA production.

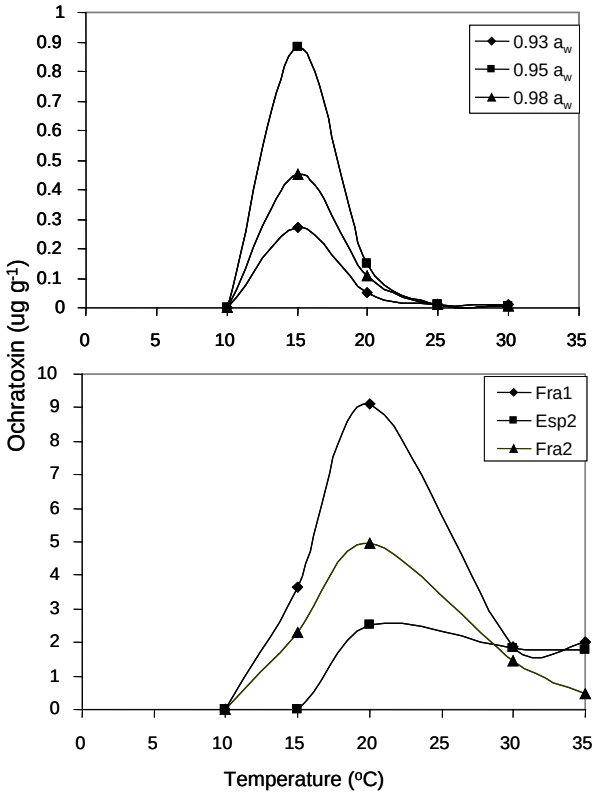

Figure 3. Effect of (a) temperature and a_w on OTA production by an isolate of *A.carbonarius* (Port1) and (b) comparison of three isolates (Esp1, Fra1, Fra2) in relation to temperature at a steady-state water activity of 0.98.

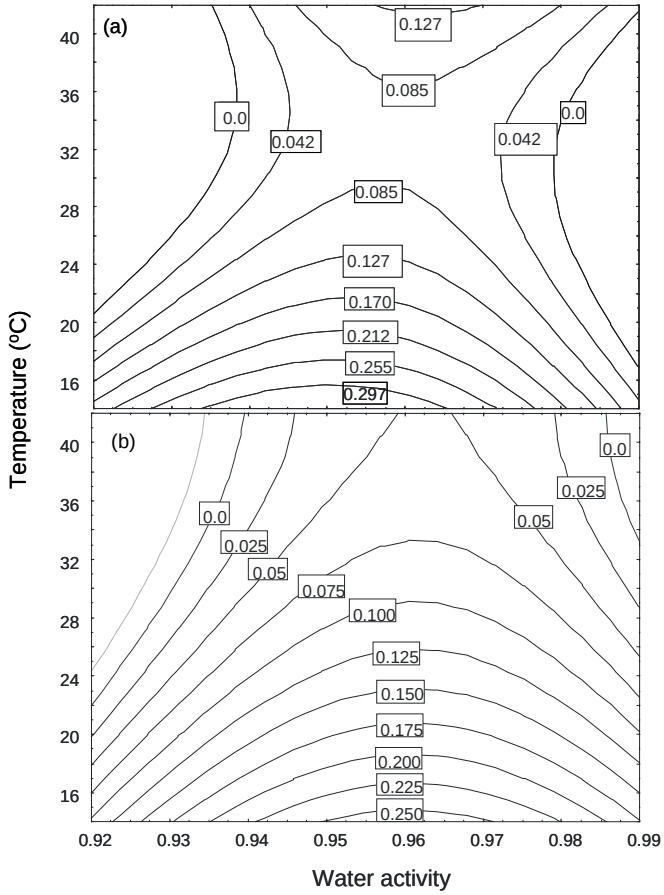

Figure 4. Surface response curves for two isolates of *A.carbonarius* from (a) Greece; Gre1 and (b) Spain; Esp1 in relation to temperature x water activity interactions. The numbers on the isopleths represent OTA production levels (µg g^{-1} medium).

The effect of three different pH levels over a range of a_w levels on OTA production is shown in Figure 5. This shows that more OTA was produced at pH 7, followed by pH 2.6, with the lowest amounts produced at pH 4 in this study at 25°C. This is in contrast to growth where very slow growth occurred at pH 2.8.

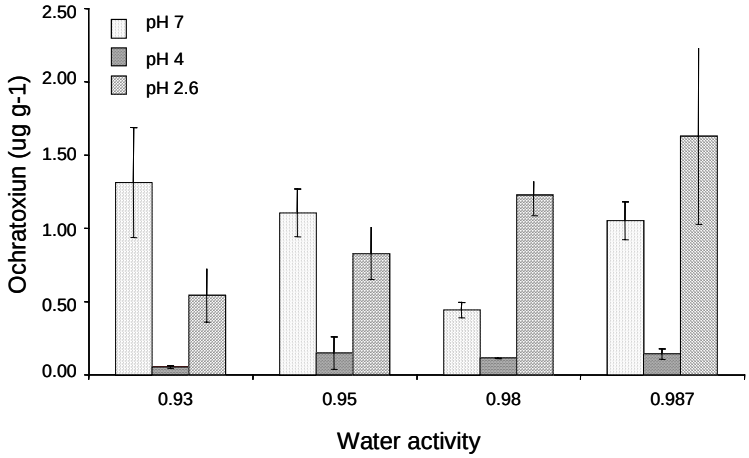

Figure 5. Effect of three pH levels on OTA production by an isolate of *A.carbonarius* (Ita1) grown on a artificial grape medium for 10 days. Bars indicate standard errors of the mean.

DISCUSSION

This study has examined, for the first time, the detailed effect of temperature x a_w conditions on germination and germ tube extension of spores of *A.carbonarius*. This is critical in defining the capacity of contaminant spores to become established on grapes and subsequently grow and produce OTA. This suggests that at >25^{o}C germination of spores is rapid and achieved in < 24 hrs. This occurs over a wide a_w range of 0.90-0.99 in the same time period. Germ tube extension of the germinated spores reflected this. This indicates that conducive conditions for germination cover a wide temperature and a_w range. This could occur easily overnight in the canopy of a vineyard should spores be present on the surface of ripening grapes. Studies of germination of *A.niger* group species prior to the taxonomic knowledge of different species in the section Nigri and their ability to produce OTA have been carried out, although they may not be strictly comparable. Ayerst (1969) and Marin et al. (1998) showed that isolates from grain germinated at 0.82-0.83 a_w at optimum temperatures with mycelial growth occurring over the range 0.83-0.995 with optimum conditions at 0.995/35^{o}C. Temperature ranges were 10-40^{o}C at optimum a_w conditions. Recent studies by Belli et al. (2004b) and Mitchell et al. (2003) showed that there are marked differences between growth of *A.carbonarius* isolates from different European countries and those of other species within the section Nigri. Interestingly, growth of the OTA producer, *Aspergillus ochraceus*, is optimal at 0.995 and 30^{o}C with a similar a_w limits of 0.83-0.85 and a temperature range of 10-35^{o}C (Ayerst, 1969; Marin et al., 1998). The other important OTA producer is *Penicillium*

verrucosum, which is common in temperate climatic conditions and grows over the range 5-35^{o}C (optimally at 25^{o}C), and the a_w range of 0.995 – 0.81 (optimum of 0.95). OTA production occurs over a narrower range than this with minimum a_w of 0.82-0.83 and optimum at 0.91 and about 16-17^{o}C (Sanchis and Magan, 2004; Cairns-Fuller et al., 2005). However, this mycotoxigenic species is not found on grapes. This demonstrates that the ecological ranges are quite different for these OTA producing species.

The effect of different pH levels on growth is important as the grape flesh and grape juice are all very acidic (< pH 4.5). It is worth noting that growth was better at pH 4 and 7 than at pH 2.6, the lowest treatment used, regardless of a_w level. This shows that very good establishment can occur in the region of pH 4, commonly achieved in grape-based products. Although we found that higher concentrations of OTA were produced at pH 7 than 4.5, the neutral pH is not a condition which normally exists in grape juice, wine or grape products. The pH of vine fruits is about 5-5.5. The higher production of OTA at pH 2.8 and 7 may reflect marginal environmental conditions for growth and this stress could explain the higher production at these pH levels than at 4.5.

This study confirms that optimum environmental conditions for OTA production by *A.carbonarius* isolates are very different from the optimum for germination and growth. Thus optimum OTA production was at 15-20^{o}C depending on isolate, with optimum a_w conditions of 0.95-0.98. The data presented in this study are for cultures analysed after 10 days growth. Previous temporal studies have suggested that optimum OTA is produced after 5 days (Belli et al., 2004a), after 5-10 days (Mitchell et al., 2003) and after 14 days (Cabanes et al., 2002). The amount of OTA produced varied markedly between isolates from different countries. For example, isolates from Spain produced 4-5 μg g^{-1}, while others produced < 1 μg g^{-1} medium (Italy, Portugal) at optimum a_w conditions.

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Skin damage, high temperature and relative humidity as detrimental factors for *Aspergillus carbonarius* infection and ochratoxin A production in grapes.

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ABSTRACT

This study investigated the impact of skin damage on visible *Aspergillus carbonarius* colonization and ochratoxin A (OTA) production in grapes at different temperatures and relative humidity. Four ochratoxigenic *A. carbonarius* strains isolated from wine grapes from four different European countries were used. Artificially damaged and undamaged table grapes were surface-disinfected and inoculated. Grapes were stored at three levels of relative humidity (80, 90 and 100 %) and at two levels of temperature (20 and 30 ºC). After seven days, the infection percentage of *A. carbonarius* was recorded and OTA accumulation in berries was analysed. Damaged grapes were more commonly infected and colonies were more developed than in undamaged ones; consequently more OTA was detected in the first ones. Temperature and relative humidity had significant influence on both infection and toxin content. The amount of OTA detected at 30 ºC was higher than at 20 ºC in most of the treatments. The highest relative humidity (100 %) led to maximum amounts of OTA while no significant differences were found between 90 % and 80 % in the OTA content. Any opening in the grape skin facilitate the penetration and development of the mould. Infection and therefore OTA production in grapes must be specially marked in hot and humid years. The results confirmed the effect of temperature and R.H. and indicate that damage of the berries contributes to infection and OTA production. The implementation of preventive measures in order to minimise berries damages in the field by controlling pathogenic fungi and insects during grape growing and removing visibly damaged grapes at harvest may significantly reduce OTA contamination in grapes.

KEY WORDS: ochratoxin A, *Aspergillus carbonarius*, grapes, skin damage, temperature, relative humidity.

Food Control **(submitted)**

INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin considered to be a possible carcinogen for humans (IARC, 1993). It has been shown to be nephrotoxic, carcinogenic, teratogenic and immunosuppressive in laboratory animals (Boorman, 1989; Dirheimer, 1998). It has been commonly found in cereals but it can also contaminate a variety of other plant and animal products. Grapes and wine contain important amounts of OTA, in particular, wine consumption could represent the 15 % of the total intake of this toxin (Codex Alimentarius Commission, 1998). Therefore, studies on these products aiming to reduce the consumer exposure to the toxin as much as possible are crucial.

OTA was originally isolated in 1965 as a metabolite from a strain of *Aspergillus ochraceus* (Van der Merwe et al., 1965). Recently, OTA production has been reported from *Aspergillus* species belonging to the *Nigri* section, which are frequently isolated from grapes (Sage et al., 2002; Battilani et al., 2003; Serra et al., 2003; Bellí et al. 2004a, 2004b; Tjamos et al., 2004). In this section, the reported OTA-producing species are *A. carbonarius* and those now included in the so-called *A. niger* aggregate (Abarca et al., 2001). *A. carbonarius* is less common than *A. niger*, but is the main species responsible for OTA in grapes because almost all strains are high OTA producers (Cabañes et al., 2002; Battilanni et al., 2003; Bellí et al., 2004b).

A preliminary study reported the ability of strains of *A. carbonarius* to colonise and penetrate intact and artificially damaged berries, finding OTA in the pulp, although berry skin was considered the major source of OTA in grapes (Battilani et al., 2001). At the present time, the effect of temperature and water availability on *A. carbonarius* growth and OTA production it has only been reported on synthetic medium (Bellí et al., 2005; Mitchell et al., 2004). These studies highlighted the need to know more about the behaviour of these species on natural substrates. In the present work, table grapes were used as the nutrient source for *A. carbonarius*. The aim was to study the influence of skin damage on visible fungal growth and on OTA accumulation in berries at different temperatures and relative humidities (R.H.)

MATERIALS AND METHODS

Fungal isolates

Four *A. carbonarius* strains isolated from naturally infected grape berries from Italy (3.161), France (3.162), Portugal (3.166) and Spain (3.168) were used in this study. All the isolates were previously found to be OTA producers and were supplied by the Faculty of Agriculture, Università Cattolica del Sacro Cuore, Piacenza, Italy; the Institut National Polytechnique de Toulouse, École Nationale Supérieure Agronomique de Toulouse, France; the Departamento Engenharia Biologica, Universidade do Minho, Braga,

Portugal; the Departament de Sanitat i d'Anatomia Animals, Facultat de Veterinària, Univ. Autònoma de Barcelona, Spain. They are held in the culture collection of the Departament de Tecnologia d'Aliments, Escola Tècnica Superior d'Enginyeria Agrària, Universitat de Lleida, Spain.

Grape decontamination

Grape berries were separated from the bunches by cutting the stem with the aid of a scissors at approximately 0.5 cm from each grape. Red table grapes (var. Red Globe) not physically damaged were used in this study. They were surface-sterilised by dipping them into a NaClO solution (0.1% Cl) for 1 min, followed by 1 more min in ethanol (70 %). Excess water was removed by placing berries on a laminar flow bench for two minutes until fungal inoculation.

Inoculation

Spore suspensions of each isolate (10^3 spores ml^{-1}) were prepared from colonies, previously grown on Synthetic Nutrient Medium (SNM) (Bellí et al., 2004d) for seven days at 25 ºC, in distilled water containing Tween (0.005 %). Berries were dipped into 100 ml of the spore suspensions for one minute. Control treatments were done in the same way, but a solution of sterile distilled water was used in the inoculation step. Half of the berries were wounded before inoculation by puncturing them 3 mm deep approx. with a sterile needle and later referred as *damaged* berries to differentiate them from the *undamaged* ones.

Incubation

For each treatment, twenty berries were placed on the top of a grate previously disinfected with ethanol (96 %), preventing any contact among them. Grates were placed into disinfected plastic boxes containing 300 ml of a glycerol-water solution to assure the R.H. of the treatment (101, 50 and 0 g glycerol/100 ml distilled water to produce 80, 90 and 100 % R.H., respectively). Filled containers were hermetically closed and incubated at different combinations of temperatures (20 and 30 ºC) and R.H. (80, 90 and 100%) for 7 days. Each treatment was performed in triplicate.

Experimental design

A full factorial design with four isolates and a control, two temperatures (20 and 30 ºC), three R.H. (80, 90 and 100%) and two berry states (damaged and undamaged) was carried out. All treatments (n= 60) were made in triplicate.

Percentage of infection

At the end of incubation, berries with visible growth were counted and were classified depending on the percentage of their surface colonised by *A. carbonarius* in order to estimate for each treatment the percentage of infection and the Infection Index (I.I.), respectively. This index ranged from 0 (healthy berries) to 1 (the surface of the twenty berries completely colonised) and was calculated as follows:

$$I.I. = \frac{(a \cdot 0.25) + (b \cdot 0.5) + (c \cdot 0.75) + (d \cdot 1)}{20}$$

a: number of berries with less than one quarter of their surface colonised with *A. carbonarius.*
b: number of berries with half of their surface colonised with *A. carbonarius.*
c: number of berries with three quarters of their surface colonised with *A. carbonarius.*
d: number of berries completely colonised with *A. carbonarius.*

OTA extraction and HPLC quantification

After 7 days of incubation, grapes in each container were analysed for OTA (Bezzo et al., 2000). The twenty berries were weighed and crushed with a hand blender machine (Opticlick Pro, Moulinex, France). The liquid extracted was centrifuged (3830 g, 15 min) and filtered (Whatman, num. 4) under vacuum. The pH of 75 ml of each sample was adjusted to 7.4 with NaOH (4M). Samples were passed through immunoaffinity columns (Ochraprep, Rhône Diagnostics Technologies, Glasgow, UK) at a flow rate of 2-3 ml min^{-1}. Afterwards, the columns were washed with 20 ml of distilled water at a flow rate of 5 ml min^{-1} and finally dried up in an air stream. Desorption was carried out with 1.5 ml of methanol/acetic acid (98/2) solution, slowly passed through the column. The eluate was then evaporated to dryness at 40 ºC under a stream of nitrogen and redissolved in 2 ml of mobile phase (Acetonitril 48 % - Sodium acetate 4mM/Acetic acid (19/1) 52 %). All solvents were of HPLC grade. A 25 µl aliquot of each final sample was injected into the HPLC system equipped with a fluorescence detector (Waters 474) (λ_{exc} 230 nm; λ_{em} 458 nm) and a C_{18} column (Waters Spherisorb 5 µm, ODS2, 4.6x250 mm, Milford, Massachusetts, U.S.A.). The analysis was performed under isocratic conditions at a flow rate of 1ml min^{-1}. The detection limit of the analysis was 0.05 µg l^{-1}, based on a signal/noise ratio of 3:1. OTA was quantified by the external standard method. OTA used as a standard was supplied by Sigma-Aldrich, Steinheim, Germany, acetonitril by Merck, Darmstadt, Germany and sodium acetate and acetic acid by Prolabo, Briare, France. The retention time of OTA under the conditions described was approximately 12 minutes.

Statistical treatment of the results

Analysis of variance of the effect of skin damage, temperature and R.H. on the percentage of *A. carbonarius* infection and OTA accumulation in berries was made with SAS software (SAS Institute, version 8.2, Inc., Cary, N.C., U.S.A.). Differences between levels of factors were determined by applying Student-Newman-Keuls multiple-range test. Statistical significance was judged at $p<0.05$ and $p<0.001$.

RESULTS

Percentage of infection and infection index

Analysis of variance revealed that temperature, R.H., damage and the interactions damage*R.H. and temperature*R.H. had a significant effect on the percentage of grapes with visible infection of *A. carbonarius* (Table 1). Interestingly, no differences were found among the four isolates, neither in their responses to the factors assayed.

Table 1. Analysis of variance of the effect of temperature, R.H., damage and isolate on the percentage of grape infection by *A. carbonarius*.

Factors	DF	Mean Square	F-value
T	1	68034.03	176.92 **
R.H.	2	18508.51	48.13 **
Damage	1	12844.44	33.40 **
Isolate	3	237.96	0.62 ns
R.H.*Damage	2	1277.26	3.32 *
Damage*Isolate	3	145.37	0.38 ns
T*Damage	1	1167.36	3.04 ns
R.H.*Isolate	6	163.14	0.42 ns
T*R.H	2	2137.67	5.56 * *
T*Isolate	3	285.88	0.74 ns
R.H.*Damage*Isolate	6	171.24	0.45 ns
T*R.H*Damage	2	231.42	0.60 ns
T*Damage*Isolate	3	28.47	0.07 ns
T*R.H.*Isolate	6	258.97	0.67 ns
T*R.H.*Damage*Isolate	6	91.14	0.24 ns

** significant ($p<0.001$); * significant ($p<0.05$); ns not significant

The percentage of infection by *A. carbonarius* in damaged berries was significantly higher than in berries not damaged initially (Figure 1). Less than 60 % of undamaged berries were symptomatic at 20 ºC irrespective of the R.H. level, whereas for damaged ones, this only happened at 20 ºC and at the lowest R.H. assayed. High R.H. contributed to increase the percentage of infection. More contaminated berries were detected at 30 ºC than at 20 ºC, with all the berries infected by *A. carbonarius* at the highest temperature and R.H. At 20 ºC, the contamination of berries incubated at 100 % R.H. was significantly higher than that at 90 % and significantly higher than at 80 %. However, no significant differences were found between 90 and 100 % R.H. in grapes incubated at 30 ºC, as almost all berries showed visible *A. carbonarius* contamination on their surface.

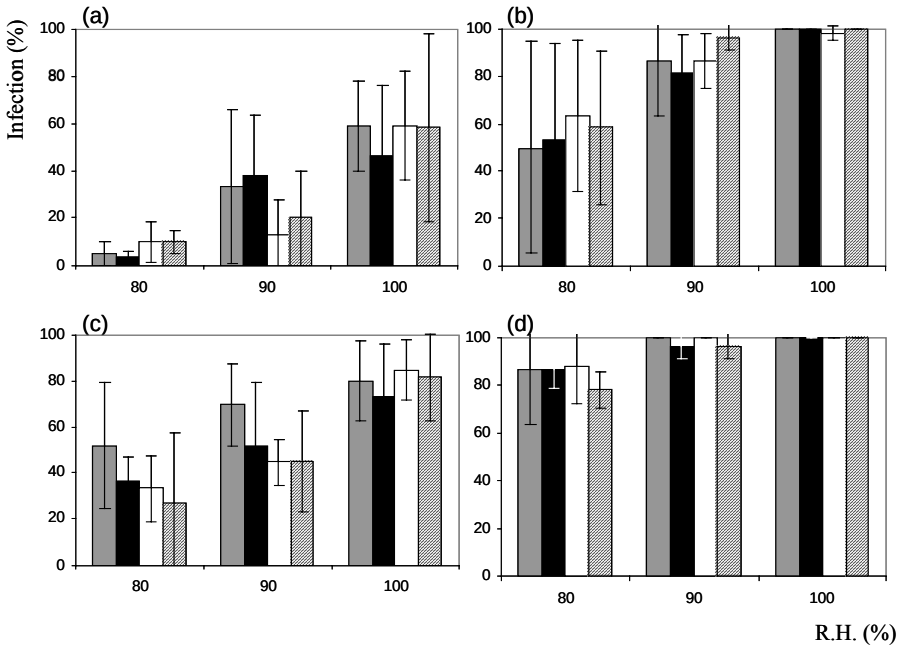

Figure 1. Percentage of damaged (c,d) and undamaged (a,b) grapes with visible growth of *A. carbonarius* (3.161, 3.162, 3.166, 3.168) after seven days of incubation at different temperatures [20 ºC (a, c); 30 ºC (b,d)] and relative humidity. Values are the mean of the three repetitions.

Fungal growth was observed nearby the puncture produced in the skin and also around the stalk of each berry. No growth was observed in control treatments. Higher infection index were obtained at 30 ºC than at 20 ºC (Figure 2). Infection index of the treatments at 20 ºC ranged from 0 to 0.4, half of the treatments between 0 and 0.1. In contrast, at 30 ºC infection index ranged from 0.2 to 1, half of the treatments presenting values higher than 0.6. In general, the highest colony development was observed in damaged grapes. Grape

colonization increased with higher values of R.H., and no differences were observed among the four isolates tested.

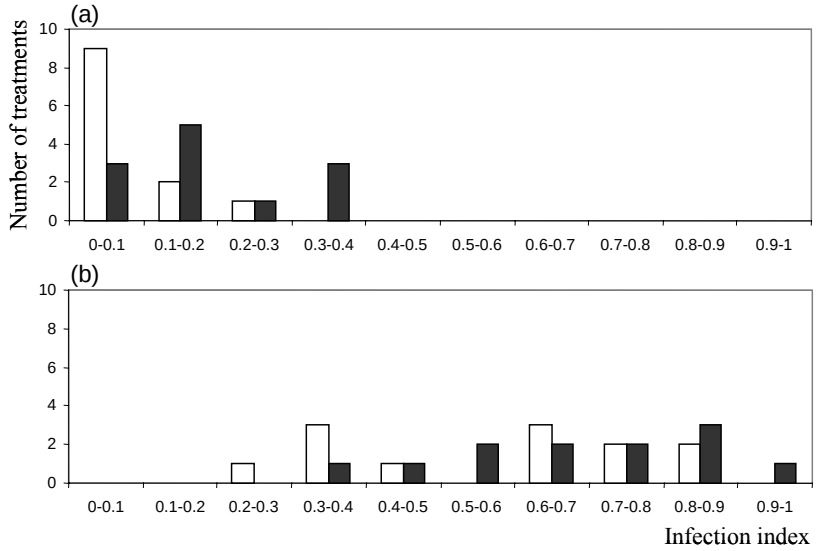

Figure 2. Infection index of each treatment at 20 ºC (a) and 30 ºC (b) for undamaged ☐ and damaged ■ grapes.

OTA accumulation

Analysis of variance revealed that all single factors (damage, temperature, R.H. and isolate) had a significant effect on the OTA levels found in grapes. Moreover, the interactions temperature*isolate and temperature*damage were also statistically significant (Table 2).

The highest amount of OTA was found in grapes inoculated with the isolate 3.168, finding up to 15 μg OTA l^{-1} must, meanwhile 3.161 and 3.166 isolates were the lowest OTA producers (Figure 3). OTA content found in damaged grapes was higher than in undamaged ones, but the difference was statistically significant only at 20 ºC. In general, grapes incubated at 30 ºC presented higher amounts of OTA than those incubated at 20 ºC. However, the mean OTA content detected at 20 ºC for damaged grapes inoculated with isolates 3.161, 3.162 and 3.168 and incubated at 90 %, 80 % and 100 % R.H, respectively, was higher than at 30 ºC. The highest R.H. (100 %) led to maximum amounts of OTA. No significant differences in OTA accumulation were detected between 90 % and 80 % R.H. Control treatments were free of OTA.

Table 2. Analysis of variance of the effect of temperature, R.H., damage and isolate on the OTA accumulation in berries.

Factors	DF	Mean Square	F-value
T	1	109.39	13.52 **
R.H.	2	70.36	8.70 **
Damage	1	54.53	6.74 *
Isolate	4	373.10	46.11 **
R.H.*Damage	2	2.43	0.30 ^{ns}
Damage*Isolate	4	14.08	1.74 ^{ns}
T*Damage	1	1.45	0.18 *
R.H.*Isolate	8	9.64	1.19 ^{ns}
T*R.H	2	4.02	0.50 ^{ns}
T*Isolate	4	14.08	1.74 **
R.H.*Damage*Isolate	8	0.51	0.06 ^{ns}
T*R.H*Damage	2	1.36	0.17 ^{ns}
T*Damage*Isolate	4	0.85	0.11 ^{ns}
T*R.H.*Isolate	8	20.64	2.55 ^{ns}
T*R.H.*Damage*Isolate	7	3.45	0.43 ^{ns}

** significant (p<0.001); * significant (p<0.05); ^{ns} not significant

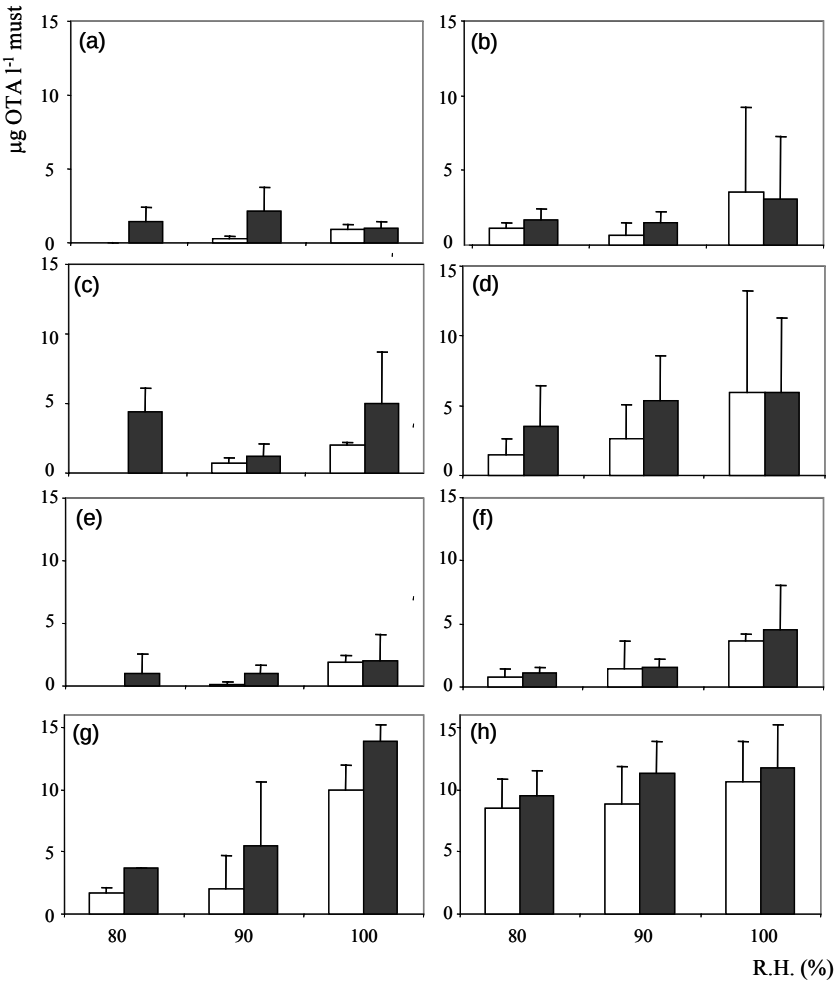

Figure 3. Ochratoxin A accumulation (µg OTA l^{-1} must) in damaged ■ and undamaged □ grapes inoculated with four isolates of *A. carbonarius* [3.161 (a, b); 3.162 (c, d); 3.166 (e, f); 3.168 (g, h)] and incubated at different temperatures [20 ºC (a, c, e, g); 30 ºC (b, d, f, h)] and relative humidity (80, 90 and 100 %) for seven days. Bars indicate standard deviation of the three repetitions.

DISCUSSION

Much of the knowledge concerning fungal development and mycotoxin production is based on work with synthetic or semisynthetic media simulating the composition of the food object of the study. There are some inconveniences when working with solid

substrates, as the difficulties in measuring fungal growth or food perishability. However, in this study the mould was inoculated directly onto the surface of grape berries, and their development could be monitored after one week. No significant differences were observed either in the percentage of infection or in the infection index among the isolates tested in this study. Most of the colonies started their growth in the puncture produced intentionally in the berry skin before the inoculation step. Moreover, berry stalk was observed to be the main route for microorganisms to entry into the berries. It is known that in most of the fruits, fungal hyphae often use natural openings to attack and to overcome their substrate (Schmidt, 1991). It is important to note that grapes used in the present study had lost the protection of the living plant system, such as the production of phytoalexins, when they were detached from the living vine.

However, significant differences in the percentage of visible infection were found between damaged and undamaged grapes, differing approximately 20 %. This is supported by Battilani et al. (2001) who found that *A. carbonarius* is a very invasive fungus, able to colonise and penetrate berries even without skin damage, as they showed differences of more than 80 % in the percentage of intact and damaged wine berries colonised both internally and superficially by *A. carbonarius* and incubated at 25 ºC for 7 days. Different grape varieties could also affect fungal invasion, as skin hardness and thickness may become a hurdle for the penetration of the mould.

The surface of berries in the field can suffer mechanical damage caused by the wheels of tractors or other machines used in field work, chemical damage when spraying the vines and splitting during rainy weather. Biological agents such as the mycoflora present on the berries and insects frequently infesting vines could also produce small injures in the berries and favour further colonization by *A. carbonarius*. Therefore, controlling the growth of this mould in grapes relies on controlling all these factors. Colonization of the berries was greater at 30 ºC than at 20 ºC and at the highest R.H. This agrees with previous results of the optimum temperature and water activity (a_w) for *A. carbonarius* growth, which has been reported between 25-35 ºC and 0.95-0.99 a_w, respectively (Mitchell et al., 2004; Bellí et al., 2005, 2004d; Leong et al., 2004).

Sometime fungal invasion occurs with no visible symptoms although when the colonization proceeds, more or less distinct alterations begin to develop. The knowledge obtained from several studies is that secondary metabolites often start to be produced after a decrease in growth rate (Häggblom, 1982). It has been supported that at 25 ºC, the highest amounts of OTA produced by *A. carbonarius*, 5 μg g^{-1} SNM, were detected between 5 and 10 days depending on the water activity of the medium (Bellí et al., 2004c). Therefore, in this study, OTA production was analysed after one week of incubation, finding a range of concentration between 0.06 and 15.60 μg l^{-1} of must, that equals to 23.8 and 6162 ng kg^{-1} of grapes. Highest contents were found when incubating grapes at 25 ºC, with maximum amounts of 25655 ng kg^{-1} detected in damaged berries, where production was 190 times greater than in intact berries (Battilani et al., 2001).

Lower although significant differences in OTA content from damaged and undamaged grapes were detected in the present study. Significant differences in OTA production among the same isolates were found in previous studies of *A. carbonarius* on synthetic nutrient medium (SNM) (Bellí et al., 2005). Isolates 3.168 and 3.162, previously coded as W120 and W38 respectively, produced the highest levels of OTA, up to 20.5 μg g-1 SNM and 9.13 μg g-1 SNM at 20 ºC, respectively. Similarly to this study, isolates 3.161 (before, W9) and 3.166 (before, W89) were the lowest OTA producers (<2 5 μg g-1 SNM) and also no significant differences among the isolates tested were observed on mycelial growth on SNM plates.

More OTA was found in undamaged grapes at 30 ºC than at 20 ºC, contrary to previous works on synthetic medium, where 15-20 ºC were reported as the optimum temperatures for *A. carbonarius* OTA production (Mitchell et al., 2003; Bellí et al., 2005). Berry skin hinders the nutrients for the mould and therefore its development and OTA production slow down when temperature is not the optimum for growth. However, when nutrients are available because of a breach in the skin, 20 ºC becomes the optimum temperature for OTA production and this could be the reason why in some cases the OTA level detected in damaged berries at 20 ºC was higher or similar to 30 ºC.

The available evidence indicates that any opening in the skin could facilitate *A. carbonarius* penetration and development in the berry. Although this fact seemed logical, it was necessary to confirm it and also to have an idea of the magnitude in OTA production increase, knowing that the responses from detached berries may be different from those attached to the vine or even in intact bunches because of the protection the plant confers. The way *A. carbonarius* penetrates the fruit in undamaged grapes when not using the portal of entry that the stem provides, is still unknown. Further microscopic studies could be of a great importance for a better understanding of colonization of grapes on the vine by this mould. Furthermore, more investigations on preventive treatments in the field, such as the application of fungicides or insecticides that minimise the damage that moulds and insects cause on the berries, are required to reduce *A. carbonarius* contamination of grapes and consequently reduce OTA contamination.

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8.4.2.2. Kinetics of OTA production

- **Incubation time and a_w effects on OTA production by *Aspergillus* section *Nigri* strains isolated from grapes**

The objective of this study was to determine the temporal OTA accumulation profile of *Aspergillus* section *Nigri* at different a_w levels. Two *A. carbonarius* and two *A. niger* aggregate strains isolated from grapes were tested in vitro for OTA accumulation at 25 ºC on SNM, over periods of 20 days at different a_w levels (0.90, 0.93, 0.95, 0.98, 0.995). Results were modelled by a multiple linear regression and response surface predictive models were obtained. High levels of a_w favoured OTA production by these moulds. Maximum amounts of OTA were found at the earlier growth states (5 days for *A. carbonarius* and 7-13 days for *A. niger* aggregate). Provided that black aspergilli, and mainly *A. carbonarius*, play the main role in OTA presence in grapes, it would be critical to adjust the harvest and processing time to significantly reduce the chances for OTA accumulation. Results are shown in the following paper:

Incubation time and water activity effects on OTA production by *Aspergillus* section *Nigri* strains isolated from grapes. *Letters in Applied Microbiology* 38, 72-77 (2004).

- **Kinetics of OTA production and accumulation by *A. carbonarius* on SNM at different temperature levels**

The effects of incubation time (up to 10 days) and temperature (7, 15, 20, 25, 30, 35 and 42 ºC) on OTA-producing capacity and OTA accumulation by four *A. carbonarius* strains isolated from Spanish and Tunisian grapes, were studied on grape-like medium. The limits of growth and OTA production have been identified. OTA production was significantly higher at 20 ºC, followed by 25, 15, 30, 35 ºC. No growth was observed at 7 ºC and 42 ºC after 10 days of incubation and consequently, no OTA was detected at these temperature levels. In general, maximum OTA-producing capacity was found earlier with increasing incubation temperatures. However, at 35 ºC, OTA was rarely detected although growth was maximum at this temperature. OTA accumulation was maximum after 10 days of incubation for all the temperatures except at 30 ºC, were the maximum was detected at earlier incubation time (6-8 days) and then remained stable. Colony diameters and OTA accumulation along time were modelled by Gompertz sigmoidal model and an estimation of maximum OTA accumulation rate and the delay till OTA production could be established. At high temperatures OTA accumulation starts earlier but increases slowly and becomes constant in a few days.

Kinetics of ochratoxin A production and accumulation by *Aspergillus carbonarius* on synthetic grape medium at different temperature levels. *Journal of Food Science* (submitted).

Incubation time and water activity effects on ochratoxin A production by *Aspergillus* section *Nigri* strains isolated from grapes.

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SUMMARY

Aims:
The objective of this study was to determine the temporal ochratoxin A (OTA) accumulation profile of *Aspergillus* section *Nigri* at different water activity (a_w) levels.

Methods and Results:
Two *A. carbonarius* and two *A. niger* aggregate strains isolated from grapes were tested *in vitro* for OTA accumulation at 25ºC on synthetic nutrient medium, over periods of 20 days at different a_w levels. Results were modelled by a multiple linear regression and response surface predictive models were obtained. High levels of a_w favoured OTA production by these moulds. Maximum amounts of OTA were found at the earlier growth states (5 days for *A. carbonarius* and 7-13 days for *A. niger* aggregate).

Conclusions:
Provided that *A.* section *Nigri*, and mainly *A. carbonarius*, play the main role in OTA presence in grapes, it would be critical to adjust the harvest and processing time to significantly reduce the chances for OTA accumulation.

Significance and Impact of the study:
Ochratoxin A production by *A.* section *Nigri* has been shown for the first time to occur optimally after as little as 5 days on a grape-like medium.

KEY WORDS: *Aspergillus carbonarius*, *Aspergillus niger* aggregate, black aspergilli, predictive models.

***Letters in Applied Microbiology* 38, 72-77 (2004)**

INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin that possesses nephrotoxic, immunosuppressive, teratogenic and carcinogenic properties (Smith and Moss, 1985). OTA is a widespread contaminant in human food and animal feed, as reviewed by International Agency for Research on Cancer (IARC, 1993). Several authors have also found OTA in beverages such as wine (Majerus and Otteneder, 1996; Zimmerli and Dick, 1996; Ospital *et al*., 1998; Burdaspal and Legarda, 1999; Cerutti *et al*., 2000; Majerus *et al*., 2000; Ottender and Majerus, 2000; Tateo *et al*., 2000; Filali *et al*., 2001; Larcher and Nicolini, 2001; Pietri *et al*., 2001; Soleas *et al*., 2001; Dumoulin and Riboulet, 2002; López de Cerain *et al*., 2002; Soufleros *et al*., 2002; Stander and Steyn, 2002; Bellí *et al*., 2003a; Stefanaki *et al*., 2003) and grape juice (Majerus *et al*., 2000; Soleas *et al*., 2001; Zimmerli and Dick, 1996; Burdaspal and Legarda, 1999; Dumoulin and Riboulet *et al*., 2002; Bellí *et al*., 2003a), being grape derivatives the most studied, since 1996. OTA has also been found in beer (Guldborg, 1997; Jørgensen, 1998; Degelmann *et al*., 1999; Bresch *et al*., 2000; Filali *et al*., 2001), and coffee (Burdaspal and Legarda, 1998; Jørgensen, 1998; Bresch *et al*., 2000; Prado *et al*., 2000; Leoni *et al*., 2001; Otteneder and Majerus, 2001; Lombaert *et al*., 2002).

OTA was isolated in 1965 from a culture of *Aspergillus ochraceus* (Van der Merwe *et al*., 1965), but subsequent studies have revealed a variety of fungal species included in the genera *Aspergillus* and *Penicillium* able to produce ochratoxins (Varga *et al*., 2001). In the last few years, the number of reports dealing with the production of OTA by members of *Aspergillus* section *Nigri* (black aspergilli) has increased. Several of these studies have highlighted *A.* section *Nigri* as the main group responsible for OTA detected in grapes, must and wines (Cabañes *et al*., 2002; Sage *et al*., 2002; Battilani *et al*., 2003; Bellí *et al*., 2003b). In this section, the reported OTA-producing species are those included in the so-called *A. niger* aggregate and *A. carbonarius* (Abarca *et al*., 2001). *Aspergillus carbonarius* has been considered the main species in OTA accumulation in grapes and wine (Cabañes *et al*., 2002). Although the effects of water activity (a_w) and temperature on growth of black Aspergilli have been investigated (Bellí et al, 2003c), little is known about the toxin production conditions of these species.

The aim of this work was to establish the temporal OTA production profile of some *A.* section *Nigri* isolates at different a_w levels in a synthetic medium which simulates grape composition, in order to provide more information when implementing preventive measures in the field. To our knowledge, this is the first study that reports the optimum time for OTA production in synthetic grape medium by *A.* section *Nigri* isolates.

MATERIAL AND METHODS

Isolates

Two *A. carbonarius* isolated from Italian (A1020, supplied by Faculty of Agriculture, Università Cattolica del Sacro Cuore, Piacenza, Italy) and Spanish (W120, Departament de Sanitat i d'Anatomia Animals, Facultat de Veterinària, Univ. Autònoma de Barcelona, Spain) grapes, and two *A. niger* aggregate species isolated from Spanish (44RJ3, Department Tecnologia d'Aliments, Escola Tècnica Superior d'Enginyeria Agrària, Universitat de Lleida, Spain) and Portuguese (01UAs203, Centro de Engenharia Biológica-IBQF, Universidade do Minho, Campus de Gualtar, Braga, Portugal) grapes, were used in this study. All isolates were found to produce OTA when tested in Czapek yeast autolysate agar (CYA) and in synthetic nutrient medium (SNM).

Medium and water activity adjustments

Studies were carried out *in vitro* using SNM, a medium similar to grape composition between véraison and ripeness (modified from Delfini, 1982). It had the following composition: D (+) glucose, 70 g; D (-) fructose, 30 g; L (+) tartaric acid, 7 g; L (-) malic acid, 10 g; $(NH_4)_2SO_4$, 0.67 g; $(NH_4)_2HPO_4$, 0.67 g; KH_2PO_4, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.75 g; NaCl, 0.15 g; $CaCl_2$, 0.15 g; $CuCl_2$, 0.0015 g; $FeSO_4 \cdot 7H_2O$, 0.021 g; $ZnSO_4$, 0.0075 g; (+) cathechin, 0.05 g; agar, 2%; water until 1000 ml; pH, 4.2 reached with KOH (2N). The a_w of this basal medium was 0.995, determined with a a_w meter (AquaLab CX-2, Decagon, Pullman, Washington, U.S.A.). SNM was modified to 0.90, 0.93, 0.95 and 0.98 a_w by adding different amounts of glycerol (N. Bellí, S. Marín, V. Sanchis and A.J. Ramos, unpublished data).

Inoculation and incubation

Suspensions (10^6 spores per ml) from 1-week-old cultures grown at 25ºC on SNM were used for inoculation. Petri plates containing 20 ml of SNM, were needle-inoculated centrally. Plates of the same a_w were enclosed in polyethylene bags and incubated at 25ºC. A full-factorial design was used where the factors were a_w and incubation time and the response was OTA content. All the treatments were repeated three times.

OTA extraction from culture

Ochratoxin A was extracted by a variation of Bragulat *et al*. (2001) method. After 5, 10, 15 and 20 days of incubation, three agar plugs (diameter 5 mm) were removed from the inner, middle and outer area of each colony. Plugs were weighed and introduced into 3-

ml vials. Methanol (1 ml) was added, and the vials were shaken for 5 s (Autovortex SA6, Surrey, UK). After being left stationary for 60 min, the extracts were shaken again, filtered (Millex^{R} SLHV 013NK, Millipore, Bedford, Massachusetts, U.S.A.) and injected into a HPLC instrument (Waters, Milford, Massachusetts, U.S.A.).

Detection and quantification of OTA by HPLC

The production of OTA was detected and quantified by HPLC with fluorescence detection (λ_{exc}, 330 nm; λ_{em}, 460 nm) (Waters 474), using a C_{18} column (Waters Spherisorb 5 μm, ODS2, 4.6x250 mm). 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 around 7 minutes. The detection limit of the analysis was 0.01 μg OTA g^{-1} SNM, based on a signal-to-noise ratio of 3:1. Quantification was achieved with a computing integrator (Millenium^{32} v.3.05 software, Milford, Massachusetts, U.S.A). OTA was quantified on the basis of the HPLC fluorimetric response compared with that of a range of OTA standards.

Statistical treatment of the results

OTA concentrations detected at each condition were evaluated by analysis of variance using SAS version 8.2 (SAS Institute, Inc, Cary, NC, USA). Incubation time and OTA production values were modelled by polynomial multiple lineal regression (MLR) and the resulting response surface models (RSM) were obtained with the Unscrambler® software, version 7.6 (CAMO ASA, Oslo, Norway), including the significant factors, interactions and quadratic terms.

RESULTS

The average OTA accumulation obtained from each isolate at the different time intervals and water activities is shown in Table 1. Analysis of variance revealed that OTA accumulation was significantly (p<0.01) higher for *A. carbonarius* strains than for *A. niger* aggregate ones. However, the significance was due to a *A. carbonarius* strain (W120) producing higher amounts than the others. Table 2 shows the results from the analysis of variance of the effects of a_w and incubation time on OTA accumulation by each of the isolates tested. Response surface contour graphs of constant yields were prepared from these data for each isolate studied and the ones for isolates 44RJ3 and W120 are shown in Fig. 1. Predicted yields of OTA in relation to time and a_w are shown. Negative values in plots should be interpreted as null values.
From the estimated response surfaces obtained for both *A. carbonarius*, a water availability level of 0.96 combined with an incubation period of 5 days resulted in the

maximum OTA accumulation. The amounts of OTA detected decreased with time, being minimum after 20 days of incubation.

No differences in the OTA accumulation profile between the two *A. niger* aggregate isolates studied were detected, although 01UAs203 produced lower amounts of OTA than 44RJ3 one. According to the proposed models, the optimum time for OTA accumulation of these strains depended on a_w, and was between 5 and 13 days. Maximum amounts of OTA for both *A. niger* aggregate isolates were found at the highest a_w (0.995) after 5 days and at 0.96, 0.94, 0.92 and 0.90 a_w after 7, 9, 11, and near 13 days, respectively.

Table 1. OTA accumulation (μg g^{-1}) (mean ± SD) by *Aspergillus carbonarius* (W120 and A1020) and *A. niger* aggregate (44RJ3 and 01UAs203) after 5, 10, 15 and 20 days of incubation at 25ºC on SNM.

		OTA (μg g^{-1}) produced after:			
	a_w	5 days	10 days	15 days	20 days
A. carbonarius (W120)	0.90	<d.l.	3.47±4.53	1.68±0.34	0.66±0.23
	0.93	2.41±1.18	3.31±0.73	1.79±0.26	1.39±0.47
	0.95	4.67±1.50	3.15±1.17	1.50±0.70	1.23±0.77
	0.98	5.06±0.62	1.63±0.93	0.67±0.16	0.47±0.10
	0.995	2.10±1.12	1.33±0.71	0.66±0.23	0.32±0.28
A. carbonarius (A1020)	0.90	<d.l.	0.03±0.03	<d.l.	<d.l.
	0.93	0.04±0.05	0.01±0.01	<d.l.	<d.l.
	0.95	0.33±0.30	0.02±0.01	<d.l.	<d.l.
	0.98	0.07±0.01	0.01±0.02	<d.l.	<d.l.
	0.995	0.10±0.02	<d.l.	0.04±0.06	<d.l.
A. niger aggregate (44RJ3)	0.90	<d.l.	0.70±1.07	0.14±0.21	<d.l.
	0.93	0.13±0.09	0.69±0.24	0.20±0.04	0.15±0.12
	0.95	0.59±0.15	1.73±0.37	0.73±0.50	0.34±0.14
	0.98	2.26±0.32	1.34±1.32	1.51±0.37	0.87±0.40
	0.995	2.12±0.41	1.24±1.14	1.06±1.10	0.74±0.78
A. niger aggregate (01UAs203)	0.90	<d.l.	<d.l.	<d.l.	<d.l.
	0.93	<d.l.	<d.l.	<d.l.	<d.l.
	0.95	<d.l.	<d.l.	0.02±0.03	<d.l.
	0.98	<d.l.	0.28±0.13	0.04±0.05	<d.l.
	0.995	<d.l.	0.30±0.18	0.05±0.04	0.05±0.02

OTA, ochratoxin A; SNM, synthetic nutrient medium; d.l. detection limit (0.01 μg g^{-1}).

Table 2. Analysis of variance of the effects of a_w and incubation time on OTA accumulation by *Aspergillus* section *Nigri* isolates, at 25 ºC on SNM medium.

FACTOR	*A. carbonarius* (W120)		*A. carbonarius* (A1020)		*A. niger* aggregate (44RJ3)		*A. niger* aggregate (01UAs203)	
	MS	F value	MS	F value	MS	F value	MS	F value
day	14.70	9.53**	0.03	7.10**	1.50	4.24*	0.04	16.24**
a_w	4.44	2.88*	0.01	2.35^{ns}	3.94	11.09**	0.02	9.51**
dia* a_w	4.33	2.80**	0.01	2.54*	0.55	1.55^{ns}	0.01	6.22**
R^2	0.648		0.605		0.654		0.801	

OTA, ochratoxin A; SNM, synthetic nutrient medium; MS, mean square; ns, not significant; *p<0.05; **p<0.001.

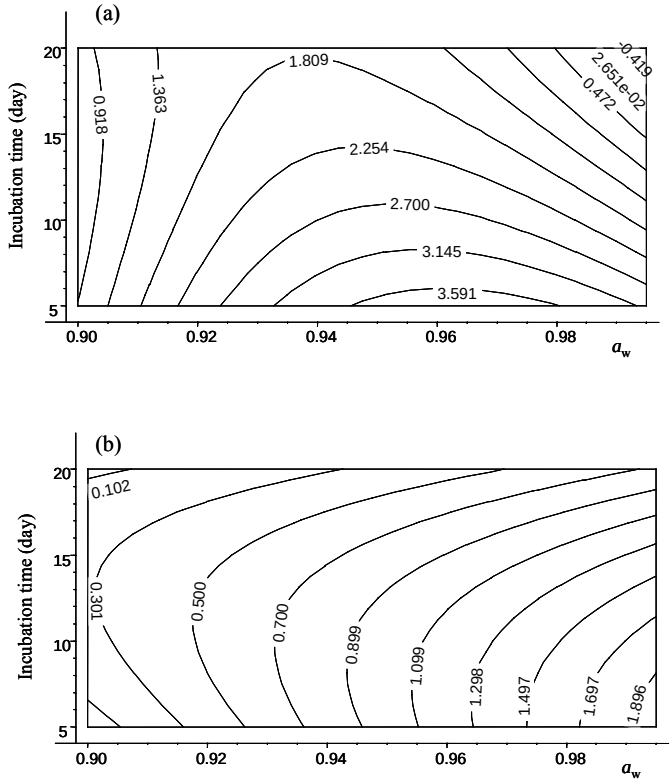

Figure 1. Response surface contour graphs showing the effect of a_w and time on ochratoxin A accumulation by (a) *Aspergillus carbonarius* (W120) (R^2= 0.602) and (b) *A. niger* aggregate (44RJ3) (R^2= 0.301) at 25 ºC on synthetic nutrient medium.

DISCUSSION

Results have shown the significant influence of two abiotic parameters (a_w and incubation time) on OTA accumulation for the *A.* section *Nigri* strains tested. High water activities seem to favour OTA production of these isolates, as well as their growth, as was pointed out by N. Bellí, S. Marín, V. Sanchis and A.J. Ramos (unpublished data) for some black Aspergilli strains. They suggested that *A.* section *Nigri* may grow at a wide range of temperatures and water activities, with optimum conditions of 25-35 ºC and 0.95-0.995 a_w (Bellí, S. Marín, V. Sanchis and A.J. Ramos, unpublished data). Therefore, the humidity conditions in the field and also during transport to wine cellars, would be an important factor to predict the possible OTA contamination of grapes.

Cabañes et al. (2002) analysed the OTA produced after 7, 14 and 21 days at 25 ºC, by two *A. niger* aggregate and five *A. carbonarius* isolates, in the central area of a three-point inoculated colonies on yeast extract sucrose agar (YES) and CYA. The amounts of OTA found were generally higher than the ones of the present study, with the different media used and the extractions done from the oldest part of the colonies being probably the cause. In their study, maximum OTA accumulation seemed to depend on the strain tested, without an evident trend by group. In general, 7 days of incubation were sufficient for OTA detection, but some isolates required 14 days to reach maximum accumulation. The fact that, in our study, maximum amounts of OTA were found at the earlier growth states of *A.* section *Nigri* isolates tested (optimum for *A. carbonarius*: 5 days) is an important hazard for OTA contamination in grapes. Consequently, carefully choosing the harvest dates and minimising the harvesting and transport time to wine cellars become crucial. Moreover, any damage on the grape skin produced at harvest or during transport may trigger the entrance of the fungi colonising the berry surface, probably enabling production of maximum amounts of OTA. Therefore, minimizing mechanical damages of grape skins during harvest and transport is a key factor in preventing OTA.

For the four strains tested, the amounts of OTA detected after 5-10 days decreased when increasing incubation time. Probably, it was degraded by the fungus itself as suggested by Varga *et al*. (2002), who found that black Aspergilli could eliminate OTA from medium resulting in degradation products such as ochratoxin α. Moreover, OTA production by the fungi could have stopped. It could be due to the limited amounts of nutrients of the synthetic media, and probably would not happen with unlimited nutrient sources in the field.

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Kinetics of ochratoxin A production and accumulation by *Aspergillus carbonarius* on synthetic grape medium at different temperature levels.

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ABSTRACT

The effects of incubation time (up to 10 days) and temperature (7, 15, 20, 25, 30, 35 and 42 ºC) on OTA-producing capacity and OTA accumulation by four *A. carbonarius* strains isolated from Spanish and Tunisian grapes, were studied on grape-like medium. The limits of growth and OTA production have been identified. OTA production was significantly higher at 20 ºC, followed by 25, 15, 30, 35 ºC. No growth was observed at 7 ºC and 42 ºC after 10 days of incubation and consequently, no OTA was detected at these temperature levels. In general, maximum OTA-producing capacity was found earlier with increasing incubation temperatures. However, at 35 ºC, OTA was rarely detected although growth was maximum at this temperature. OTA accumulation was maximum after 10 days of incubation for all the temperatures except at 30 ºC, were the maximum was detected at earlier incubation time (6-8 days) and then remained stable. Colony diameters and OTA accumulation along time were modelled by Gompertz sigmoidal model and an estimation of maximum OTA accumulation rate and the delay till OTA production could be established. At high temperatures OTA accumulation starts earlier but increases slowly and becomes constant in a few days.

KEY WORDS: *Aspergillus carbonarius*; temperature; incubation time; ochratoxin A; grapes.

INTRODUCTION

Ochratoxin A (OTA) is a secondary metabolite produced by some fungal species belonging to *Aspergillus* and *Penicillium* genera. The presence of ochratoxin A in foods is of current interest owing to its toxic effects, as it has been shown to be teratogenic, immunotoxic and nephrotoxic (Creppy 1999). OTA has been suspected to be implicated in an endemic kidney disease in South-Eastern Europe that became known as Balkan Endemic Nephropathy (Vrabcheva and others 2004). Recently it has been considered to be a causal agent of a chronic interstitial nephropathy discovered in Tunisia (Abid-Essefi and others 2003; Bacha and others 1993; Maaroufi and others 1995, 1996). Daily intake of OTA was estimated in European countries between 0.15 and 2.5 ng kg^{-1} of body weight $^{-1}$ (Miraglia and others 2002). OTA can be ingested from different food sources. In fact, this mycotoxin is widely distributed in food products and its occurrence has been reported in cereals, coffee, beans, soya, cacao, nuts, milk, and beer. In 1996, OTA was detected in wine by Zimmerli and Dick (1996), and subsequent surveys have confirmed the presence of OTA on grape-derived products (Bellí and others 2002, 2004a; Larcher and Nicolini 2001; Soufleros and others 2002; Stander and Steyn 2002).

Wine is considered, after cereals, the second major source of OTA intake (Anonymous 1998). There is a great concern on this metabolite in the Mediterranean countries because many results show that wines from the Mediterranean area contain higher levels of OTA than wines from Northern areas in Europe (Majerus and Otteneder 1993; Zimmerli and Dick 1996). Therefore wine production has an important impact on both Tunisian and Spanish economies. Concerning grape derived products, since January 2005, the European Union legislation authorities have set up a maximum level for OTA in wine, musts and grape juices (2 μg l^{-1}) and dried vine fruits (10 μg kg^{-1}) (Anonymous 2005).

It is necessary to control OTA in grapes and therefore to study the sources of production of this mycotoxin. The occurrence of OTA in wine results from the contamination of grapes with ochratoxigenic fungi during the maturation of the berries. OTA was initially believed to be produced only by *Aspergillus ochraceus* and closely related species from the section *Circumdati* (Hesseltine and others 1972) and by *Penicillium verrucosum* (Pitt 1987). As *P. verrucosum* occurs only in cool temperate zones and *A. ochraceus* does not appear to be spread in the tropics (Pitt 1997), many surveys carried out in different countries from Southern Europe, with Mediterranean climate, have reported that *Aspergillus* section *Nigri* (black aspergilli), in particular *A. carbonarius*, is the main responsible for OTA production in grapes (Battilani and others 2003; Bellí and others 2004b, 2005a; Cabañes and others 2001).

The purposes of this study were to determine the effect of incubation time, especially at early growth stages, on OTA-producing capacity and OTA accumulation at different incubation temperatures. Previous work at 25 ºC has been carried out (Bellí and others 2004c). Synthetic grape-like medium was used to inoculate four *A. carbonarius* strains

isolated from Spanish and Tunisian grapes. The limits of growth and OTA production of these strains have been identified.

MATERIAL AND METHODS

Isolates

Four ochratoxigenic *A. carbonarius* strains were used in this study, two (47S and 56S) isolated from North-East Tunisian wine grapes and two (23N and 234N) from North-East Spanish wine grapes, all isolated in 2004. Isolates are held in the culture collection of the Department of Food Technology of the University of Lleida (Spain).

Inoculation and incubation

Isolates were grown on Czapek yeast extract (CYA) medium at 25 ºC for 5 days. Conidia were collected by scraping in an aqueous 0.005 % Tween 80 solution to 10^6 spores ml^{-1}, determined by a Thoma chamber, and used for inoculation. Synthetic Nutrient Medium (SNM) agar plates (20 ml, 9 cm in diameter), with similar composition to grapes [12], were needle inoculated centrally and incubated at seven temperatures (7, 15, 20, 25, 30, 35 and 42 ºC) for 10 days. Previously, 135 g of glycerol were added to each litre of medium to get 0.96 a_w, the optimum a_w reported for *A. carbonarius* OTA production (Bellí and others 2004d). During incubation, Petri dishes were examined every two days and the diameter of the colony was measured. All the assays were repeated three times.

Ochratoxin A extraction

OTA was extracted by a variation of Bragulat and others (2001) methodology, after 2, 4, 6, 8 and 10 days of incubation. Three plugs (6 mm) were removed from the inner, middle and outer area of the colony, were weighed and introduced in a vial with 1 ml of methanol. After 1 hour, the extracts were vortexed for 5 seconds, filtered (Millex^R SLHV 013NK, Millipore, Bedford, MA, U.S.A.) and analysed by high-performance liquid chromatography (HLPC). The equipment was fitted with a fluorescent detector set (λ_{exc} 330 nm; λ_{em} 460 nm) (Waters 474, Milford, MA, U.S.A.) and a C_{18} column (Waters Spherisorb 5 μm, ODS2, 4.6 mm x 250 mm). The mobile phase consisted of acetonitril-water-acetic acid (57:41:2). The injection volume was 25 μl, flow rate 1 ml min^{-1} and retention time 7.1 min. Standard OTA curves were established with an ochratoxin standard (Sigma-Aldrich, Steinheim, Germany). The limit of detection was 0.005 μg OTA g^{-1} SNM, based on a signal-to-noise ratio of 3:1. Quantification was achieved with a computing integrator (Millenium^{32} v.3.05 software, Mildford, MA., U.S.A.). It was a full factorial experimental design and 420 assays were carried out as extractions were done in triplicate.

Statistical processing of results

The amount of toxin extracted by removing part of the colony from different zones – centre, middle and outer area of the colony- was used to estimate the OTA-producing capacity (μg OTA g^{-1} agar) of each colony at all the conditions assayed. It is known that OTA production depends on the age of the mycelium. Shorter distances from the inoculum point resulted in maximum OTA concentrations (Valero and others 2005). Consequently, extracting the toxin from three different zones of the colony would give an approximation of the ochratoxigenic *A. carbonarius* ability. OTA accumulation (μg OTA $colony^{-1}$) was calculated taking into account the area of each colony at each time of extraction and the area of the extracted plug. Given a certain OTA-producing capacity, bigger colonies will present higher OTA accumulation.

OTA-producing capacity and OTA accumulation were evaluated by an analysis of variance ANOVA using SAS Enterprise Guide, version 2.0 (SAS Institute Inc., Cary, NC, USA). Duncan's multiple range test was used to assess the differences among the levels studied. Statistical significance was judged at $P<0.05$. In addition, both colony diameters and OTA accumulation were plotted against time and a non-linear regression was carried out using the Gompertz sigmoidal model modified by Zwietering and others (1990) (Statgraphics v.2.1).

RESULTS

Analysis of variance of OTA-producing capacity revealed that all single factors and their two-way interactions were significant (Table 1). The significance of the factor strain was due to the higher OTA-producing capacity of the strain 234N, with values up to 27 μg g^{-1} SNM (Fig. 1), while the other strains produced less than 10 μg g^{-1} SNM under the same conditions. OTA production was significantly higher at 20 ºC for all the strains, followed by 25, 15, 30, 35 ºC, with no significant differences among them. Only for the strain 234N OTA-producing capacity at 25 ºC was significantly higher than that at 15, 30 and 35 ºC. No growth was observed at 7 ºC and 42 ºC after 10 days of incubation and consequently, no OTA was detected at these temperature levels. At the lowest temperature allowing fungal growth, 15 ºC, maximum OTA production was found after 10 days of incubation, followed by 8, 6, 4 and 2 days. Similarly occurred at 20 ºC, except for 56S strain, which was able to produce more OTA after 8 than 10 days. In general, the peak of OTA-producing capacity was found earlier with increasing incubation temperatures. At 25 ºC, maximum OTA was detected after 6 days and at 30 ºC after 4 days. However, at 35 ºC, OTA was rarely detected although growth was maximum at this temperature.

Table 2 shows the analysis of variance for the effect of strain, temperature and incubation time on OTA accumulation. The significant factors were found to be all single factors and

the interactions time x strain and temperature x strain. OTA accumulation was maximum after 10 days of incubation for all the temperatures except at 30 ºC, were the maximum was detected at earlier incubation time (6-8 days) and then it remained stable (Fig. 2). Increasing colony radius fitted the Gompertz model with R^2 ranging from 70% to 100%. OTA accumulation however, despite following a sigmoid increase showed low R^2 values (30-90%) mainly due to the differences among replicates. In particular, strain 234N followed an exponential trend for most temperatures, heading to poor estimation of OTA production parameters. Estimation of maximum rate of OTA accumulation and delay till OTA production are shown in table 3. The different accumulation of OTA at the different temperatures was due to higher OTA rates of production observed at 20 ºC. Regarding delay before production, it was shorter at the higher temperature where OTA was detected (30 ºC) and increased as temperature decreased until 15 ºC. Consequently, at high temperatures OTA accumulation starts earlier but increases slowly and becomes constant in a few days.

Table 1. Analysis of variance of OTA-producing capacity (µg OTA g^{-1} SNM) at different temperatures by four *A. carbonarius* strains after several days growing on Synthetic Nutrient Medium (SNM).

Source	DF	Mean Square	F Value
Time	4	77.07	8.54**
T	6	212.58	23.56**
Strain	3	151.82	16.83**
Time*Strain	12	21.77	2.41**
T*Strain	18	58.63	6.50**
Time*T	24	46.56	5.16**
Time*T*Strain	72	11.49	1.27^{ns}

** significant (P<0.001); ns, not significant.

Table 2. Analysis of variance of OTA accumulation (µg OTA colony^{-1}) at different temperatures by four *A. carbonarius* strains after several days growing on Synthetic Nutrient Medium (SNM).

Source	DF	Mean Square	F Value
Time	4	844.69	4.70**
T	6	745.22	4.15**
Strain	3	1102.39	6.14**
Time*Strain	12	402.78	2.24*
T*Strain	18	372.56	2.07**
Time*T	24	254.22	1.41^{ns}
Time*T*Strain	72	139.16	0.77^{ns}

* significant (P<0.05); ** significant (P<0.001); ns, not significant.

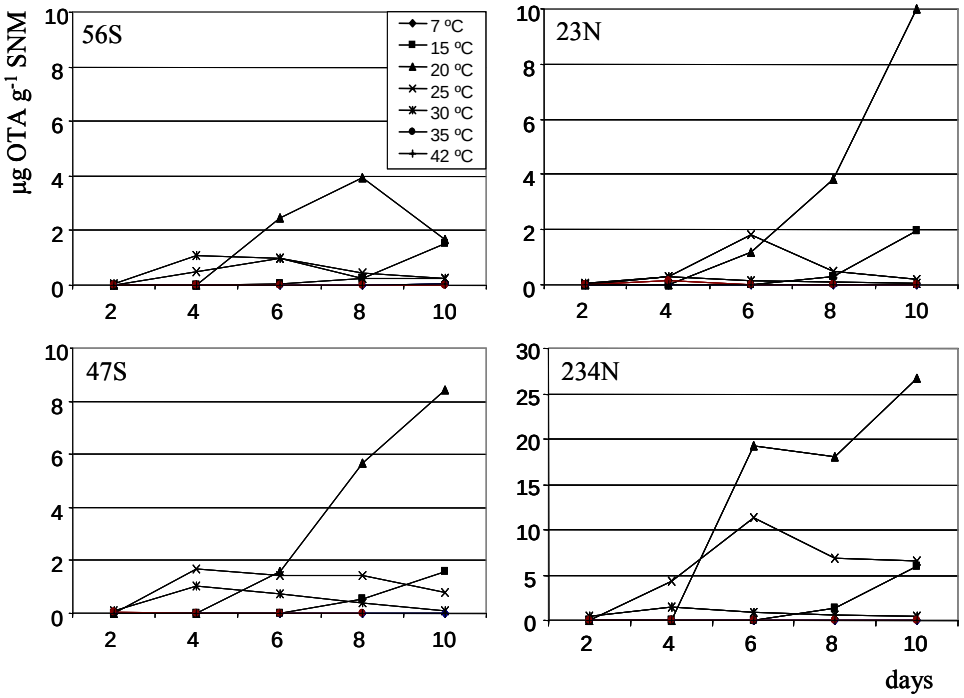

Fig. 1. OTA-producing capacity (µg OTA g^{-1} SNM) at different temperatures (7, 15, 20, 25, 30, 35 and 42 ºC) by four strains of *A. carbonarius* after several days (2, 4, 6, 8 and 10 days) growing on Synthetic Nutrient Medium (SNM).

Table 3. Estimated maximum rates of OTA accumulation and delay till OTA production through the Gompertz model for *A. carbonarius* strains ± SD.

	Maximum OTA rate (µg OTA day^{-1})			Delay (days)		
Temp.	**23N**	**47S**	**56S**	**23N**	**47S**	**56S**
7 ºC	ND[a]	ND	ND	ND	ND	ND
15 ºC	1.6 ± 2.1	3.6 ± 5.9	1.3 ± 0.1	8.1 ± 0.1	8.2 ± 0.1	8.3 ± 0.1
20 ºC	2.5 ± 1.1	3.7 ± 3.8	2.2 ± 3.8	5.9 ± 0.8	6.1 ± 1.9	5.8 ± 6.7
25 ºC	1.4 ± 3.4	3.5 ± 3.5	0.5 ± 0.2	3.9 ± 0.9	5.4 ± 0.9	3.3 ± 0.7
30 ºC	0.7 ± 3.3	2.8 ± 11.4	1.6 ± 6.4	2.2 ± 3.8	3.4 ± 26.5	2.8 ± 6.9
35 ºC	ND	ND	ND	ND	ND	ND
42 ºC	ND	ND	ND	ND	ND	ND

[a]ND, not detected.

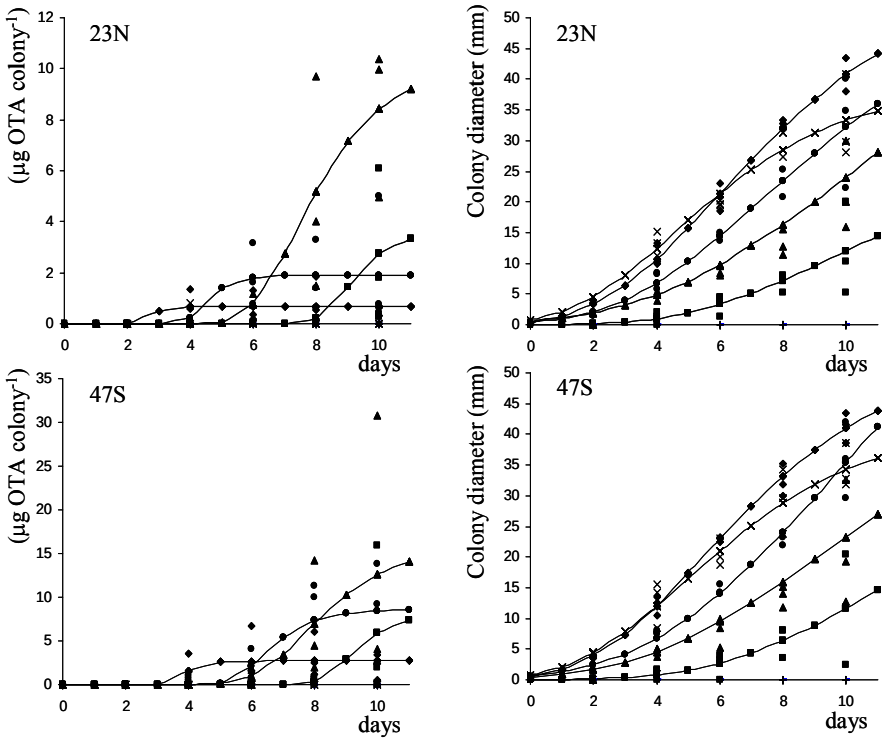

Fig. 2. OTA accumulation (µg OTA colony^{-1}) and colony diameters of two *A. carbonarius* strains (23N and 47S) at different temperatures (7, 15, 20, 25, 30, 35 and 42 ºC) and after several days (2, 4, 6, 8 and 10 days) growing on Synthetic Nutrient Medium (SNM). Values of three replicates are plotted and adjusted using the Gompertz sigmoidal model modified by Zwietering et al. (1990).

DISCUSSION

A wide range of temperature (15-35 ºC) at 0.96 a_w was demonstrated to let *A. carbonarius* growth, although previous studies on SNM enlarged that range to 10-40 ºC with longer incubating periods (Bellí and others 2004d; Mitchell and others 2004). In order to find maximum and minimum temperatures allowing growth, 7 ºC and 42 ºC were assayed in the present study proving no growth in any of them after 10 days. *A. carbonarius* growth on other substrates has been studied previously. Esteban and others (2004) reported temperature ranges for *A. carbonarius* growth on yeast extract sucrose (YES) medium between 10 and 40 ºC and narrower on CYA (15-30/40 ºC). Optimum thermal conditions found in the present study, 30-35 ºC, agree with previous works suggesting the optimal growth at 25-30 ºC (Leong and others 2004), 30 ºC (Bellí and others 2004d, 2005b), 25-35 ºC (Mitchell and others 2004) and 35 ºC (Mitchell and others 2003).

Temperature ranges for OTA production (15-30 ºC) were narrower than those reported for growth. It is known that OTA production depends on the water activity of the medium. This explains that in previous work *A. carbonarius* was able to produce OTA at higher temperatures (35-37 ºC), although little amounts, as the study was carried out at 0.99 a_w (Bellí and others 2005b). Maximum OTA-producing capacity on SNM was detected at 20 ºC, similarly to Bellí and others (2005b). Other studies suggested temperatures between 15-20 ºC (Esteban and others 2004; Mitchell and others 2003, 2004) as the optimum ones.

Few studies have report mycotoxin production along time. Previous work with different *A. carbonarius* strains incubated at 25 ºC on SNM suggested that a level of water activity of 0.96 a_w combined with an incubation period of 5 days resulted in the maximum OTA-producing capacity (Bellí and others 2004c). The present study confirmed that result and enlarge the range of temperatures studied, finding out that at higher levels, OTA was produced very soon after fungal inoculation (2-4 days). However, at temperatures below 25 ºC, maximum OTA-producing capacity was detected latter, after 10 days.

At 15 ºC and 20 ºC, OTA-accumulation during the first ten incubation days increased exponentially meanwhile at higher temperatures (25 ºC, 30 ºC and 35 ºC) it reached a maximum at earlier growth stages (4-6 days) and then it remained constant or slightly decreased. At this point, a balance between production and degradation may take place, as some authors suggested the ability of *A. carbonarius* to degrade OTA to ochratoxin α (Abrunhosa and others 2002). At highest temperatures, fungal growth was very fast, consuming maximum amounts of substrate in few days. Probably, *A. carbonarius* uses some of the nutrients of the substrate to produce the toxin and therefore at high temperatures, OTA-producing capacity decreases. This could explain why at 35 ºC, where maximum growth rates were obtained, few OTA was produced.

Few studies have reported the effect of incubation time on the amount of OTA produced by *A. carbonarius* and none referred to OTA accumulation. Esteban and others (2004) found maximum amounts of OTA after 30 days of incubation at minimum temperature (15 ºC). Considerable amounts of OTA were detected by them at 35 ºC in comparison with the present study and the optimum incubation time reported for OTA production was strongly related to the strain tested.

To our knowledge this is the first attempt to primary model OTA accumulation. Besides Gompertz model, any other sigmoidal model could have been used. The main challenge is the production of repeatable and reproducible data, as there is a well-known intrinsic biological variability in mycotoxins accumulation. However, the estimation of time till OTA production and rate of OTA production, would give a crucial knowledge in order to prevent them in food substrates. Accurate primary models are needed before beginning secondary modelling of OTA as a function of abiotic/processing factors in food substrates.

As a conclusion OTA production and accumulation may occur in the temperature range 15-30 ºC. Thus, temperature levels taking place during sun-drying (T >30 ºC) which has been considered an important hazard as black aspergilli can quickly grow, would not be conducive to OTA accumulation. Finally, OTA accumulation in grapes in the field which has been reported near harvest (average of minimum, mean and maximum temperatures in Spain at harvest = 15.5, 22.0, 31.5 ºC, respectively) (INM 2003), must occur during the night when temperature may decrease to optimum levels for OTA production.

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8.4.2.3. Effect of photoperiod and day night temperature

The effect of light and temperature regimes simulating day and night in the field in the months preceding grape harvest on *A. carbonarius* growth and OTA production were investigated in the following paper. Twelve-hour photoperiod affected positively *A. carbonarius* growth with no differences between incubating the mould at day temperature (28 ºC) or alternating day/night temperatures (28 ºC/20 ºC). Slower growth, however, was observed at constant incubation at 20 ºC. Under 12h-alternation periods of day and night temperatures, growth was faster at continuous darkness than under continuous light conditions. Light did not cause any morphological changes in the aspect of the colonies.

No significant differences on OTA production were detected due to either fluctuating temperature or photoperiod. However, as photoperiod enhanced the growth of colonies, it would also enhance OTA accumulation. The well-known ability of *A. carbonarius* to produce OTA reported in previous laboratory studies has been demonstrated to be stimulated in field conditions.

Effect of photoperiod and day-night temperatures simulating field conditions on growth and OTA production of *Aspergillus carbonarius* strains isolated from grapes. *Food Microbiology* (in press).

Effect of photoperiod and day-night temperatures simulating field conditions on growth and ochratoxin A production of *Aspergillus carbonarius* strains isolated from grapes.

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ABSTRACT

The effect of light and temperature regimes simulating day and night in the field in the months preceding grape harvest on *A. carbonarius* growth and ochratoxin A production were investigated. Twelve-hour photoperiod affected positively *A. carbonarius* growth with no differences between incubating the mould at day temperature (28 ºC) or alternating day/night temperatures (28 ºC/20 ºC). Slower growth, however, was observed with constant incubation at 20 ºC. Under 12h-alternation periods of day and night temperatures, growth was faster at continuous darkness than under continuous light conditions. Light did not cause any morphological changes in the aspect of the colonies.

No significant differences on ochratoxin A production were detected due to either fluctuating temperature or photoperiod. However, as photoperiod enhanced the growth of colonies, it also enhance ochratoxin A accumulation. The ability of *A. carbonarius* to produce OTA reported in previous laboratory studies has been demonstrated to be stimulated in field conditions.

KEY WORDS: *Aspergillus carbonarius*, growth, ochratoxin A, light, darkness, photoperiod, temperature.

INTRODUCTION

Ochratoxin A (OTA) is the most studied mycotoxin of a structurally related group of compounds, the ochratoxins, both for its high presence and its toxicological importance in foodstuffs. OTA is a potent toxin affecting mainly the kidneys, in which it can cause both acute and chronic lesions. It is also a potent teratogen, genotoxic and immunotoxic agent in animals (JECFA, 1995; Pfohl-Leszkowick, 1999). Therefore, the International Agency for Research on Cancer has classified OTA in group 2B as a possible human carcinogen (IARC, 1993). Because human exposure to ochratoxin A has been clearly demonstrated by its detection in blood and breast milk (Gareis et al., 2000; Thuvander, et al., 2001), the presence of OTA in foodstuffs is clearly undesirable. Recent investigations on grapes showed that OTA is produced by *Aspergillus* section *Nigri* (black aspergilli). Among them, *A. carbonarius* is the primary OTA producer, with a small percentage of isolates of the closely related *A. niger* aggregate group also being ochratoxigenic (Battilani et al., 2003a; Serra et al., 2003; Bellí et al., 2005a). Because of their black spores, these moulds are highly resistant to sunlight and survive sun-drying (Rotem and Aust, 1991). Apart from fresh grapes, they are the source of OTA in dried vine fruits, musts and wine (Sage et al., 2002; Battilani et al., 2003b; Leong et al., 2004).

Ecophysiological studies carried out so far, have been developed under constant conditions of temperature, light or darkness. *A. carbonarius* growth was found to occur optimally between 25 ºC and 35 ºC (Mitchell et al., 2003; Bellí et al., 2004a, 2005b; Leong et al., 2004), meanwhile optimum temperature for OTA production has been reported at 20 ºC (Bellí et al., 2004a, 2005b) and 25 ºC (Mitchell et al., 2003).

The effect of photoperiod and alternation of temperatures, both on black aspergilli growth and OTA synthesis is unknown. The present work was conducted to determine the effect of a 12-hours photoperiod and 12-hours alternation of temperature simulating day (20 ºC) and night (28 ºC) field conditions, on the growth and ochratoxin A production by *Aspergillus carbonarius* strains isolated from grapes.

MATERIAL AND METHODS

Fungal isolates

Three ochratoxigenic *A. carbonarius* strains, isolated from Italian (W9), French (W38) and Spanish (W120) wine grapes were used in this study. Cultures were provided by the Faculty of Agriculture, Università Cattolica del Sacro Cuore, Piacenza, Italy, the Institut National Polytechnique de Toulouse, École Nationale Supérieure Agronomique de Toulouse, France, and the Departament de Sanitat i d'Anatomia Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona, Spain, respectively. Isolates are held in

the culture collection of the Departament de Tecnologia d'Aliments, Escola Tècnica Superior d'Enginyeria Agrària, Universitat de Lleida, Spain.

Preparation of spore suspensions, inoculation and incubation

Spores from 7-day old cultures grown at 25 ºC on Czapek yeast extract agar (CYA) medium were harvested in sterile distilled water containing 0.005 % of Tween 80, counted using a Thoma chamber and adjusted to a final concentration of 10^6 spores ml^{-1}. Nine-cm diameter Petri dishes containing 20 ml of Synthetic Nutrient Medium (SNM) plates were prepared (0.99 a_w) and were needle-inoculated centrally with the *A. carbonarius* spore suspensions. SNM simulates grape composition between veraison and ripeness and its composition is detailed in Bellí et al. (2004b).

Petri dishes were incubated upside down under different combinations of controlled light and dark conditions and day (28 ºC) and night (20 ºC) temperatures (Table 1).

Table 1. Combinations of light and temperature assayed on *A. carbonarius* growth and OTA production.

Treatment	Studied effect	Period 1 (12 h)	Period 2 (12 h)
I (Field conditions)	-	Light, 28 ºC	Darkness, 20 ºC
II (Control)	Effect of fluctuating temperatures	Light, 28 ºC	Darkness, 28 ºC
III (Control)		Light, 20 ºC	Darkness, 20 ºC
IV (Control)	Effect of photoperiod	Light, 28 ºC	Light, 20 ºC
V (Control)		Darkness, 28 ºC	Darkness, 20 ºC

Illumination conditions

Petri dishes were placed at a distance of approximately 40 cm from the light source. White light (Mazda, 23w Eureka[3] Electronic bulbs; 230-240 V; 50-60 Hz; 1500 lumen; 175 mA) was used when required. Periods of 12-hours alternating light and darkness were analysed in order to study the influence of photoperiod. The heat emitted by the bulb did not increase the temperature proposed for incubation. Darkness was achieved placing the plates into a cardboard box.

Day and night temperatures

The effect of alternating temperature in the growth and OTA production was studied. Two average temperatures (28 ºC and 20 ºC) were chosen to represent day and night in the field. Both values were the mean of day and night temperatures, respectively, of the two months preceding harvest in North and North-East Spanish wine-making regions during years 2002 and 2003 (INM, 2003). Temperatures were cycled at 12-hours intervals.

Growth measurement

Diameters of the growing colonies were measured 3, 5 and 10 days after inoculation with the aid of a binocular magnifier. Treatments were repeated three times.

OTA measurement

OTA was determined after 3, 5 and 10 days of incubation at different conditions depending on the treatment, with a modification of the procedure described by Bragulat et al. (2001). Three agar plugs (diameter: 6 mm) were removed from the inner, middle and outer area of each colony. Plugs were weighed and introduced into 3-ml vials. Methanol (1 ml) was added, and the vials were shaken for five seconds (Autovortex SA6, Surrey, UK). After being left stationary for 60 minutes, the extracts were shaken again, filtered (Millex^{R} SLHV 013NK, Millipore, Bedford, Massachusetts, U.S.A.) and injected into a high-performance liquid chromatography (HPLC) instrument (Waters, Milford, Massachusetts, U.S.A.) with fluorescence detection (λ_{exc}, 330 nm; λ_{em}, 460 nm) (Waters 474). Acetonitrile-water-acetic acid (57:41:2) (1.0 ml min^{-1}) was the mobile phase and a C_{18} column (Waters Spherisorb 5 μm, ODS2, 4.6x250 mm) was used. The injection volume was 25 μl and the retention time was 6.8 minutes. The detection limit was 0.01 μg OTA g^{-1} SNM, based on a signal-to-noise ratio of 3:1. Quantification was achieved with a computing integrator (Millenium^{32} v.3.05 software, Milford, Massachusetts, U.S.A). OTA was quantified on the basis of the HPLC fluorimetric response compared with that of a range of OTA standards (Sigma-Aldrich, Steinheim, Germany). Three replicates per treatment were carried out.

Statistical analysis

Colony diameters and OTA concentrations detected in each treatment were evaluated by analysis of variance followed by a Duncan test, using SAS version 8.2 (SAS Institute, Inc, Cary, NC, USA). Statistical significance was judged at $P<0.01$.

RESULTS

Effect on mycelial growth

As no significant differences were found either among isolates or in their responses to photoperiod/alternating temperatures treatments, the mean diameter of the three *A. carbonarius* strains tested is shown in Table 2. Continuous light exposure, darkness or photoperiod conditions did not cause visible morphological changes in the colonies.

Photoperiod (treatment I, light 28 ºC/darkness 20 ºC) enhanced significantly the growth of *A. carbonarius* compared to continuous dark (treatment V, darkness 28 ºC/darkness 20 ºC) or light conditions (treatment IV, light 28 ºC/light 20 ºC). In addition, incubation under darkness (treatment V) was in general more suitable for *A. carbonarius* growth than light conditions (treatment IV). Regarding temperature fluctuation, no significant differences were found between growth under 12-h switching between 28 and 20 ºC (treatment I) and growth under a constant 28 ºC regime (treatment II, light 28 ºC/darkness 28 ºC). Slower growth, however, was observed at constant incubation at 20 ºC (treatment III, light 20 ºC/darkness 20 ºC). Overall, treatment I and II, both under photoperiod and either constant temperature of 28 ºC or alternating between 20 and 28 ºC, led to the faster growth of these species.

Table 2. Mean diameters (mm) ± standard errors of three *A. carbonarius* isolates after 3, 5 and 10 days of incubation at different conditions of light and temperature (treatments: I, light 28ºC/darkness 20 ºC; II, light 28 ºC/darkness 28 ºC; III, light 20 ºC/ darkness 20 ºC; IV, light 28 ºC/light 20 ºC; V, darkness 28 ºC/ darkness 20 ºC). Letters show the significance of the treatments within each incubation period.

	Colony diameter (mm)		
TREATMENT	**3 days**	**5 days**	**7 days**
I	9.36 ± 0.09^{a}	28.10 ± 1.80^{a}	75.70 ± 3.30^{a}
II	10.70 ± 1.16^{a}	30.93 ± 2.26^{a}	82.33 ± 6.60^{a}
III	5.73 ± 0.82^{c}	20.76 ± 2.35^{b}	59.33 ± 6.51^{b}
IV	6.46 ± 0.85^{c}	22.30 ± 0.66^{b}	58.63 ± 3.20^{b}
V	8.66 ± 0.72^{b}	24.50 ± 1.14^{b}	70.03 ± 4.09^{b}

Effects on OTA accumulation

The identify of the isolate, the length of the incubation period, and the interaction of these two parameters were the factors that significantly affected (Table 3). Fluctuating

temperature and photoperiod did not have any significant effect on the toxin production of the isolates, although there were a number of non-significant trends that may warrant more detailed studies in the future. In general, continuous exposure to light (treatment IV, light 28 ºC/light 20 ºC) or darkness (treatment V, darkness 28 ºC/darkness 20 ºC) increased the levels of OTA detected in comparison with the application of photoperiod (treatment I, light 28 ºC/darkness 20 ºC), in the treatments where temperature was variable (Figure 1). When alternating light and darkness, low temperatures seemed to increase OTA production measured after 10 days (treatment III, light 20 ºC/darkness 20 ºC), meanwhile no general trend was observed after 3 or 5 days of incubation. Longer periods of incubation produced OTA degradation.

Table 3. Analysis of variance of OTA production by *A. carbonarius* isolates on SNM medium, incubated under different conditions of light and temperature.

Source	DF	Mean Square	F Value	Pr > F
Isolate	2	63.77	41.79	*
Treatment	4	0.19	0.13	n.s.
Day	2	26.27	17.21	*
Isolate*day	4	14.42	9.45	*
Treatment*day	8	1.14	0.75	n.s.
Isolate*treatment	8	0.91	0.60	n.s.
Isolate* treatment*day	16	1.75	1.15	n.s.

n.s. not significant; * P<0.01.

Maximum OTA at the shortest period of incubation tested (3 days) was detected at the optimum conditions for growth (treatment II, light 28 ºC/darkness 28 ºC) and the lowest values were detected in treatment III (light 20 ºC/darkness 20 ºC) for all the isolates. W120 was the maximum OTA producer of the three isolates tested, with mean contents up to 6 μg g^{-1} SNM. Maximum amounts of OTA were detected after 5 days of incubation in most of the cases, followed by 10 days of incubation. No significant differences were found between the amount of OTA detected after 5 and 10 days for the isolate W120, and between 10 and 3 days for W9 and W38 ones.

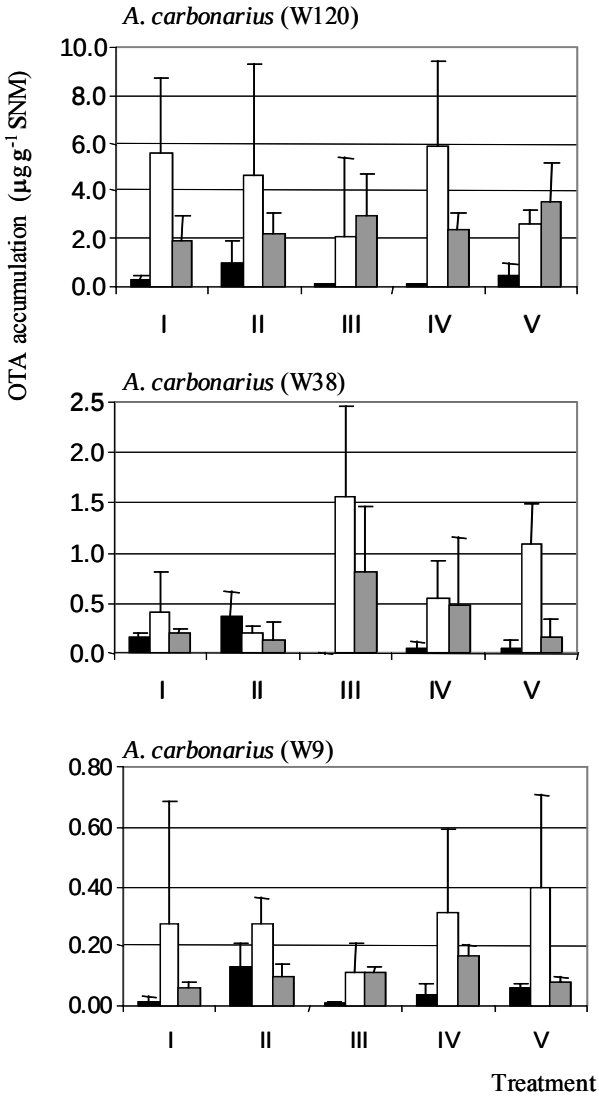

Figure 1. OTA accumulation (µg g^{-1} SNM) after 3 , 5 and 10 days of incubation at different conditions of light and temperature (treatments: I, light 28ºC/darkness 20 ºC; II, light 28 ºC/darkness 28 ºC; III, light 20 ºC/ darkness 20 ºC; IV, light 28 ºC/light 20 ºC; V, darkness 28 ºC/ darkness 20 ºC). Each value represents the mean of three replicates ± SD.

DISCUSSION

Alternation of light and darkness and day and night temperatures simultaneously (treatment I) is the situation more similar to the field conditions. However, it is difficult to estimate fungal growth and toxin production under field conditions in which temperatures fluctuate daily and seasonally, being able to reach higher values than 28 ºC and lower than 20 ºC before harvest. Moreover, photoperiod could vary in length, and other factors such as humidity, viticulture practices, etc. could also affect fungal growth and OTA production in field.

A. carbonarius growth was favoured by photoperiod (Table 2). When simulating an alternation of day and night temperatures, maximum growth rates were detected in treatment I. Few articles are published reporting the effect of photoperiod or alternating temperatures on filamentous fungi and any on black aspergilli. Similarly to our results, Reiss (1975) reported a slight growth-promoting effect in the growth of *A. parasiticus* in the absence of light. Furthermore, the mycelial growth of *A. alternata*, a fungi commonly isolated from grapes, although not being significantly inhibited by white light exposure (180 W/m^2), trended to be higher in dark conditions (Söderhäll et al., 1978).

The highest growth in our study was detected in treatments II and I, no significant differences being found between them. This means that photoperiod is the reason for the stimulation in growth and not the variation of temperature, although the temperature had an important effect on *A. carbonarius* growth. The maximum growth was observed in treatment II, as the temperature selected for representing day, 28 ºC, was close to 30 ºC, the optimum temperature reported for *A. carbonarius* growth *in vitro* (Bellí et al., 2004a, 2005b). Minimum growth was detected under treatment III and IV as continuous night temperature and light exposure, respectively, were detrimental factors for *A. carbonarius* growth.

Continuous presence of light did not reduce the yields of OTA in this study, similar to aflatoxin production by *A. parasiticus* reported in bread (Reiss, 1975). However, the effect of light on aflatoxin production is unclear. Some studies reported an inhibitory effect of light on the aflatoxin production by *A. flavus* (Joffe and Lisker, 1969) and by *A. parasiticus* (Bennett et al., 1971). Others showed a greater production under light exposure (Aziz and Moussa, 1997), meanwhile no effect was detected by Benett et al. (1978). In contrast, twelve-hours of light exposure were sufficient to decrease significantly the production of some secondary metabolites of *A. alternata*, such as alternariol and alternariol monomethyl ether, probably due to a change in the metabolism preceding the production of toxins (Söderhäll et al, 1978). On the contrary, the formation of ochratoxin A produced by *A. ochraceus* (Aziz and Moussa, 1997) and patulin produced by *Penicillium expansum* (Reiss, 1975), was considerably higher in light than in darkness. Häggblom and Unestam (1979) analysed the effect of different sources of light on mycotoxins produced by *A. alternata*. Blue light inhibited alternariol and alternariol

monomethyl ether production by 69 and 77 %, respectively, meanwhile red light gave no reduction of toxin levels.

The amount of OTA detected in this study was not statistically affected by alternating temperatures. Previous studies on OTA production by *A. ochraceus* in raw coffee reported a higher production under alternating temperature (25 ºC/14 ºC) than at constant temperature (Palacios-Cabrera et al., 2004). Similarly, fumonisin B_1 production by *Fusarium moniliforme* and *F. proliferatum* has been reported to be affected by cycling temperatures (Ryu et al., 1999).

Similar to this study, W120 isolate was previously considered as a higher OTA producer (Bellí et al., 2004a, 2005b). Maximum amounts of OTA detected after 5 days of incubation were also in agreement with previous work reporting the optimum time for *A. carbonarius* OTA production on SNM at 25 ºC (Bellí et al., 2004b).

OTA detected after 3 days of incubation was the lowest in treatment III, as growth of the colonies at 20 ºC was minimal in such a short time. However, when the mould started growing, incubation at night temperature became a good condition for OTA production. This result agrees with previously reported optimum temperature (20 ºC) for *A. carbonarius* OTA production (Bellí et al., 2004a). At the highest temperature, OTA formation was restricted as metabolism was used for growing.

Predicting OTA accumulation in grapes in the field is far from possible. This study gives a general overview of the influence of field factors such as photoperiod and day/night temperature in the ochratoxigenic capacity of the main OTA-producing fungi in grapes. The ability of *A. carbonarius* to produce OTA reported in previous laboratory studies has been demonstrated to be stimulated in field conditions.

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8.4.2.4. Effect of fungicides

Fumigation is a treatment measure intended to eliminate the maximum percentage of pests infesting agricultural commodities. Good fumigants should have the following qualities:

- Must have a sufficiently low boiling point so it can easily be converted to gaseous state and remain in that state during fumigation.

- Be insoluble in water, since those that are highly soluble in water are highly sorbed by treated commodities, and high sorption rates of a commodity are positively correlated to an excess amount of residue.

- Must not leave an excess amount of toxic residue.

- Must not cause external injury as burns, pitting or blackening.

- Must not cause internal injury as discoloration of fruit or malfunction of plant systems.

- Must not prevent uniform maturation and softening of fruits and vegetables or retard or excite growth of plant or affect seed germination.

- Must not cause off flavour in fruits, vegetables or foodstuff.

- Must not shorten shelf life of commodities.

▪ **Impact of fungicides on *A. carbonarius* growth and OTA production on SNM and on grapes**

A study to evaluate the impact of the application of several fungicide treatments used in Spanish vines, on *A. carbonarius* growth and OTA production was developed. Three trials were designed in order to:

- Screen 26 fungicides at the doses recommended by manufacturers on SNM medium at 20 ºC and 30 ºC.

- Find out the minimum inhibitory concentration (MIC) of each fungicide for *A. carbonarius* growth on SNM.

- Investigate the effect of several fungicides on *A. carbonariu*s-inoculated grapes.

In synthetic medium, nine fungicides significantly reduced *A. carbonarius* growth rate, meanwhile 13 completely inhibited its growth. In general, growth was faster at 30 ºC than at 20 ºC, contrary to OTA production. Fungicides that stopped fungal growth also inhibited OTA production, but not all the fungicides that reduced growth reduced the OTA synthesis. In general, fungicides that contained copper or strobilurin reduced both growth and OTA production, contrary to sulfur fungicides. At the optimum temperature for *A. carbonarius* growth, 30 ºC, higher amounts of fungicide were needed to prevent fungal growth than at 20 ºC. Among the fungicides that inhibited *A. carbonarius* growth on SNM at the initial doses, cyprodinil seemed to be the active ingredient more effective to stop fungal growth when testing reduced doses. Fungicide effect on grapes was similar than that on synthetic medium. Both infection and OTA production were reduced when using cyprodinil 37.5 % + fludioxonil 25 % and azoxystrobin. Penconazole 10 % also showed a clear reduction of the OTA production at both temperatures, although infection was only reduced at 20 ºC. OTA reduction was strain and temperature-dependent. In general, fenhexamid 50 %, mancozeb 80 % and copper 50 % enhanced infection and OTA production.

Impact of fungicides on *Aspergillus carbonarius* growth and ochratoxin A production on synthetic grape-like medium and on grapes. *Food Additives and Contaminants* (submitted).

- **Effect of chemical treatments on ochratoxigenic fungi and common mycobiota of grapes in the field**

The effect of the application of two fungicides (cyprodinil and cyprodinil+fludioxonil) was studied on the mycoflora of grapes, especially on ochratoxigenic fungi. Different doses and application times were analysed. Grape mycobiota was isolated and identified and a classification of black aspergilli was carried out, finding 81.7 % of the isolates belonging to *Aspergillus niger* aggregate and 18.3 % to *A. carbonarius*. The ability to produce ochratoxin A was studied on CYA medium in 238 isolates. Most of *A. carbonarius* (97.2 %) produced detectable amounts of OTA, while only 2.9 % of *A. niger* aggregate were OTA producers. Most of the isolates (58 %) produced less than 2.5 $\mu g\ g^{-1}$ CYA. That, together with the highest levels of black aspergilli detected near harvest, proved the reported theory that they are the main responsible for OTA in grapes. The fungicides studied had a significant effect on black aspergilli in three of the four vineyards sampled, as the natural increase of black aspergilli when approaching harvest was in general lower in all the fields treated with fungicides, in comparison with the control treatment. A mixture of cyprodinil (37.5 %) and fludioxonil (25 %) applied at veraison and 21 days before harvest, was the most effective treatment to prevent black aspergilli in grapes, together with a single application of this mixture at veraison followed by an application 21 days before harvest of cyprodinil (50 %). No OTA was

detected in musts (n=112) produced from either the control treatment or the treated grapes.

Effect of chemical treatments on ochratoxigenic fungi and common mycobiota of grapes (*Vitis vinifera*). *Journal of Food Protection* (submitted).

Impact of fungicides on *Aspergillus carbonarius* growth and ochratoxin A production on synthetic grape-like medium and on grapes.

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ABSTRACT

A study to evaluate the impact of the application of several fungicide treatments used in Spanish vines, on *Aspergillus carbonarius* growth and ochratoxin A (OTA) production was developed. Three trials were designed in order to: i) screen 26 fungicides at the doses recommended by manufacturers on grape-like synthetic medium (SNM) at 20 ºC and 30 ºC; ii) find out the minimum inhibitory concentration (MIC) of each fungicide for *A. carbonarius* growth on synthetic medium, and iii) investigate the effect of several fungicides on *A. carbonarius*-inoculated grapes. In synthetic medium, nine fungicides significantly reduced *A. carbonarius* growth rate, meanwhile 13 completely inhibited its growth. In general, growth was faster at 30 ºC than at 20 ºC, contrary to OTA production. Fungicides that stopped fungal growth also inhibited OTA production, but not all the fungicides that reduced growth reduced the OTA synthesis. In general, fungicides that contained copper or strobilurins reduced both growth and OTA production, contrary to sulfur fungicides. At the optimum temperature for *A. carbonarius* growth, 30 ºC, higher amounts of fungicide were needed to prevent fungal growth than at 20 ºC. Among the fungicides that inhibited *A. carbonarius* growth on SNM at the initial doses, cyprodinil seemed to be the active ingredient more effective to stop fungal growth when testing reduced doses. Fungicide effect on grapes was similar than that on synthetic medium. Both infection and OTA production were reduced when using cyprodinil 37.5% +fludioxonil 25% and azoxystrobin. Penconazole 10% also showed a clear reduction of the OTA production at both temperatures, although infection was only reduced at 20 ºC. OTA reduction was strain and temperature-dependent. In general, fenhexamid 50%, mancozeb 80% and copper 50% enhanced infection and OTA production.

KEY WORDS: fungicides, *Aspergillus carbonarius*, ochratoxin A, grapes.

***Food Additives and Contaminants* (submitted)**

INTRODUCTION

Ochratoxins are fungal secondary metabolites produced mainly by fungi from the genera *Aspergillus* and *Penicillium*, which are present in a wide variety of foods. Ochratoxin A is one of the more studied mycotoxins in wines nowadays, having a recent legislation in the European Union (Commission Regulation 123/2005), mainly due to its high toxicity and its presence in wines over the world. Its production in grapes from Mediterranean area is associated with different *Aspergillus* spp., mostly black aspergilli and among them, *A. carbonarius* (Cabañes *et al*. 2001, Battilani *et al*. 2003, Bellí *et al*. 2004a).

Prevention of growth of mycotoxin-producing fungi is the most effective strategy for controlling the presence of mycotoxins in foods. This could be achieved by knowing the critical limits of different ecophysiological factors affecting fungal infection and mycotoxin synthesis, but in many cases, the use of fungicides is the only efficient, cost-effective, and often successful way to prevent the mould growth (Munimbazi *et al*. 1997).

The aim of this study was to evaluate the impact of the application of several fungicides to grapes, on *A. carbonarius* growth and OTA production. The experimental design was divided in three parts in order to: i) screen the main fungicides used in Spanish vines, to test their efficiency against *A. carbonarius* growth and OTA production on synthetic nutrient medium (SNM); ii) find out the minimum inhibitory concentration (MIC) of each fungicide for *A. carbonarius* growth on SNM; iii) investigate the effect of several fungicides on growth and OTA production of *A. carbonarius* inoculated on grapes.

MATERIAL AND METHODS

Screening of the main fungicides used in vines on synthetic grape-like medium

Three ochratoxigenic *A. carbonarius* strains (3.161, 3.162 and 3.168, grape-isolated from Italy, France and Spain, respectively), were used to centrally inoculate (106 spores ml-1) Petri dishes containing 20 ml of synthetic nutrient medium (SNM), which had a composition similar to grapes, and a water activity (a_w) level of 0.99 a_w (Bellí et al. 2004b). Strains are held in the culture collection of the Food Technology Department, University of Lleida, Spain.

Twenty-six fungicides commonly used in Spanish vines, were added to the medium at the doses recommended by manufacturers (Table 1). Each fungicide was aseptically added to the autoclaved medium before plating it. No fungicides were added to control plates. Plates were incubated at 20 ºC and 30 ºC inside plastic bags. Colony diameters were measured after 3, 5 and 7 days and OTA was extracted after 7 days following the method of Bragulat *et al*. (2001).

Table 1. Fungicides used in the study, their composition, origin and doses recommended by the manufacturers.

Code	Fungicide	Company	Composition	Dose
F1	TOPAS	Syngenta	Penconazole 10% p/v	0.35 ml l^{-1}
F2	SHIRLAN 500SC	Syngenta	Fluazinam 50% p/v	2 ml l^{-1}
F3	QUADRIS	Syngenta	Azoxystrobin 25 % p/v	2.25 ml l^{-1}
F4	Experimental product	Syngenta	CGA302130	2 ml l^{-1}
F5	CUPROCOL	Syngenta	Copper oxychloride 70% p/v	2 ml l^{-1}
F6	GEOXE	Syngenta	Fludioxonil 50%	0.5 g l^{-1}
F7	Experimental product	Syngenta	CGA379438	1 g l^{-1}
F8	THIOVIT JET	Syngenta	Sulfur 80% WG	4 g l^{-1}
F9	SWITCH	Syngenta	Cyprodinil 37.5% + Fludioxonil 25%	1.8 g l^{-1}
F10	CHORUS 50 WG	Syngenta	Cyprodinil 50%	2 g l^{-1}
F11	SUMISCLEX 50WP	Masso	Procymidone	1 g l^{-1}
F12	RIDOMIL GOLD COMBI	Syngenta	Folpet 40% + Mefenoxam 5% WP	2 g l^{-1}
F13	QUADRIS DUO	Syngenta	Azoxystrobin 18.7% + Cymoxanil 12% WG	2.25 g l^{-1}
F14	TELDOR	Bayer	Fenhexamid 50% p/p	2 g l^{-1}
F15	EUPAREN M	Bayer	Tolyfluanid 50% p/p	1.75 g l^{-1}
F16	FOLICUR 25EW	Bayer	Tebuconazole 25% p/v	0.70 ml l^{-1}
F17	FLINT	Bayer	Trifloxystrobin 50% p/p	0.13 g l^{-1}
F18	CAPLUQ-50	Luqsa	Captan 50% p/p	3.5 g l^{-1}
F19	CARBENLUQ-50	Luqsa	Carbendazim 50% p/p	0.6 g l^{-1}
F20	COBRELUQ-50	Luqsa	Copper oxychloride 50% p/p	3.5 g l^{-1}
F21	CUPROLUQ	Luqsa	Cuprous oxide 75% p/p	2 g l^{-1}
F22	LUQSAZUFRE	Luqsa	Sulfur 80% p/p	5 g l^{-1}
F23	MANCOZEB 80	Luqsa	Mancozeb 80% p/p	3 g l^{-1}
F24	TMTD 80	Luqsa	Tiram 80% p/p	2.5 g l^{-1}
F25	ZICOLUQ 320	Luqsa	Copper oxychloride 22% p/p + Mancozeb 17.5% p/p	5 g l^{-1}
F26	HIDROXILUQ 800	Luqsa	Copper hydroxide 80% p/p + Copper 50 % pp	2 g l^{-1}

High performance liquid chromatography (HPLC) with fluorescence detection (Waters 474, Milford, Massachusetts, U.S.A.) (λ_{exc} 330 nm; λ_{em} 460 nm) was used for OTA analysis. The mobile phase was acetonitrile-water-acetic acid (57:41:2) (1.0 ml min^{-1}) and a C_{18} column (Waters Spherisorb 5 μm, ODS2, 4.6x250 mm) was used. The injection volume and the retention time were 25 μl and 7.1 minutes, respectively. The detection limit of the analysis was 0.02 μg OTA g^{-1} of SNM, based on a signal-to-noise ratio of 3:1. The ochratoxin A standard was from *A. ochraceus* (Sigma-Aldrich, Steinheim, Germany). The standard solution was made in methanol and concentration confirmed by using an UV spectrophotometer. Three repetitions were carried out, both for growth and OTA studies.

Minima doses preventing A. carbonarius growth on synthetic grape-like medium

All the fungicides that prevented *A. carbonarius* growth at the doses recommended by the manufacturers in the previous experiment (n=13) were selected for this study in order to find the minimum concentration that inhibits the growth of this mould (MIC). Spore suspensions of 3.162 and 3.168 *A. carbonarius* strains were adjusted to contain approximately 10^6 spores ml^{-1} for use as inoculum. SNM plates (0.99 a_w) with decreasing concentrations of those fungicides (D, dose recommended by the manufacturer; d1, 0.75xD; d2, 0.5xD; d3, 0.25xD; d4, 0.1xD; d5, 0.01xD and d6, 0.005xD) were single-point inoculated and incubated at 20 ºC and 30 ºC. Growth was measured daily during a period of 30 days. Three repetitions were carried out.

Effect of fungicides on grapes

The effect of six fungicides applied directly to grapes was investigated. F3, F14 and F26 were used at the dose recommended by the manufacturer and F1, F9 and F23 were used at d4 (0.1xD). Table grapes (Red Globe var.) were surface disinfected by dipping them in NaClO (0.1 % Cl) and ethanol (70 %) solutions for 30 seconds and excess of moisture was aseptically removed. Afterwards, 20 grapes were dipped in a fungicide solution for 30 seconds and were placed onto a grid inside plastic boxes containing 300 ml of water to keep a high relative humidity (90-100%) throughout the experiment. In control treatments, grapes were dipped in water instead of fungicide. For culturing, 10^3 spore suspensions of two *A. carbonarius* strains (3.162 and 3.168), were sprayed onto the grapes. After 7 days of incubation at 20 ºC and 30 ºC, the percentage of grapes infected by *A. carbonarius* was assessed and OTA was extracted from the whole set of grapes of each treatment (Bezzo *et al*. 2000). Twenty-five μl of each sample were injected into the HPLC system equipped with a fluorescence detector (Waters 474, Milford, Massachusetts, U.S.A.) (λ_{exc} 230 nm; λ_{em} 458 nm) and a C_{18} column (Waters Spherisorb 5 μm, ODS2, 4.6x250 mm). The analysis was performed under isocratic conditions, with acetonitril 48% -sodium acetate 4mM/acetic acid (19/1)- 52%, as the mobile phase,

pumped at a flow rate of 1 ml min^{-1}. The injection volume and the retention time were 25 µl and 12 min, respectively. The detection limit of the analysis was 0.05 µg l^{-1}, based on a signal/noise ratio of 3:1. OTA was quantified by the external standard method. The ochratoxin standard was from *Aspergillus ochraceus* (Sigma-Aldrich, Steinheim, Germany). The standard solution was made in methanol and confirmed by using an UV spectrophotometer.

Statistical treatment of the results

The regression lines of colony diameters against days after inoculation were calculated for each fungicide and were used to obtain the growth rate under each treatment conditions. Fungicide effect on mycelial growth on SNM and OTA production, both in medium and in natural grapes, were statistically analysed with SAS Enterprise Guide software (SAS Institute, version 2.0, Inc., Cary, N.C., U.S.A.) by analysis of variance followed by either LSMEAN or Duncan multiple range tests. Statistical significance was judged at $P<0.001$.

RESULTS

Screening of the main fungicides used in vines on synthetic grape-like medium

Significant differences were detected for the single factors temperature and fungicide and their interaction, while all the strains showed statistically similar growth (Table 2). Growth was faster at 30 ºC than at 20 ºC, except for F2 and F11. Nine fungicides significantly reduced *A. carbonarius* growth rate at both temperatures (F2, F5, F7, F11, F13, F14, F20, F21 and F26) in comparison with the control treatment, meanwhile thirteen fungicides completely inhibited fungal growth at the dose assayed (F1, F4, F6, F9, F10, F12, F15, F16, F18, F19, F23, F24 and F25) (Table 3). No significant effects were observed for the remaining four fungicides on growth.

The fungicides had a significant effect on OTA production by *A. carbonarius*, because the 13 fungicides that prevented fungal growth also inhibited OTA production (Table 4). Among the remaining fungicides, analysis of variance showed that none of them reduced significantly OTA production. The interaction fungicide x temperature was also significant. Contrary to the growth pattern, OTA production was in general higher at 20 ºC than at 30 ºC. Although not having a significant weight, general trends can be drawn from the results. Mean levels of OTA production showed that most of the fungicides that reduced *A. carbonarius* growth, also reduced OTA production, with the exception of F2, F5 and F11, which favoured toxin production at both temperatures. OTA was also favoured by the addition of F7 at 20 ºC and F14, F21 and F26 at 30 ºC. OTA was also higher than the control under the effect of F8 at both temperatures although growth was

only stimulated at 30 ºC. Contrary, F17 reduced OTA production although growth was favoured at 20 ºC. OTA was favoured at 20 ºC and 30 ºC under the effect of F22, the unique fungicide that increased fungal growth at both temperatures. Figure 1 compares the growth rate and the amount of OTA detected at both temperatures after the application of each fungicide with the control treatment, which is represented at the origin of coordinates. Control growth and OTA production detected after the application of each fungicide has been substracted from growth and OTA production by the control treatment. Thus, fungicides in the third quadrant of the graphic resulted in a reduction of both growth and OTA production.

Table 2. Analysis of variance of the effect of fungicide on the growth of three strains of *A. carbonarius* (3.161, 3.162 and 3.168) at two temperatures (20 ºC and 30 ºC).

Source	DF	Mean Square	F Value
T	1	265.29	16.77*
fungicide	26	52.34	3.31*
strain	2	2.26	0.14^{ns}
T x fungicide	26	35.26	2.23*
T x strain	2	2.66	0.17^{ns}
fungicide x strain	52	7.27	0.46^{ns}
T x fungicide x strain	52	10.13	0.64^{ns}

*significant ($P<0.001$); ^{ns} not significant.

Table 3. Mean growth rates (mm day^{-1}) and OTA production on Synthetic Nutrient Medium (µg g^{-1} SNM) at two temperatures (20 ºC and 30 ºC) by three strains of *A. carbonarius* (3.161, 3.162 and 3.168). Values are the mean of the three strains and three replicates of each ± standard deviation. Data in each column followed by different letters are significantly different in the Duncan test.

	Growth rate (mm day^{-1})		OTA production (µg g^{-1} SNM)	
Fungicide	20 ºC	30 ºC	20 ºC	30 ºC
Control	5.46 ± 0.16^{ab}	7.98 ± 0.32^{ab}	5.68 ± 5.13^{bc}	1.14 ± 1.17^{c}
F1	N.G.	N.G.	<d.l.^{d}	<d.l.^{d}
F2	1.73 ± 0.15^{ed}	1.67 ± 0.52^{fg}	19.66 ± 16.24^{a}	14.20 ± 0.81^{b}
F3	4.50 ± 0.32^{bc}	7.79 ± 1.77^{ab}	2.84 ± 4.70^{bc}	0.22 ± 0.39^{c}
F4	N.G.	N.G.	<d.l.^{d}	<d.l.^{d}
F5	1.53 ± 0.63^{ed}	3.00 ± 0.43^{ef}	13.34 ± 20.29^{abc}	2.58 ± 3.01^{c}
F6	N.G.	N.G.	<d.l.^{d}	<d.l.^{d}
F7	3.91 ± 0.35^{c}	6.77 ± 0.10^{bc}	14.41 ± 17.98^{ab}	0.64 ± 0.59^{c}
F8	5.24 ± 0.65^{ab}	8.65 ± 0.99^{a}	8.03 ± 7.00^{abc}	8.92 ± 9.55^{bc}
F9	N.G.	N.G.	<d.l.^{d}	<d.l.^{d}
F10	N.G.	N.G.	<d.l.^{d}	<d.l.^{d}
F11	2.26 ± 1.56^{d}	1.59 ± 0.18^{g}	11.29 ± 12.74^{abc}	58.61 ± 18.23^{a}
F12	N.G.	N.G.	<d.l.^{d}	<d.l.^{d}
F13	1.91 ± 0.42^{ed}	2.66 ± 0.22^{efg}	0.96 ± 1.56^{c}	0.34 ± 0.59^{c}
F14	3.92 ± 0.29^{c}	6.34 ± 0.43^{c}	4.28 ± 3.73^{bc}	1.96 ± 3.27^{c}
F15	N.G.	N.G.	<d.l.^{d}	<d.l.^{d}
F16	N.G.	N.G.	<d.l.^{d}	<d.l.^{d}
F17	5.53 ± 0.05^{ab}	7.58 ± 0.83^{abc}	1.24 ± 2.14^{c}	0.95 ± 1.64^{c}
F18	N.G.	N.G.	<d.l.^{d}	<d.l.^{d}
F19	N.G.	N.G.	<d.l.^{d}	<d.l.^{d}
F20	2.20 ± 0.70^{d}	4.41 ± 0.89^{d}	1.05 ± 1.72^{c}	0.94 ± 1.62^{c}
F21	0.85 ± 0.46^{e}	2.96 ± 0.35^{ef}	0.07 ± 0.12^{c}	4.06 ± 2.77^{c}
F22	5.72 ± 0.81^{a}	8.71 ± 1.04^{a}	6.82 ± 6.94^{bc}	3.96 ± 5.43^{bc}
F23	N.G.	N.G.	<d.l.^{d}	<d.l.^{d}
F24	N.G.	N.G.	<d.l.^{d}	<d.l.^{d}
F25	N.G.	N.G.	<d.l.^{d}	<d.l.^{d}
F26	2.64 ± 0.30^{d}	3.56 ± 0.06^{ed}	1.97 ± 2.52^{bc}	1.86 ± 3.22^{c}

N.G. no growth; <d.l. below detection limit.

Table 4. Analysis of variance of the effect of fungicide on OTA production capacity of three *A. carbonarius* strains (3.161, 3.162 and 3.168) at two temperatures (20 ºC and 30 ºC).

Source	DF	Mean Square	F Value
T	1	0.33	0.00^{ns}
fungicide	26	318.25	3.15*
strain	2	291.63	2.88^{ns}
T x fungicide	26	471.85	4.67*
T x strain	2	223.00	2.21^{ns}
fungicide x strain	52	133.12	1.32^{ns}
T x fungicide x strain	52	110.88	1.10^{ns}

*significant (P<0.001); ^{ns} not significant.

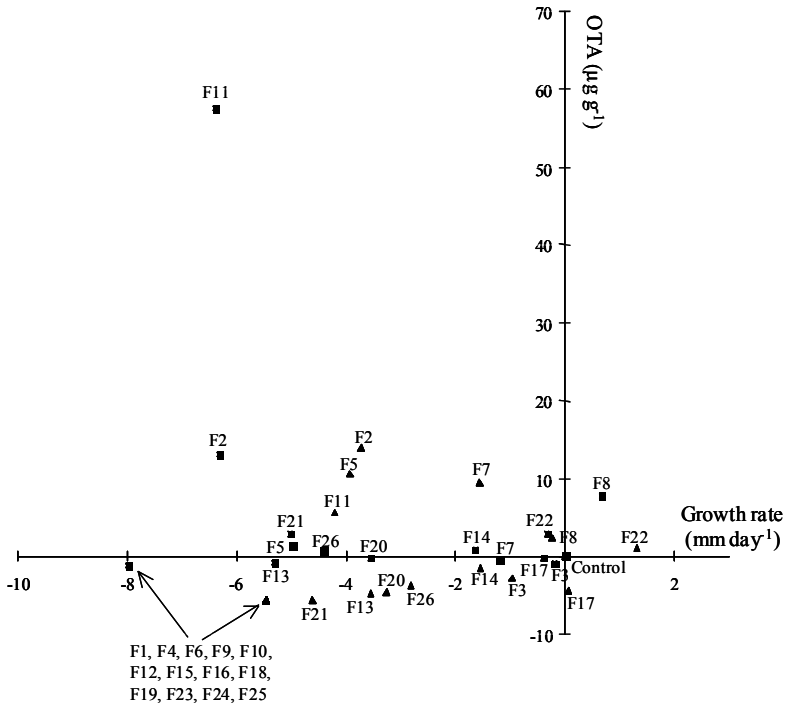

Figure 1. Growth rate (mm day^{-1}) and OTA production (µg g^{-1}) at ▲20 ºC and ■30 ºC of three strains of *A. carbonarius* (3.161, 3.162 and 3.168) after the addition of several fungicides (F1-F26) to the SNM medium. Values are the mean of the three strains. No fungicide was added to the control treatment and growth and OTA production was considered as zero.

Minima doses preventing A. carbonarius growth on synthetic grape-like medium

No significant differences were found between the two strains of *A. carbonarius* tested (data not shown). At the optimum temperature for *A. carbonarius* growth, 30 ºC, higher concentration of fungicide was needed to prevent fungal growth than at 20 ºC. Each fungicide had a different effect on *A. carbonarius* growth, but most of them were effective at doses around $1/4^{th}$ (d3) of the dose recommended by the manufacturer (D) (Table 5). Only three fungicides did not prevent growth at this dose: F4 at 0.75xD (d1) and F1 and F16 at 0.10xD (d2), in the assays at 30 ºC. $1/10^{th}$ of D (d4), was the MIC of fungicides F4 and captan 50% (F18) at 20 ºC, and fludioxonil (F6) and tolyfluanid (F15) at both temperatures. A mixture of cyprodinil 37.5% and fludioxonil 25% (F9) and cyprodinil alone (F10), were the most effective fungicides, as they hinder growth at the minimum doses assayed (d5 and d6, respectively).

Table 5. *A. carbonarius* growth at 20 ºC and 30 ºC on SNM containing different fungicides at different doses: D, dose recommended by the manufacturer; d1, 0.75xD; d2, 0.5xD; d3, 0.25xD; d4, 0.1xD; d5, 0.01xD; d6, 0.005xD.

	D		d1		d2		d3		d4		d5		d6	
Fung.	**20**	**30**	**20**	**30**	**20**	**30**	**20**	**30**	**20**	**30**	**20**	**30**	**20**	**30**
Control	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F1	-	-	-	-	-	-	-	+	+	+	+	+	+	+
F4	-	-	-	+	-	+	-	+	-	+	+	+	+	+
F6	-	-	-	-	-	-	-	-	-	-	+	+	+	+
F9	-	-	-	-	-	-	-	-	-	-	-	+	+	+
F10	-	-	-	-	-	-	-	-	-	-	-	-	-	+
F12	-	-	-	-	-	-	-	-	+	+	+	+	+	+
F15	-	-	-	-	-	-	-	-	-	-	+	+	+	+
F16	-	-	-	-	-	-	-	+	+	+	+	+	+	+
F18	-	-	-	-	-	-	-	-	-	+	+	+	+	+
F19	-	-	-	-	-	-	-	-	+	+	+	+	+	+
F23	-	-	-	-	-	-	-	-	+	+	+	+	+	+
F24	-	-	-	-	-	-	-	-	+	+	+	+	+	+
F25	-	-	-	-	-	-	-	-	+	+	+	+	+	+

+ *A. carbonarius* growth; – no growth.

Effect of fungicides on grapes

Three fungicides that completely inhibited *A. carbonarius* on SNM: penconazole 10% (F1), cyprodinil 37.5%+fludioxonil 25% (F9) and mancozeb 80% (F23), and three fungicides that reduced its growth at the initial doses assayed: azoxystrobin (F3), fenhexamid 50% (F14) and copper 50% (F26), were chosen for this study. The factors fungicide and temperature were significant in both grape infection and OTA production experiments, and the factor strain only in the OTA production trial (data not shown). The percentage of grapes infected by *A. carbonarius* was calculated for each treatment. Infection was significantly higher at 30 ºC than at 20 ºC. A percentage of reduction of the percentage of infection for each treatment was determined by comparison with the control. The average of the percentage of reduction of both strains is shown in Figure 2. Infection was reduced at both temperatures with azoxystrobin and cyprodinil 37.5%+fludioxonil 25% , and with penconazole 10% at 20 ºC. Maximum reduction (> 95 %) was achieved with cyprodinil 37.5%+fludioxonil 25% at 20 ºC, followed by azoxystrobin (65 %) and penconazole 10% (59 %) at the same temperature. Around 10 % reduction in the infection percentage was also detected with mancozeb 80% at 20 ºC. In general, fenhexamid 50%, mancozeb 80% and copper 50% enhanced *A. carbonarius* grape infection, especially at 30 ºC.

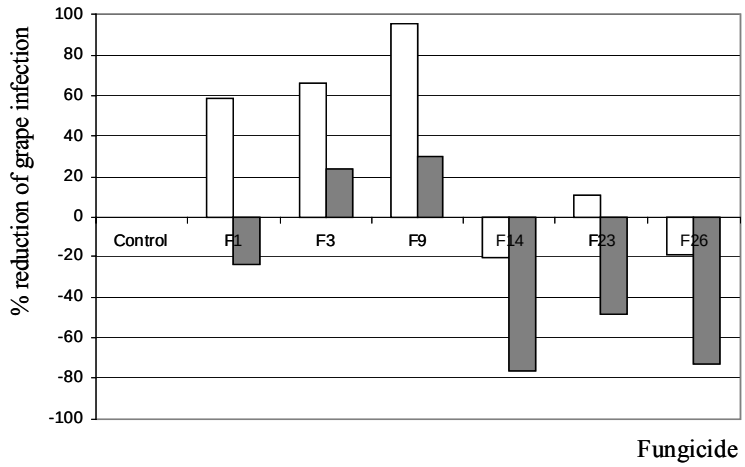

Figure 2. Percentage of reduction in the percentage of grape infection by *A. carbonarius* treated with different fungicides and incubated at □20 ºC and ■30 ºC. Values are the mean of two strains.

Half of the fungicides (penconazole 10%, mancozeb 80% and cyprodinil 37.5%+fludioxonil 25%) showed a clear reduction of the OTA production at both temperatures, in comparison with the control treatment (Figure 3). For these fungicides, reduction was higher at 20 ºC than at 30 ºC, except for penconazole 10% for 3.162 strain. Differences in the percentage of reduction were also observed between both strains, especially at 30 ºC, where fungicides were more effective against strain 3.168. At 20 ºC, fenhexamid 50%, azoxystrobin and copper 50% reduced the OTA production for strain 3.168 meanwhile they produced the opposite effect on strain 3.162, increasing OTA production more than 200 % sometimes. At 30 ºC, these three fungicides increased up to 20 % OTA production of both strains.

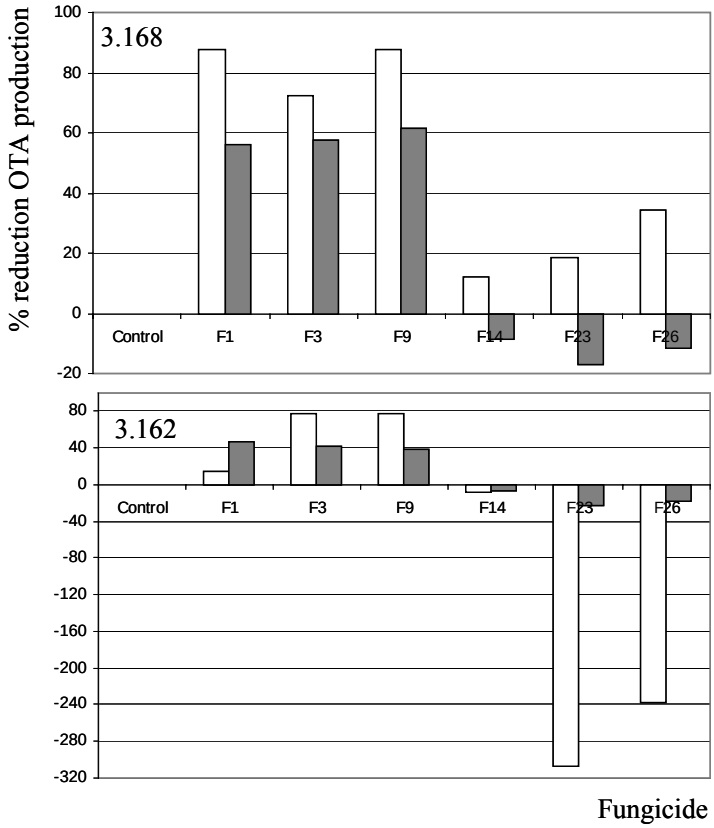

Figure 3. Percentage of reduction of OTA production by two *A. carbonarius* strains (3.162 and 3.168) inoculated on grapes (µg ml^{-1} must) treated with different fungicides and incubated at □20 ºC and ■30 ºC.

DISCUSSION

A significant effort has been concentrated on the development and use of fungicides for the control of other food-spoilage fungi like *Fusarium* spp. (Mathies and Buchenauer 1996, Moss and Frank 1985), *Aspergillus flavus* and *A. ochraceus* (Munimbazi *et al*. 1997), *Botrytis cinerea* (Slawecki et al., 2002), etc, but any on grapes. Data on resistance of *A. carbonarius* to fungicide treatments is inexistent so far. Therefore, the efficiency against this mould of a range of fungicides designed to control other species infecting vines, has been tested in this study. Moreover, it has to be underlined, that the doses proposed by the manufacturers and used in the present study, were also designed for the control of other moulds.

To discuss the results obtained, the different fungicides may be grouped according to their active ingredients. *A. carbonarius* growth and OTA production were minimised when using fungicides with copper in their composition (F20, F21, F24, F25 and F26). However, copper oxychloride was also present in F5, which although limiting *A. carbonarius* growth, did not decrease OTA production. The same OTA-enhancing effect was detected for fluazinam (F2) and procymidone (F11), which were classified as dinitro aniline and dicarboximide fungicides, respectively. It is known that fluazinam (F2), together with azoxystrobin (F3, F13 and F17), thifluzamide and carboxin can interfere with respiration processes (Corbett *et al*. 1984, Guo *et al*. 1991, Sauter *et al*. 1995). Both *A. carbonarius* growth and OTA production were increased, although not significantly, when adding inorganic fungicides containing sulfur to the medium (F8 and F22). Similar effects were detected in a study of the OTA content in red wines produced from vineyards treated with different pesticides (Lo Curto *et al*. 2004). The level of OTA in wines from sulfur-treated grapes was higher than in the other samples. Furthermore, those authors reported azoxystrobin as a fungicide able to reduce OTA concentration in wine, with 96.5 % of reduction. In the present study, the total amount of OTA produced by *A. carbonarius* growing with sulfur fungicides increased due to both the higher OTA-producing capacity detected and the bigger diameters of the colonies. However, it is not clear whether the total amount of OTA accumulated by *A. carbonarius* treated with fluazinam and procymidone increased or not because OTA production stimulation occurred but, in contrast, smaller colonies were observed.

Little is known about the mechanism of action of the active ingredients of the fungicides assayed. For many compounds, spore germination is the growth stage that is most sensitive to inhibition (Slawecki *et al*. 2002). In the present study, fungicides that completely inhibited germination were enclosed in several groups according to their active ingredients: amide (F12 –one component-, F15) and dicarboximide fungicides (F12 –one component-, F18), triazol fungicides (F1 and F16), benzimidazole (F19) and dithiocarbamate fungicides (F23, F24 and F25 –one component-), pyrimidine fungicides (F9 -one component- and F10), phenylpyrrole fungicides (F6 and F9 –one component-), etc. It would be interesting to study growth for longer periods in order to know if this last

group of fungicides at the doses assayed, totally inhibited growth or only prolonged the lag phase of the mould. Doses of these fungicides were reduced in a subsequent experiment in order to find the threshold dose preventing *A. carbonarius* growth. In general, fungicides with the same active ingredients seemed to have similar effects when reducing the doses. Triazol fungicides (F1 and F16) were the less effective fungicides against *A. carbonarius* growth, as just reducing up to one quarter the initial dose the inhibitory growth effect disappeared. Fungicides with the MIC at one quarter of the initial one (F19, F23, F24 and F25), were classified as carbamate and dithiocarbamate fungicides. Cyprodinil seemed to be the active ingredient more suitable to stop fungal growth, as it was a component of the pyrimidine fungicides F9 and F10, which showed the minimum threshold concentrations.

Sauter *et al*. (1995) noted that the group of fungicides containing the strobilurins, blocked electron transport at the cytochrome bc_1 complex of the mitochondrial electron transport chain, and therefore were extremely potent inhibitors of spore germination, but much less active as inhibitors of mycelial growth. No germination-inhibitory effect of fungicides grouped as strobilurin fungicides (F3, F13 and F17) was noticeable in this study, as the three of them allowed *A. carbonarius* growth, although less than the control treatment. Other reported fungicides that typically acted after germination in filamentous fungi by strongly inhibiting mycelial growth, included antimicrotubule agents (carbendazim and N-phenylcarbamates, which inhibited nuclear division (Suzuki *et al*. 1984)), and inhibitors of ergosterol biosynthesis (Buchenauer, 1987). However, in this study, benzimidazol (F19) and dithiocarbamate fungicides (F23, F24 and F25–one component-) showed a completely inhibition of germination as mentioned before.

On grapes, a mixture of cyprodinil (37.5 %) and fludioxonil (25 %) (F9) seemed the best fungicide that controlled *A. carbonarius* growth and OTA production together with penconazole 10% (F1) and azoxystrobin (F3). All three were also restrictive fungicides in terms of growth and mycotoxin production when tested on SNM medium. Penconazole, was previously reported as a synthetic pesticide able to reduce around 90 % the level of OTA in wines made from grapes treated with this fungicide (Lo Curto *et al*. 2004). Fenhexamid 50% (F14) and copper 50% (F26) showed the same effects on synthetic nutrient medium than on grapes, as they increased OTA production, especially at 30 ºC. Temperature was a determinant factor and could influence in the effectiveness of the fungicides. Results are in accordance with previous work, reporting optimum temperatures for *A. carbonarius* growth and OTA production at 30 ºC and 20 ºC, respectively (Bellí *et al*. 2005). Propitious levels of other environmental factors, such as humidity, could also interfere in the efficacy of the fungicides assayed, together with the reiterative application of the same fungicide, as it could modify the equilibrium in the ecosystem, enhancing other microorganisms development, as competing fungi are removed.

Additional *in vitro* studies on grapes testing the whole range of fungicides are needed in order to find out the best active ingredients against *A. carbonarius* development and mycotoxin production. Afterwards, further studies of the *in situ* efficiency of pesticide treatments against *A. carbonarius* infection and OTA production in vines would be required.

ACKNOWLEDGMENTS

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Effect of chemical treatments on ochratoxigenic fungi and common mycobiota of grapes (*Vitis vinifera*)

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ABSTRACT

The effect of the application of two fungicides (cyprodinil and cyprodinil+fludioxonil) was studied on the mycoflora of grapes, especially on ochratoxigenic fungi. Different doses and application times were analysed. Grape mycobiota was isolated and identified and a classification of black aspergilli was carried out, finding 81.7 % of the isolates belonging to *Aspergillus niger* aggregate and 18.3 % to *A. carbonarius*. The ability to produce ochratoxin A was studied on CYA medium in 238 isolates. Most of *A. carbonarius* (97.2 %) produced detectable amounts of OTA, while only 2.9 % of *A. niger* aggregate were OTA producers. Most of the isolates (58 %) produced less than 2.5 μg g^{-1} CYA. That, together with the highest levels of black aspergilli detected near harvest, proved the reported theory that they are the main responsible for OTA in grapes. The fungicides studied had a significant effect on black aspergilli in three of the four vineyards sampled, as the natural increase of black aspergilli when approaching harvest was in general lower in all the fields treated with fungicides, in comparison with the control treatment. A mixture of cyprodinil (37.5 %) and fludioxonil (25 %) applied at veraison and 21 days before harvest, was the most effective treatment to prevent black aspergilli in grapes, together with a single application of this mixture at veraison followed by an application 21 days before harvest of Cyprodinil (50 %). No OTA was detected in musts (n=112) produced from either the control treatment or the treated grapes.

KEY WORDS: *Aspergillus carbonarius*; ochratoxin A; fungicide; insecticide; grapes; *Vitis vinifera*; must.

Journal of Food Protection **(submitted)**

INTRODUCTION

Ochratoxins are mycotoxins which exhibit nephrotoxic, immunosuppressive, teratogenic and carcinogenic properties (1). Ochratoxin A (OTA), the most potent, chlorinated derivative was discovered in 1965 as a secondary metabolite of an *Aspergillus ochraceus* strain (2). *A. niger* var. *niger* was first reported as an OTA-producing species in 1994 (3). Since then, the ability to produce OTA by members of *Aspergillus* section *Nigri* (black aspergilli) has been demonstrated and they have been reported as the main responsible for the OTA levels found in grapes and wine. A three-group classification of this section (uniseriates, *A. niger* aggregate and *A. carbonarius*), according to their morphological characteristics, has been proposed (4, 5). *A. carbonarius* is widely accepted as the main OTA-producing species in grapes in the Mediterranean area. A maximum OTA content legislated in wine and must (6), have led to focus on the strategies to control *A. carbonarius in situ*. This study has emerged in order to contribute to that, by assaying the effect of two fungicides in field. Little literature exists about pesticides on black aspergilli. Recently, a study of the effect of 26 fungicides on *A. carbonarius in vitro* has been reported (7), and previously, Lo Curto et al. (8) had studied the OTA content in wine produced in three Italian regions treated with different pesticides, and Tjamos et al. (9) had evaluated the efficiency of several pesticides in restricting the population of *Aspergillus* spp. in Greek grapes.

Many insecticides and fungicides are commonly used in vines, but none was designed to prevent ochratoxigenic fungi. To our knowledge, only the fungicide Switch (Syngenta-Agro, S.A.) has been recently registered against *Aspergillus* on grapes in Spain. Their active ingredients, fludioxonil and cyprodinil, originally designed to prevent *Botrytis* infection, have shown to prevent *A. carbonarius* growth and OTA production when tested *in vitro* (7), and also seemed to be effective when tested in the field (9). Therefore, two commercial fungicides containing these two active ingredients (Switch and Chorus) were used in the present study in order to determine their effects *in vivo* on the whole mycobiota present in grapes, especially on ochratoxigenic fungi, in four different vineyards located in three different regions in Spain. Furthermore, the amount of OTA in musts elaborated with grapes from the treated vineyards was also analysed.

MATERIAL AND METHODS

Field sampling

Four Spanish vineyards dedicated to grape cultivation for winemaking were selected, two in Turis (Valencia, V1 and V2), one in Villarubia de los Ojos (Ciudad Real, CR) and one in Vendrell (Tarragona, T) (Figure 1). Those vineyards presented high black aspergilli infection in the previous years and consequently they were chosen for the present study. Grape varieties cultivated in V1, V2, CR and T were Tempranillo, Malvasía, Airén and

Macabeo, respectively. Tempranillo is the Spanish most widely planted red vine variety, and the others are white grapes, very common in the respective regions. Four plots (repetitions) were considered in each vineyard, with two different extensions, 30 m^2 in V1 and CR and 70 m^2 in V2 and T. Sampling was carried out in two sampling dates in 2004: the first at veraison, just before the first fungicide application (2-30 August 2004), and the second one (5-24 September 2004) before harvest and after the second fungicide application. Five bunches from each plot were collected in paper bags to reduce handling and prevent external contamination, and kept at 4 ºC until laboratory analysis. Bunches were collected from the ten central vines of each plot.

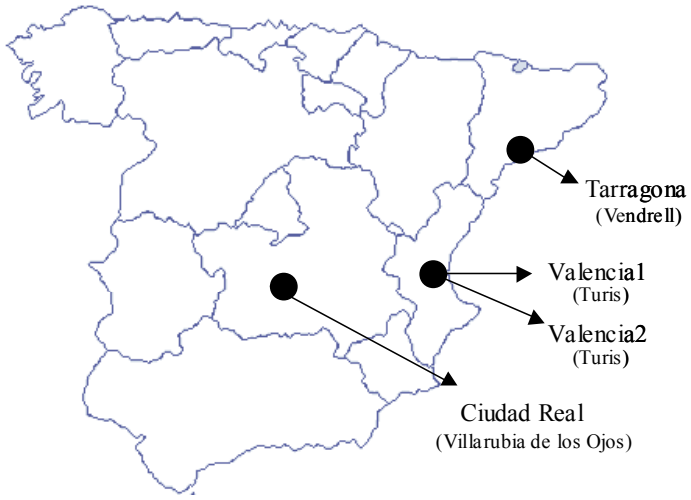

Figure 1. Geographical location of the four different selected vineyards in Spain.

Pesticide treatments

Two commercial fungicides (Switch and Chorus) were sprayed under seven different pest management strategies (treatments) with a manual sprayer onto each plot (Table 1), Switch is a preformulated mixture (37.5 % cyprodinil + 25 % fludioxonil) commercialized in Spain and other countries. Cyprodinil is an anilinopyrimidine fungicide that has some systemic properties, which is taken up into the cuticle and waxy layers of leaves and fruits. Fludioxonil is a Phenylpyrrole fungicide residual component of Switch, which stays on the leaf and fruit surfaces to provide contact activity. Switch is registered in Spain against *Botrytis* and *Sclerotinia* in several crops, and against *Botrytis* and *Aspergillus* in grapes; Chorus (50 % Cyprodinil) is an anilinopyrimidine fungicide with protective activity against Scab, Powdery Mildew and *Botrytis* spp. in apples, and Blossom Blight and Brown rot (*Monilia* spp.) in peaches and nectarines. State registration is pending in Spain. Moreover, high and low doses of an insecticide (Lufox)

were applied in order to evaluate the possible synergistic effect of the pesticides together. This preventive insecticide combines two active ingredients (fenoxycarb 75 g l^{-1} + lufenuron 30 g l^{-1}) for the control of *Lobesia botrana* in vines, as it is designed to destroy the insect eggs and first larval stages, minimizing the risk of damages in the skin of the grape caused by these insects. Nowadays it is in the way to receive governmental registration in Spain.

The doses, the number and timing of applications of each pesticide are indicated in Table 1. No pesticides were used in treatment one which was used as the control. Basic cover treatments were also applied in the fields selected for this experiment, including the control (Table 2), as it was important to reduce the apparition of other pest and diseases in vines that could interfere with *Aspergillus* or *Botrytis*. All the chemicals were obtained from Syngenta-Agro (Spain).

Table 1. Pesticide treatments (dose, number and time of application) on grapes used in this study.

Treatment	Pesticide (commercial name)	Dose	Number and period of application
T1	- (Control)	-	-
T2	Lufox[a] Chorus[b]	1 l ha^{-1} 0.75 kg ha^{-1}	1 1 at harvest
T3	Lufox Chorus	1 l ha^{-1} 0.75 kg ha^{-1}	6 1 at harvest
T4	Lufox Switch[c] Chorus	1 l ha^{-1} 1 kg ha^{-1} 0.75 kg ha^{-1}	1 1 at veraison 1 at harvest
T5	Lufox Switch Chorus	1 l ha^{-1} 1 kg ha^{-1} 0.75 kg ha^{-1}	6 1 at veraison 1 at harvest
T6	Lufox Switch	1 l ha^{-1} 1 kg ha^{-1}	1 2 (veraison and harvest)
T7	Lufox Switch	1 l ha^{-1} 1 kg ha^{-1}	6 2 (veraison and harvest)

[a] Fenoxycarb 75 g l^{-1} + Lufenuron 30 g l^{-1} ; [b] Cyprodinil 50 %; [c] Cyprodinil 37.5% + Fludioxonil 25%.

Table 2. Basic treatments (active ingredients, dose and time of application) applied to all the vineyards studied.

Commercial name	Active ingredients	Dose	Application
Ridomil Gold MZ[a]	4% Mefenoxam 64% Mancoceb	2.5 kg ha^{-1}	14 June
Quadris F[a]	9.35% Toxistrobin 50% Folpet	1.4 l ha^{-1}	14 June
Quadris F[a]	9.35% Toxistrobin 50% Folpet	1.8 l ha^{-1}	22 June
Neptune 310EC[a]	25% Dinocap 6% Difenoconazol	0.6 l ha^{-1}	29 June
Neptune 310EC[a]	25% Dinocap 6% Difenoconazol	0.68 l ha^{-1}	14 July
Neptune 310EC[a]	25% Dinocap 6% Difenoconazol	0.69 l ha^{-1}	27 July
Thiovit[a]	80 % Sulfur	0.4 kg hl^{-1}	6 August

[a] Syngenta-Agro, Spain

Mycobiota analysis

Five grapes were randomly chosen from each bunch and plated directly in Petri dishes containing Dichloran Rose Bengal Chloramphenicol medium (DRBC) under sterile conditions. Plates were incubated for seven days at 25 ºC and colonies of developing fungi were examined and classified into genera (10). The difference in the percentage of each mould between harvest and veraison was calculated and it was statistically compared with the control treatment, in order to know how the fungicide treatment affected. Furthermore, most of the potential OTA producers were isolated onto Czapek Dox agar (CZ) for classification, onto Czapek Yeast Extract agar (CYA) for the OTA production analysis, and incubated at 25 ºC for seven days. All media are described in Pitt and Hocking (10). Black aspergilli were classified according to the morphology of their conidia and conidial heads into three groups: uniseriates, *A. niger* aggregate (biseriates excluding *A. carbonarius*) and *A. carbonarius*.

Screening of fungi for OTA production

The method used was adapted from Bragulat et al. (11) and described in Bellí et al. (12). Briefly, it consisted of an extraction of part of the colony grown in CYA with methanol, filtration of the extract and analysis by high performance liquid chromatography with

fluorescence detection (λexc 330 nm; λem 460 nm) (detection limit 0.01 μg OTA g^{-1} of CYA).

OTA in musts

A total of 112 musts extracted from the five bunches of each treatment collected in the last sampling period (28 musts from each vineyard), were analysed following the method proposed by the International Office of Wine and Vineyard (13).

Statistical analysis

A randomised complete block design was used with a total of 1120 bunches of grapes analysed (5 bunches x 7 treatments x 4 replicates x 4 vineyards x 2 samplings). Analysis of variance and Duncan's multiple range test ($P<0.05$) were carried out with the SAS Enterprise Guide version 2.0 software (SAS Institute, Inc., Cary, N.C., U.S.A.), to determine the influence of the fungicide treatment on the difference between the percentage of the main microorganisms detected in grapes at harvest and at veraison.

RESULTS

Percentage of infection

The main genera isolated from grapes were *Alternaria*, *Aspergillus*, *Botrytis*, *Cladosporium*, *Epicoccum*, *Fusarium*, *Penicillium*, *Rhizopus*, *Trichoderma* and yeasts. Other genera like *Mucor*, *Phoma*, *Ulocladium*, etc. were also isolated although in lower amounts. The percentage of grapes infected by *Cladosporium*, *Alternaria*, *A. ochraceus* and *A. flavus* were significantly higher in the first sampling than in the second one, similarly to *Botrytis* in T vineyard (Table 3).

For other fungi like *Penicillium*, *Trichoderma*, *Fusarium* and *Epicoccum* this was variable according to the vineyard sampled. On the contrary, the number of black *Aspergillus*, *Rhizopus* and yeasts was significantly higher at harvest, except black aspergilli in CR vineyard, where no significant differences between both sampling dates were observed. *Alternaria* was frequently isolated, mainly in the first sampling (48-90 % infection). The percentage of *A. ochraceus* was different in each vineyard, although always less than 5 % . The incidence of *Botrytis* was lower in all the vineyards, and only grapes from T at veraison presented an infection percentage slightly higher than 2 %.

Table 3. Percentage of grapes infected with different fungi from a total of 700 berries analysed in each vineyard (T, Tarragona; V1, Valencia 1; V2, Valencia 2 and CR, Ciudad Real) and sampling period.

Region	Sampl.	Black aspergilli	*Penic.*	*A. ochr.*	yeast	*Clad.*	*Altern.*
T	1[a]	25.6	31.9	3.4	3.9	21.4	65.8
	2[b]	66.0	42.6	0.6	21.4	7.6	9.6
V1	1	9.2	16.9	0.8	4.8	16.4	88.4
	2	39.6	4.0	0.4	53.7	1.6	10.1
V2	1	5.4	12.5	1.7	6.6	23.0	90.1
	2	83.5	1.6	0	8.4	1.5	2.1
CR	1	22.8	6.7	1.6	2.1	19.7	48.9
	2	25.9	20.7	1.6	9.5	17.9	32.5

Region	Sampl.	*Rhiz.*	*Trichod.*	*Fus.*	*A. flavus*	*Botr.*	*Epic.*
T	1	2.6	0.6	6.8	1.6	2.1	4.0
	2	9.7	1.6	2.7	0.7	0.8	1.6
V1	1	0.9	0.6	1.2	0.6	0	2.4
	2	3.8	0.5	1.6	0.3	0.2	0.7
V2	1	0.3	0.5	1.8	0.6	0	1.7
	2	20.9	0	0.9	0	0	2.9
CR	1	3.8	0.1	5.7	1.0	0	3.8
	2	12.6	0	2.2	0.6	0.1	2.7

[a]at veraison; [b]at harvest.

Fungicide treatment had a significant effect on the infection of black *Aspergillus* (Table 4) *Alternaria*, *Epicoccum*, *Cladosporium*, *Rhizopus* (Table 5) in some of the vineyards. Although treatment 5 and 6 reduced significantly the number of *Alternaria* in CR, and treatment 2 and treatment 5 the percentage of *Epicoccum* in T region, it seems that the fungicides did not alter the composition and the rates of the common mycoflora of grapes.

Table 4. Percentage of grape berries infected by ochratoxigenic fungi in each vineyard, sampling (s1, at veraison; s2, before harvest) and treatment (T1-T7), expressed as a decimal. Values are the mean of four repetitions. Letters means significant differences (P<0.05) between the treatments in each region. No letters means that no significant differences were detected.

Region		Ciudad Real			Tarragona			Valencia1			Valencia2		
	T	s1	s2	s2-s1	s1	s2	s2-s1	s1	s2	s2-s1	s1	s2	s2-s1
Black aspergilli	1	0.25	0.51	0.26 a	0.31	0.91	0.60 a	0.19	0.46	0.27	0.12	0.31	0.19 b
	2	0.18	0.20	0.02 ab	0.20	0.75	0.55 a	0.08	0.45	0.37	0.04	0.50	0.46 ab
	3	0.11	0.11	0.01 b	0.32	0.80	0.48 ab	0.04	0.50	0.46	0.05	0.46	0.41 ab
	4	0.20	0.22	0.02 ab	0.30	0.57	0.27 ab	0.06	0.11	0.05	0.01	0.90	0.89 a
	5	0.14	0.17	0.03 ab	0.21	0.57	0.36 ab	0.14	0.32	0.18	0.05	0.70	0.65 ab
	6	0.36	0.06	-0.30 c	0.26	0.59	0.33 ab	0.07	0.36	0.29	0.04	0.84	0.80 ab
	7	0.31	0.07	-0.24 c	0.19	0.43	0.24 b	0.04	0.18	0.14	0.04	0.67	0.63a
A. ochraceus	1	0.02	0.04	0.02	0.05	0.00	-0.05	0.00	0.00	0.00	0.02	0.00	-0.02
	2	0.02	0.00	-0.02	0.05	0.01	-0.04	0.04	0.00	-0.04	0.01	0.00	-0.01
	3	0.00	0.00	0.00	0.01	0.00	-0.01	0.01	0.00	-0.01	0.05	0.00	-0.05
	4	0.03	0.00	-0.03	0.02	0.03	0.01	0.00	0.00	0.00	0.00	0.00	0.00
	5	0.03	0.05	0.02	0.02	0.00	-0.02	0.00	0.00	0.00	0.02	0.00	-0.02
	6	0.01	0.00	-0.01	0.04	0.00	-0.04	0.01	0.03	0.02	0.00	0.00	0.00
	7	0.00	0.02	0.02	0.05	0.00	-0.05	0.00	0.00	0.00	0.01	0.00	-0.01

Table 5. Percentage of grape berries infected by the main mycoflora in each vineyard, sampling (s1, at veraison; s2, before harvest) and treatment (T1-T7), expressed as a decimal. Values are the mean of four repetitions. Letters means significant differences (P<0.05) between the treatments in each region. No letters means that no significant differences were detected.

Region		Ciudad Real			Tarragona			Valencia1			Valencia2		
	T	s1	s2	s2-s1	s1	s2	s2-s1	s1	s2	s2-s1	s1	s2	s2-s1
Cladosporium	1	0.11	0.08	-0.03	0.19	0.00	-0.19	0.16	0.02	-0.14	0.24	0.00	-0.24
	2	0.21	0.24	0.03	0.30	0.06	-0.24	0.08	0.00	-0.08	0.14	0.02	-0.12
	3	0.21	0.16	-0.05	0.19	0.08	-0.11	0.14	0.00	-0.14	0.27	0.00	-0.27
	4	0.26	0.21	-0.05	0.22	0.11	-0.11	0.14	0.00	-0.14	0.16	0.00	-0.16
	5	0.20	0.04	-0.16	0.09	0.15	0.06	0.16	0.03	-0.14	0.17	0.02	-0.15
	6	0.17	0.15	-0.02	0.32	0.10	-0.22	0.12	0.02	-0.10	0.30	0.04	-0.27
	7	0.17	0.33	0.16	0.19	0.03	-0.16	0.31	0.03	-0.28	0.22	0.01	-0.21
Alternaria	1	0.23	0.29	0.06a	0.60	0.06	-0.54	0.89	0.04	-0.85	0.86	0.00	-0.86b
	2	0.41	0.38	-0.03a	0.58	0.10	-0.48	0.89	0.04	-0.85	0.70	0.00	-0.70a
	3	0.41	0.52	0.11a	0.63	0.05	-0.58	0.91	0.13	-0.79	0.95	0.02	-0.93ab
	4	0.69	0.49	-0.20abc	0.67	0.11	-0.56	0.87	0.02	-0.85	0.95	0.03	-0.92b
	5	0.60	0.10	-0.50bc	0.77	0.14	-0.63	0.59	0.14	-0.45	0.67	0.00	-0.67ab
	6	0.58	0.13	-0.45c	0.62	0.09	-0.53	0.84	0.16	-0.69	0.92	0.01	-0.91b
	7	0.39	0.28	-0.11ab	0.74	0.12	-0.62	0.98	0.09	-0.89	0.81	0.05	-0.76ab
Rhizopus	1	0.05	0.11	0.06	0.05	0.07	0.02	0.00	0.01	0.01	0.02	0.03	0.01c
	2	0.03	0.13	0.10	0.02	0.19	0.17	0.02	0.01	-0.01	0.00	0.11	0.11bc
	3	0.06	0.13	0.08	0.02	0.14	0.12	0.01	0.06	0.05	0.00	0.05	0.05c
	4	0.03	0.16	0.13	0.02	0.09	0.07	0.01	0.02	0.01	0.00	0.29	0.29ab
	5	0.04	0.27	0.23	0.02	0.09	0.07	0.02	0.04	0.02	0.00	0.16	0.16bc
	6	0.03	0.05	0.02	0.03	0.06	0.03	0.00	0.02	0.02	0.00	0.34	0.34a
	7	0.02	0.00	-0.02	0.02	0.04	0.02	0.00	0.04	0.04	0.00	0.12	0.12bc
Epicoccum	1	0.03	0.04	0.01	0.02	0.05	0.03a	0.03	0.01	-0.02	0.04	0.03	-0.01
	2	0.02	0.04	0.02	0.06	0.01	-0.05b	0.03	0.01	-0.02	0.01	0.05	0.04
	3	0.01	0.03	0.02	0.02	0.01	-0.01ab	0.02	0.01	-0.01	0.01	0.01	0.00
	4	0.04	0.03	-0.01	0.02	0.00	-0.02ab	0.01	0.00	-0.01	0.00	0.01	0.01
	5	0.05	0.00	-0.05	0.07	0.00	-0.07b	0.01	0.01	0.00	0.03	0.00	-0.03
	6	0.04	0.01	-0.03	0.04	0.01	-0.03ab	0.04	0.00	-0.04	0.01	0.04	0.03
	7	0.06	0.03	-0.03	0.05	0.03	-0.02ab	0.02	0.00	-0.02	0.01	0.01	0.00

Regarding black *Aspergillus*, the fungicides effect was significant in three of the four sampled vineyards: CR, T and V2. Although the natural trend of black aspergilli is to increase when approaching harvest, the rise was lower in the fields treated with fungicides, in comparison with the control treatment, except in V2. This means that the fungicides studied had an effect on black aspergilli. Treatment 7, involving a high application of insecticide and a double application of Switch, showed a high inhibition of black aspergilli infection in T and CR. Treatment 6, which differs only from treatment 7 by a lower insecticide application, was also effective in CR, as the increment in the percentage of black aspergilli between harvest and veraison when applying this treatment, was significantly lower than the control. In all the vineyards except V2, the rise in the black aspergilli infection under treatment 4 and 5 was also lower than in the control.

Black aspergilli classification and OTA-producing capacity

A total of 1648 *Aspergillus* section *Nigri* isolates were identified, 290 of them were isolated on CZ for further classification, 237 of them (81.7 %) belonged to *A. niger* aggregate group, while 53 (18.3 %) were *A. carbonarius*. No black aspergilli uniseriates were detected. The ability to produce OTA of 238 strains of black *Aspergillus* (36 *A. carbonarius* and 202 *A. niger* aggregate) was analysed. *A. carbonarius* resulted the highest OTA producer as 35 strains (97.2 %) were able to produce OTA, while OTA was only detected above the limit of detection in 6 (2.9 %) of the *A. niger* aggregate isolates. Figure 2 shows the amount of OTA produced by each black aspergilli group. Maximum amounts of OTA detected both in *A. carbonarius* and *A. niger* aggregate group were higher than 16 μg g^{-1} CYA. Most of the isolates (58 %) produced low amounts of OTA (<2.5 μg g^{-1} CYA).

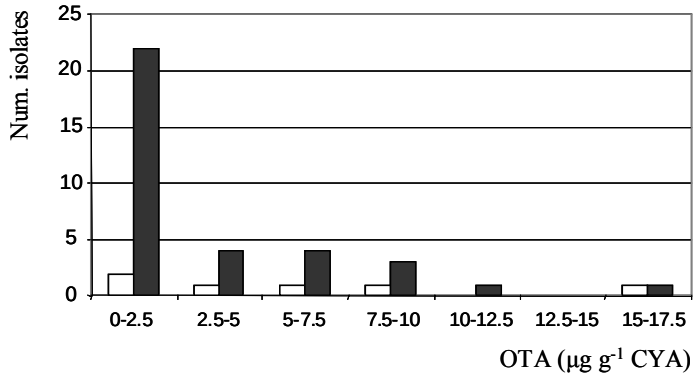

Figure 2. OTA-producing ability of □ *A. niger* aggregate and ■ *A. carbonarius* isolates. Detection limit: 0.01 μg OTA g^{-1} CYA.

OTA in musts

The levels of OTA found in the musts obtained from the treated grapes were below the detection limit of the analysis (0.05 μg l^{-1}). Therefore no correlation between the levels of OTA detected in musts and the incidence of ochratoxigenic fungi in grapes could be established.

DISCUSSION

The main genera isolated from grapes was similar to that reported in previous studies of Spanish grape mycoflora (14, 15). The present study corroborate that *Alternaria* was the principal component of the mycoflora characteristic of grapes, reducing its infection along time as other competing fungi increased their presence when reaching harvest. Although in the present study grapes were treated with different fungicides, black aspergilli were still dominant at harvest, and among them, *A. carbonarius*, showed a high ability to produce OTA, corroborating this species as the main responsible for the OTA found in grapes and grape-derived products.

Fungicide Switch seems to be active against black aspergilli as in most cases vineyards treated with a single (Treatments 4 and 5) or a double application of this fungicide (Treatments 6 and 7), showed the minimum percentage of black aspergilli on grapes at harvest. The reduction in the number of black aspergilli observed in Treatments 2 and 3 was lower than the treatments with Switch and therefore, we conclude that Chorus applied at harvest was less effective than Switch. Maximum reduction in the percentage of *A. carbonarius* grape-infection and OTA production when adding Switch to the medium, was also detected in a previous *in vitro* study (7). Moreover, in that study, cyprodinil was also reported as one of the active ingredients more effective to stop *A. carbonarius* growth. It seems that there are between cyprodinil and fludioxonil some synergistic effects against *A. carbonarius* in the field. It would be interesting to find out how both fungicides affect the different species in the section *Nigri*, and better investigate which active ingredient is the best one to minimize *A. carbonarius* grape-infection, as they were the most OTA-producer species in this fruit. The number of insecticide application seems that did not interfere in the efficiency of the fungicides assayed, although the prevention of skin damages caused by insects, could led to think in a further reduction in the mould infection. However, more studies have to be carried out in order to clarify this aspect and contribute in the search of the best fungicide treatments against black aspergilli in grapes.

Drawing general conclusions from field studies is difficult as many factors such as meteorological conditions, cultural practices, etc. would interfere in the process of grape infestation by the different fungal species. It is known that berry splitting caused by *Botrytis* provided the opportunity for infection by *Aspergillus* spp. (16). In the present study, this factor may not contributed significantly in black aspergilli infection, as the percentage of *Botrytis* was irrelevant in most of the regions studied. Rain damages could

also favour the infection of all species (17). Other factors affecting the effectiveness of fungicides include dose-response, maintenance of the use and time of application. But preventing mycotoxin contamination could be contradictory due to resistance and shift in mould populations (18).

OTA was no found in any sample of the must produced, neither in the control treatment. Therefore, it was not possible to correlate its presence with the pesticide treatments.

Although the use of pesticides seems one of the simplest and most effective methods to control different microorganisms of vine, there is also an increasing concern regarding the impact of fungicides on human health and environment but many studies and strict controls evidence that every time pesticides are more safety for the user, the environment and the consumer. Moreover, it is possible to predict the residual levels of pesticides on bunch grapes and leave them in safety levels, and most of the times, the residual levels in vinification are below the detection limit. However, combination of preventing measures for both infection and mycotoxin production, have to be considered.

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8.5. Monitoring of grape infection using transgenic green fluorescence protein (GFP) strains of *A. carbonarius*.

Knowledge of the infection cycle of *Aspergillus* and *Penicillium* is scarce and completely absent as far as grapes are concerned. A study of the first transformation of *A. carbonarius* introducing the reporter gene green fluorescent protein (*gfp*) into the fungal genome was carried out.

Until recently, the introduction of foreign DNA into filamentous fungi was achieved using either polyethylene glycol (PEG)-mediated transformation, electroporation, or particle bombardment. An alternative of these methods is the *Agrobacterium*-mediated transformation system, which was originally developed for plants, but has been applied successfully in recent years to several fungi, most of them plant pathogenic (De Groot et al. 1998; Covert et al., 2001; Malonek and Meinhardt 2001; Mullins et al. 2001, Leclerque et al., 2004). This method presented a number of advantages as a method for transforming fungi as for example, high efficiency transformation, elevated frequency of homologous recombination, a low copy number of inserted T-DNA per genome and an ability to transform spores, hyphae and also other fungal tissues (Dobinson et al., 2004). Few transformation studies with members of *Aspergillus* section *Nigri* have been reported and none regarding *A. carbonarius*. The first transformation system for *A. niger*, using PEG, was described in 1985 (Buxton et al., 1985) and the first successful expression of the *gfp* gene in this fungus appeared in 1999 (Siedenberg et al., 1999).

Green fluorescent protein (GFP) is a spontaneously fluorescent protein isolated from coelenterates, such as the Pacific jellyfish *Aequorea victoria* (Morin and Hastings, 1971). This protein was introduced into cell biology and biotechnology shortly after the *gfp* gene was cloned (Prasher et al., 1992). It was shown that the gene could be expressed in other organisms and detected as green fluorescence following excitation with UV or blue light (Chalfie et al., 1994).

Three ochratoxigenic strains of *A. carbonarius* were stably transformed with the *gfp* gene using *Agrobacterium tumefaciens*-mediated transformation and Hygromycin B (*hygB*) resistance as selection marker. The transformation protocol was adjusted for *A. carbonarius* obtaining the highest number of transformants (average number of transformants 1-50 per 106 spores) with an optical density at 600 nm (OD_{600}) of 1.5 followed by 2 days of cocultivation. Southern hybridization confirmed the incorporation of the *gfp* gene with single or double copies into the fungal genome. Transformants were mitotically stable for at least one year. Replacement of the *hygB* and *gfp* genes did not noticeably alter either growth or mycotoxin producing in culture. The *gfp* expressing V.v transformants allowed Confocal Laser Scanning Microscopy (CLSM) infection studies on and inside the grape skin. Results and images of the infection are shown in the following paper:

Visualisation of grape (*Vitis vinifera*) infection using transgenic green fluorescence protein (GFP) of strains of *Aspergillus carbonarius*. *FEMS Microbiology Letters* (submitted).

Visualisation of grape (*Vitis vinifera*) infection using transgenic green fluorescence protein (GFP) strains of *Aspergillus carbonarius*

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ABSTRACT

Three ochratoxigenic strains of *A. carbonarius* were stably transformed with the reporter gene green fluorescent protein (*gfp*) using *Agrobacterium tumefaciens*-mediated transformation and Hygromycin B (*hygB*) resistance as selection marker. The transformation protocol was adjusted for *A. carbonarius* obtaining the highest number of transformants (average number of transformants 1-50 per 10^6 spores) with an optical density at 600 nm (OD_{600}) of 1.5 followed by 2 days of cocultivation. Southern hybridization confirmed the incorporation of the *gfp* gene with single or double copies into the fungal genome. Transformants were mitotically stable for at least one year. Replacement of the *hygB* and *gfp* genes did not noticeably alter either growth or mycotoxin producing in culture. The *gfp* expressing V.v transformants allowed confocal laser scanning microscope (CLSM) infection studies on and inside the grape skin.

KEY WORDS: *Aspergillus carbonarius*, *Agrobacterium tumefaciens*, fungal transformation, green fluorescent protein, hygromycin B, nucleus, fluorescence microscopy, confocal microscopy, grapes.

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INTRODUCTION

Ochratoxins are secondary metabolites produced by moulds belonging to several species of the genera *Aspergillus* and *Penicillium*. The most extensively studied compound of this group, ochratoxin A (OTA), has been shown to be a nephrotoxic, immunosuppressive, teratogenic and carcinogenic agent (JECFA, 1991).

Fungi belonging to *Aspergillus* section *Nigri*, commonly known as black aspergilli, are the main OTA producers in grape-derived products. In addition, recent studies have shown that the three major black species, *A. carbonarius*, *A. niger* aggregate, and *A.* section *Nigri* uniseriates, are very common in grapes at harvest (Battilani et al., 2003, Bellí et al., 2004a). Within *Aspergillus* section *Nigri*, *A. carbonarius* has been confirmed as the major OTA-producing species in grapes (Battilani et al., 2003, Bellí et al., 2004a). The extent of *A. carbonarius* natural occurrence in food is very difficult to establish from the literature because it, together with other black aspergilli, were commonly regarded as *A. niger* (Abarca et al., 2004). Most of the studies for identification and quantification of ecological factors affecting *A. carbonarius* have so far been carried out on synthetic substrates (Mitchell et al., 2003; Bellí et al. 2004b; 2004c; 2005a). The expression of a vital marker such as GFP would allow studies on natural substrates, which would provide more effective control ot these fungi.

Few transformation studies with members of *Aspergillus* section *Nigri* have been reported and none regarding *A. carbonarius*. The first transformation system for *A. niger*, using PEG, was described in 1985 (Buxton et al., 1985) and the first successful expression of the green fluorescent protein (*gfp*) gene in this fungus appeared in 1999 (Siedenberg et al., 1999). *Agrobacterium* mediated transformation have proven efficient for several plant pathogenic fungi and due to the reported advantages such as high efficiency, elevated frequency of homologous recombination, low copy number per genome and an ability to transform spores, hyphae and also other fungal tissues (Dobinson et al., 2004) we chose to base the transformation on this method.

In the present study, we report the first transformation of *A. carbonarius*. We describe the introducing of the *gfp* gene into the fungal genome and show that the transformants are mitotically stable. In a second step, we demonstrate how the stable fluorescing transformed strains were used to visualize *A. carbonarius* grape colonization and infection with epi-, stereo- and confocal fluorescence microscopy.

MATERIALS AND METHODS

Strains

Three ochratoxigenic strains of *A. carbonarius* originally isolated from Italian (W9), French (W38) and Spanish (W120) grapes, were used throughout this work. Strains are held in the culture collection of the Food Technology Department of the University of Lleida (Spain). The fungi were cultivated on Czapek yeast extract agar (CYA) (Pitt and Hocking, 1997) plates at 25 °C.

Construction of transformation plasmid pPZP201-GG-BH

The vector pPZP201-GG-BH was originally developed for expression in *Blumeria graminis* f.sp. *hordei* and thus promoter regions from this fungus have been used. The promoter region of the *B. graminis GPD* gene was used to regulate the *GFP* gene and the *B. graminis* β-tubulin promoter region to regulate the *hygB* gene encoding hygromycin B resistance. The *B. graminis GPD* promoter region was isolated from pEghGPD (Christiansen et al., 1997) by PCR using degenerate primers (GPD3: 5´GCCCATGGATTCTGTTGATAAACCTT 3´ and GPD6: 5´GCGTCGACTATTAAGACCGGAAGGAC 3´) with the addition of *Nco*I and *Sal*I restrictions sites, respectively. As the resulting PCR product contained an internal *Nco*I site a partially digestion was performed and the complete promoter region was inserted in the *Nco*I/*Sal*I sites upstream the *GFP* gene in blue-SGFP-TYG-nos SK (Spellig et al., 1996) resulting in pGG. The *B. graminis* β-tubulin promoter region was isolated from pBTEG (Sherwood and Sommerville, 1990) using a combination of the universal primer T7 and primer BTEG9. The product was *Sac*I/*Bam*HI digested and inserted in the *Sac*I/*Bam*HI site of pGG resulting in pGG-B. The *hph* gene was isolated from pAN7-1 (Punt et al., 1987) using the primers HYG1 and M13 Universal. The PCR product was *Bam*HI digested and inserted in the *Bam*HI site of pGG-B under the control of the *B. graminis* β-tubulin promoter region. The whole insertion was excised from pGG-BH by a *Sac*I/*Sal*I digestion and transferred to the the *Sac*I/*Sal*I sites of the *A. tumefaciens* vector pPZP-201 (Hajdukiewicz et al., 1994). All fusions were verified by sequence analysis.

A. tumefaciens AGL1 competent cells were transformed with the plasmid pPZP-201-GG-BH and kept on Luria Broth agar containing 20 ppm rifampicin + 50 ppm kanamycin. Plasmid propagation was performed according to Sambrook et al. (1987) while plasmid purification was carried out using the Qiagen plasmid purification procedure according to the manufacturers instruction (Qiagen, Chatsworth, CA, USA).

Agrobacterium-mediated transformation of *Aspergillus carbonarius*

Hygromycin B sensitivity of *A. carbonarius*

Resistance level of each *A. carbonarius* strain to the antibiotic hygromycin B (hygB) was tested plating 100 µl of a spore suspension (10^6 spores ml^{-1}) on solidified CYA medium containing 0, 50, 100, 150, 200, 250 and 300 µg ml^{-1} hygB. Plates were incubated at 25 ºC for 7 days and emerging colonies were counted.

Nuclear staining of *A. carbonarius* spores

A loop of young *A. carbonarius* spores grown on CYA plates at 28 ºC for 3 days, was deposited onto a slide and stained for 15 minutes in the dark with a drop of DAPI solution (4',6-Diamidino-2-phenylindole dihydrochloride; 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride) (25 µg ml^{-1} H_2O). Nuclei of 100 spores of each strain were counted in triplicate by the aid of an epifluorescence light microscope (Olympus BX60) (Filter WU; λexc. 330-385; dicrotic mirror DM400; barrier filter >420).

Fungal transformation

W9, W38 and W120 *A. carbonarius* were used as recipient strains for transformation. Spores were harvested from 7 day-old CYA plates incubated at 25 ºC and dissolved into 3 ml induction medium (IM) tubes (Bundock et al., 1995) until a final concentration of 10^6 spores ml^{-1}, counted in a Thoma haemocytometer.

Agrobacterium tumefaciens AGL1 cells containing the plasmid pPZP-201-GG-BH were prepared largely as described by Malonek and Meinhardt, (2001), and grown to an OD_{600} of 1.5.

For cocultivation 100 µl of the mixture of an equal volume of the fungal spore suspension and *Agrobacterium* culture was plated on sterile black cellulose filters (AGF 220-75 mm, Frisenette ApS, Ebeltoft, DK) on IM± acetosyringone (AS) (200µM AS) plates and incubated at 25 °C for 2 days. Filters were transferred to selective medium plates (CYA supplemented with 50 µg ml^{-1} hygB and 300 µg ml^{-1} cefotaxime to inhibit *A. tumefaciens* growth), over-layed with selective medium and incubated at 25 °C for 10 days. HygB-resistant colonies were analysed for expression of GFP using a stereomicroscope (Leica MZ FLIII).

Mitotic stability of the transformants

Transformed colonies were transferred to fresh selective plates and purified to mitotic stability by 4-5 rounds of single-spore isolation.

Genomic DNA extractions of freeze dried mycelium were performed following the protocol of Nucleon extraction and purification kit (Amersham Biosciences Corp., NJ, USA). DNA hybridizations were carried out to determine the copy number of the *gfp* gene and the site of integration into the genome of *A. carbonarius* transformants (Southern, 1975). Total genomic DNA (approx. 2.2 µg) were digested with either *Hin*dIII or *Bgl*II restriction enzymes, fractionated through 0.7 % agarose gels, and transferred by capillary blotting to Hybond N+ membranes (Amersham, Biosciences, UK) using standard methods (Sambrook et al., 1987). Genomic DNA was probed with the 700bp *gfp* fragment from *Nco*I+*Pst*I digested gGFP (Maor et al. 1998). DIG-high prime DNA labelling and detection starter Kit II (Roche Diagnostics GmbH, Germany) were used for labelling of probe and subsequent chemiluminescence detection.

Ecophysiological studies

Two green fluorescing *A. carbonarius* transformants and the wild type of each strain (W9wt, W9a, W9b; W38wt, W38a, W38b; W120wt, W120a, W120b) were inoculated (10^6 spores ml^{-1}) on synthetic medium (SNM) with composition similar to grapes (Bellí et al. 2004c) and incubated at 25 ºC. The diameter of the colonies were measured daily up to 15 days and the growth rate (mm day^{-1}) was calculated. OTA-producing capacity of each strain was analysed as described in Bellí et al. (2004b) after 4 and 7 days of incubation at 25 ºC. Three independent replicates were carried out.

Statistical analysis

Analysis of variance was performed for number of nuclei, growth rate and for OTA production experiments (SAS Enterprise Guide, version 2.0, SAS Institute Inc., Cary, N.C.). Treatment means in all the analysis were compared by Duncan test ($P<0.001$).

Infection studies

Three different microscopes were used for fluorescence detection; (i) epifluorescence light microscope (ELM) (Olympus BX60) equipped with a mercury lamp (Olympus U-RFL-T), two filter sets with the corresponding excitation wavelengths (WU 330-385 nm, WIB 460-490 nm), dicrotic mirrors (DM400 and DM505), barrier filters (>420, 515-550) and a digital camera (Olympus C-200Z); (ii) stereomicroscope (SM) (Leica MZ FLIII), with a mercury lamp (Leica ebq100), two green fluorescent filters GFP2 and GFP3 with excitation and barrier filters 480/40 – 510 nm long pass (LP) and 470/40 nm – 525/50 nm, respectively (Leica Microsystems, Wetzlar, Germany) and a digital camera (Leica DC 300F); (iii) confocal laser scanning microscope (CLSM). Images were handled with

software (i) analySIS, Soft Imaging System GmbH and (ii) Leica IM500 version 1.20 release 19.

Expression of the GFP protein in the transformants was detected by the fluorescence SM. Red and white table grapes were disinfected by dipping in 70 % ethanol for 15 seconds and dried on a laminar flow bench and inoculated with a 0.2 µl drop of spore suspension (10^6 spores ml^{-1}) and incubated at 25 ºC. The developing mycelium growth was observed using ELM, SM and CLSM.

RESULTS

Agrobacterium-mediated transformation of *A. carbonarius*

Hygromycin B sensitivity of *A. carbonarius*

Fifty µg ml^{-1} of hygromycin B (hygB) was the minimum concentration inhibiting *A. carbonarius* natural growth and was chosen for selection of hygB-tolerant transformants. However, unspecific fungal growth on the cellulose filter, was not sufficiently inhibited and therefore a layer of molten selective media was poured onto the filter for efficient selection of transformants.

Nuclear staining of *A. carbonarius* spores

The number of nuclei in spores of wild type *A. carbonarius* strains varied between 1 and 10 (Figure 1). The majority of spores held 4-6 nuclei, and an average of 2 % contained 10 or more nuclei. No mononucleate spores were detected in strains W9 and W120, and only 1 % of W38 spores had a single nucleus. Spores of strain W38 contained a significantly lower number of nuclei, although the mean number of nuclei was similar: 5.75, 4.77 and 5.62 in W9, W38 and W120 strains, respectively. DAPI stained spores of W9 strain is shown in figure 2.

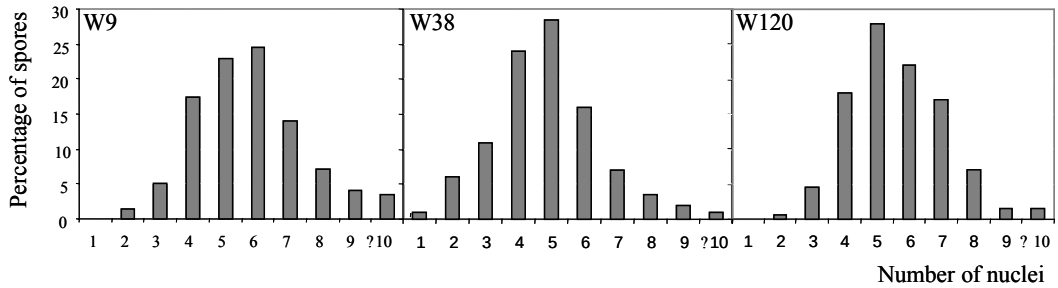

Figure 1. Nuclei distribution in the spores of three strains of *A. carbonarius* (W9, W38 and W120). Spores were stained with DAPI ($C_{16}H_{15}N_5$·2HCl) solution and observed under fluorescent light.

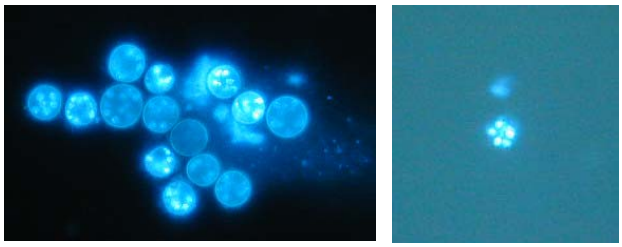

Figure 2. Epifluorescence light microscopy images of *A. carbonarius* spores stained with DAPI ($C_{16}H_{15}N_5$·2HCl) to monitor their number of nuclei.

Transformation

After 2 days of cocultivation, filters were completely covered with a mycelial lawn. Therefore, a layer of molten selective medium was poured on top to facilitate the identification of the transformants from the background. In the absence of acetosyringone (AS) (control plates) during cocultivation, no transformants were formed (Figure 3A1). HygB-resistant transformants arose after approx. 2-3 days of incubation in selective medium, but expression of *gfp* was not observed until 10 days after transferring the filters (Figure 3A2, 3A3). The transformation frequency was in the range of 1 to 50 transformants in 10^6 spores (Table 1), although numbers varied depending on the conditions used (data not shown).

From a preliminary study on the influence of different parameters on transformation the following results were found. No differences were detected in the efficiency of the methods using pre-incubated or fresh *A. carbonarius* spores. Three different levels of *Agrobacterium tumefaciens* OD_{600} (0.5, 1 and 1.5) were assayed and the highest seemed

most efficient. One day of cocultivation was not sufficient to transform any of the *Aspergillus carbonarius* strains. Maximum number of transformed colonies were detected with 48 h of cocultivation (1-50 transformants per 10^6 spores), while an average of 3 transformants per 10^6 spores appeared in plates cocultivated 72h. Different volumes (100 and 200 µl) and proportion of the mixture *A. carbonarius*/*Agrobacterium tumefaciens* (1:1, 0.5: 1.5 and 1.5: 0.5) on each plate didnot affect the results. An initial concentration of 10^6 spores ml^{-1} of *Aspergillus carbonarius* resulted in a higher number of transformants than higher concentrations (10^7 and 10^8 spores ml^{-1}).

Table 1. Number of *A. carbonarius* transformed colonies (hygB-resistant and *gfp* expressed) after *Agrobacterium tumefaciens*-mediated transformation. Values are the mean of three replicates ± standard deviation.

Strain	Medium	No. of spores	No. of transformants
W9	-AS	$1.0x10^6$	0
	+AS	$1.0x10^6$	2 ± 1.5
W38	-AS	$1.0x10^6$	0
	+AS	$1.0x10^6$	29 ± 22
W120	-AS	$1.0x10^6$	0
	+AS	$1.0x10^6$	5 ± 4

Mitotic stability of transformants

Between 10 and 20 transformants from each strain were used to study their stability. No morphological differences were observed between transformants and wild type (data not shown). The mitotic stability of hygB resistance and *gfp* expression over at least four life cycles of single-spore-derived isolates on hygB was demonstrated. All GFP transformants continued to express the green fluorescent protein after 12 months of several subcultures also in non-selective medium.

DNA hybridisation analyses of two randomly chosen transformants of each strain were performed with *Hin*dIII that cut once in the T-DNA and the non-cutter *Bgl*II. Genomic DNA hybridizations confirmed that *gfp* was integrated into the fungal genome in one or two sites (Figure 4). One of the transformants (W120b) appeared to have a single integrated copy of the gene at a single site in the genome as a single band was detected in both blots, The other four transformants carried at least two copies of the *gfp* gene, three (W9a, W9b, W120b) at a single site and one (W38b) at two sites in the genome. No hybridizing band was detected in any of the three wild type strains (only strain W9 is shown in figure 4).

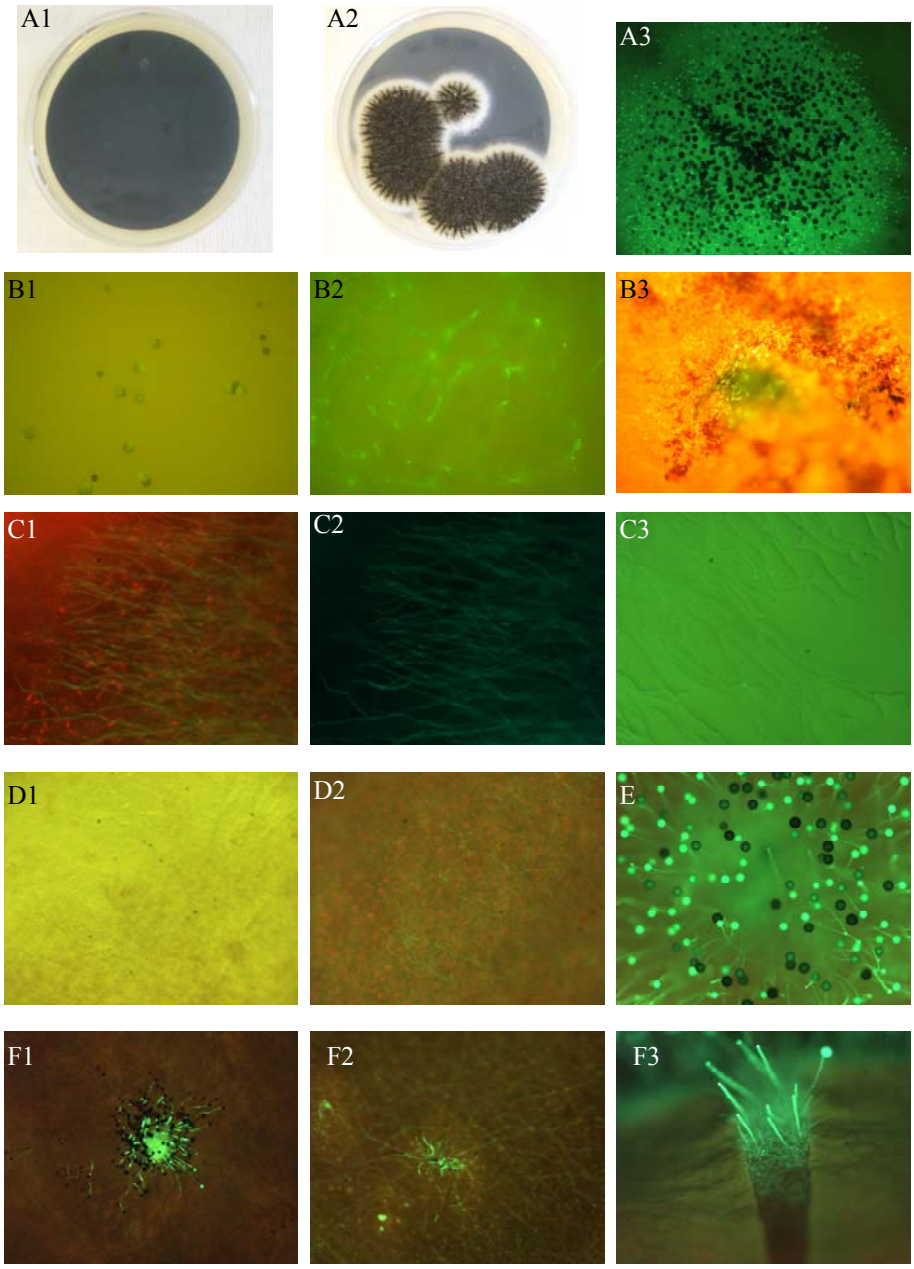

Figure 3. (Next page).

Figure 3. Fluorescence stereomicroscope images. A: Transformed colonies of *A. carbonarius* with *Agrobacterium*-mediated transformation system after two days of cocultivation and 10 days of incubation at 25 ºC; A1: Control plate (without acetosyringone); A2: Five transformed colonies on selective media; A3: Transformed colony under GFP2 filter. Epifluorescence light microscopy images. B1: Germinating *A. carbonarius* spores on CYA; B2: Fluorescing mycelium on CYA after 12 hours of inoculation. B3: Early fluorescing growth mycelium onto red grapes. Fluorescence stereomicroscope images. C1: Mycelium under GFP2 filter; C2. Mycelium under GFP3 filter; C3. Mycelium under normal light. D: mycelium onto white grape. D1: under normal light; D2: under GFP2 filter. E. Young and old conidia under GFP2 filter. F. Infection on damaged grapes. F1: Grapes artificially damaged; F2, F3: Natural openings onto the skin.

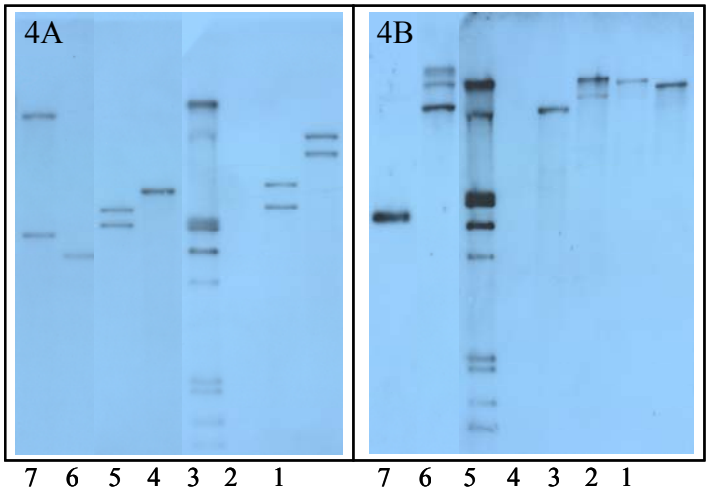

Figure 4. DNA hybridization analysis of the *gfp* gene in *A. carbonarius*. Genomic DNA was digested with (4a) *Hin*dIII, a restriction enzyme that cut once in the T-DNA, and (4b) *Bgl*II that did not cut in the T-DNA. DIG-labelled molecular size marker is indicated to the right of the figure. Equivalent amounts of genomic DNA from each *A. carbonarius* strains were in each lane (Lane 1, wild type; 2, W9a; 3, W9b; 4, W120a; 5, W120b; 6, W38b; 7, positive control).

Growth and OTA production of the transformed strains

Table 2 shows the growth rates and OTA detected after 4 and 7 days of incubation at 25 ºC. No significant differences in the growth rates and OTA production were found between wild type and the two transformants of each strain. All the strains maintained their ability to produce OTA after being transformed.

Table 2. *A. carbonarius* growth rates (mm day^{-1}) and OTA accumulation (µg g^{-1} SNM) at 25 ºC of the transformants and wild type strains. Values are the mean of three replicates ± standard deviation. No significant differences were found between wild type and both transformants of each strain (P<0.001).

Strain	Growth rate (mm day^{-1})	OTA Day 4	OTA Day 7
W9wt	3.17 ± 0.13	1.98 ± 0.81	0.56 ± 0.56
W9a	2.85 ± 0.15	0.43 ± 0.39	1.25 ± 1.76
W9b	2.99 ± 0.11	1.05 ± 1.46	0.19 ± 0.13
W38wt	3.01 ± 0.24	6.88 ± 3.33	0.89 ± 0.29
W38a	2.98 ± 0.17	2.59 ± 2.99	8.17 ± 7.29
W38b	2.55 ± 0.21	3.02 ± 1.49	10.77 ± 13.1
W120wt	2.74 ± 0.37	3.42 ± 3.62	3.29 ± 2.14
W120a	2.68 ± 0.20	4.14 ± 2.48	14.35 ± 5.80
W120b	2.56 ± 0.32	2.25 ± 1.05	4.86 ± 2.52

Infection studies

Transformed spores inoculated on CYA presented a weak fluorescence being detectable with an EFM when they started germinating and further monitoring on this medium could be done (Figure 3B1, 3B2).

Red grapes showed some green auto-fluorescence in the epidermis cell wall that was difficult to distinguish from the green fluorescent hyphae. White table grapes were used for infection studies as the auto-fluorescence was less and the fluorescent hyphae visible.

Fluorescence was weak in the hyphae and strong in the conidiophores until the black pigmentation of the spores restricted the fluorescence signal (Figure 3C1, 3C2, 3C3, 3E). The green colour of transformed hyphae onto the grape was clearly visible within the first two days of incubation at 28 ºC while wild type strains at the same conditions were barely visible with normal light (Figure 3D1, 3D2). The infection developed considerably faster when grapes were previously damaged and a dense mat of hyphae was formed within the first 36 h (Figure 3F1). Natural opening on the skin placed under the inoculation point had the same effect (Figure 4F2, 4F3).

After approx. 3 days of incubation at 28 ºC (colony height 0.5-1 cm) grapes were hand-sectioned by the middle of the colony in order to study fungal colonization in the inside part of the grape. No green hyphae were detected in the pulp with the SM (data not shown), although they were detected at the first epidermal cells under the skin with the

aid of the CLSM. Figure 5A1 shows the location of different points onto the grape skin were confocal images were taken. A brown rot was produced around the point of infection of *A. carbonarius*. Fluorescent hyphae were detected outside the brown part with the SM (Figure 5 A2 and A3), although internal infection was located only under the rot. Green hyphae was observed inside the grape growing from the center of the wound or crack to the edge of the brown spot. Hyphae were never observed in the green pulp as if fungal secreted enzymes had to digest some cell wall components restricting hyphal growth.

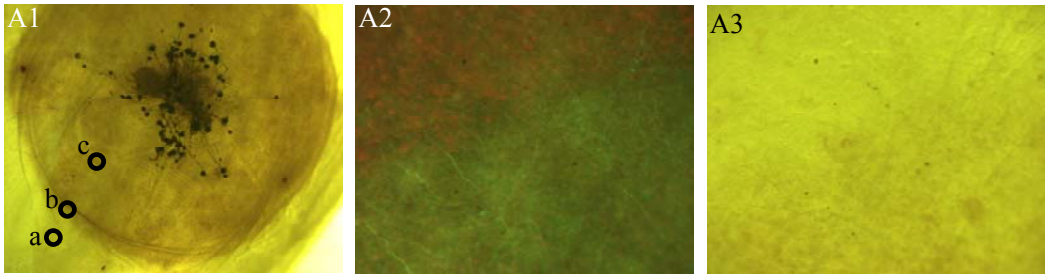

Figure 5. Fluorescence stereomicroscope images. A. Brown rot caused by *A. carbonarius* infection onto white grapes. A1: Sites where confocal images where taken (a, b and c); A2: mycelium growing at the edge of the brown rot under GFP2 filter; A3: same as A2 under normal light.

DISCUSSION

A. carbonarius presented a high sensitivity to hygB, as wild type colonies of the three strains only grew in control plates, in comparison with most filamentous fungi which presented a natural relatively resistance to this antibiotic (Goettel et al., 1990; Pfeifer and Khachatourians, 1994; Leclerque et al., 2004). Results from Peberdy et al. (1990) confirmed the hygB sensitivity of another black Aspergilli species, *A. niger*, finding the minimum tolerable amount of hygB between 10 and 50 μg ml^{-1}.

DNA transfer from *A. tumefaciens* to *A. carbonarius* was dependant on the presence of the *vir* inducer AS in the medium, as also observed in transformation of other fungi (Bundock et al., 1995). The average number of transformants obtained for *A. carbonarius* was in the same range as the reported for *A. niger* (5 hygB-resistant transformants per 10^7 spores) (Groot et al., 1998) and *Botrytis cinerea* (15 transformants per 10^6 spores) (Rolland et al., 2003), but lower in comparison with that reported for other filamentous fungi (Groot et al., 1998).

Four-hours of pre-incubation did not increase the transformation efficiency of *A. carbonarius*. In the case of *Aspergillus giganteus*, germination time up to 22 h decreased the number of transformants by around one order of magnitude (Meyer et al., 2003). However, the ability of *A. tumefaciens* to introduce DNA into hyphal tissue of other filamentous fungi has been reported (Groot et al., 1998).

Cocultivation time of 2 days was the optimum for A. *carbonarius* transformation and was also recommended for other fungi (Mullins et al., 2001), although it was not enough for *Beauveria bassiana* (Leclerque et al., 2004). Similarly to our results, transformation frequencies of this fungus were independent of the ratio of bacterial to fungal cells for *B. bassiana*, as long as the agrobacteria were in excess (Leclerque et al., 2004). However, a positive correlation between transformation efficiency of other fungi and the amount of *A. tumefaciens* cells present during cocultivation has been reported (Mullins et al., 2001; Meyer et al., 2003). Zeilinger (2004) tested different concentrations of *Trichoderma atroviride* conidia (10^6, 10^7 and 10^8 ml^{-1}) to optimize the *Agrobacterium*-mediated transformation of this fungus and most transformants were obtained using 10^7 conidia (30-50 transformants).

It is known that transformation efficiency also depends on temperature, obtaining the best results when cocultivation is done at the optimal growth temperature for the fungi (Combier et al., 2003; Rolland et al., 2003). In the present study, cocultivation was carried out at 25 ºC as 25-30 ºC has been reported as the optimum thermal range for *A. carbonarius* growth (Leong et al. 2004; Bellí et al. 2004c; 2005a).

Although the predominance of monolocal integration that *A. tumefaciens* presents, several copies of the gene in the fungal genome were detected in this study. The high number of nuclei in the spores of *A. carbonarius* seemed to do not interfere in the stability of the transformants. Similarly, Rolland et al. (2003) reported that conidiogenesis was not affected in the selected transformants of *Botrytis cinerea* although more than 65 % of conidia housed between 3 and 5 nuclei.

Parallel infection studies of wild type and transformed strains of *A. carbonarius* on grapes showed a similar development, although monitoring transformants was much easier due to their green fluorescence. A slight autofluorescence of both red and white grape skin complicated the monitoring tasks at the early infection stages. Preliminary CLSM studies allowed the detection of emerging hyphae of transgenic *A. carbonarius* under the grape skin. Similarly to what has been reported for *Alternaria alternata* grape infection (Swart et al., 1995) we detected *A. carbonarius* mycelium in the epidermal cells and in the cells surrounding wounds. In comparison to the development of mycelium on the grape surface the development inside was slow. As previously reported we also found faster growth on naturally or artificially injured grapes and correlated to an increase on OTA production in damaged grapes (Bellí et al., 2005b).

These results show that GFP is a suitable marker for following different stages of the development of *A. carbonarius* inside grape. It is important to know the distribution of internal fungal mycelium in grapes infected by *A. carbonarius* as OTA has been previously reported in grape pulp (Battilani et al., 2002). OTA content reported in the pulp was lower than in the skin, but the location of the toxin production is still unknown. A better knowledge of the way *A. carbonarius* penetrates inside the fruit could help to clarify it.

The transformants obtained in this study will be useful for further work in fungal ecology, as they maintained the fluorescence for long periods and continued producing OTA in the same range as the wild type. They could be also useful for further studies of interaction with fungi labelled with other markers as *DsRed* gene (Mikkelsen et al., 2003). The *Agrobacterium*-mediated transformation system proposed can be considered a promising tool for similar transformation of other fungi present in grapes.

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