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D MITCHELL

Ecological factors affecting growth and ochratoxin A production of *Aspergillus* section *Nigri* species on grapes

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This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

This study investigated the effects of interacting environmental factors on the ecophysiology of *Aspergillus carbonarius* and ochratoxin A (OTA) production, to facilitate the building of a database of knowledge for risk prevention. Spore germination and germ tube extension was faster on grape flesh than *in vitro* on grape-based media, or grape skin. Under optimum conditions (25-35°C; 0.90-0.99 water activity (a_w)) 50% of conidia germinated in < 4.5 hours. Two dimensional profiles in relation to $a_w \times$ temperature were constructed for rate of germination and germ tube extension for the first time. Mycelial growth was also influenced by a_w, temperature, pH and modified atmospheres. Two dimensional temporal profiles of growth and OTA production were constructed based on information on strains from four different European countries. This showed that the optimum a_w and temperature, and range were 0.98 a_w, 30°C and 0.99-0.88 a_w and 15-40°C, respectively. In contrast, for OTA production these were 0.95 a_w, 15-20°C, and 0.99-0.93 a_w and 15-35°C respectively. Environmental factors also influenced mycelial interactions between *A. carbonarius* and nine other common grape fungi. An Index of Dominance showed that *A. carbonarius* was very competitive against these species over a range of a_w levels and temperatures. OTA production was generally suppressed by interactions at 0.95 a_w with some inhibition of OTA production at 30°C at 0.99 and 0.95 a_w. At 0.99 and 20°C all strains except two species produced a stimulation in OTA production.

The efficacy of fungicides (5) and anti-oxidants (2) on growth and OTA production was limited at the concentration used. The only two fungicides that did suppress

growth were Switch (cyprodinil and fludioxonil) and carbendazim. OTA production was stimulated at sub-optimal concentrations regardless of a_w level used. Anti-oxidants were ineffective under all conditions tested. Modified atmospheres (0.03, 25 and 50% $C0_2$) at different a_w levels showed that there was very little long term effect on spore germination and germ tube extension, even at 50% CO_2. Regardless of a_w, OTA production was influenced more by the a_w itself than CO_2 concentrations. The efficacy of Sodium metabisulphite (NaMBS) at different a_w levels (0.99, 0.97, 0.93 a_w) on grape juice-based media at 25°C on germination and germ tube extension showed inhibition by >500 mg kg^{-1} of NaMBS. Low concentrations (100, 250 mg kg^{-1}) stimulated growth and OTA production. LD_{50} and LD_{90} ranges were determined for both growth and OTA production for the first time.

Field sampling found that black aspergilli were present on grapes from setting to harvest with a significant increase in the population with grape ripening, and higher temperatures. The most commonly isolated OTA species from grapes was *A. carbonarius* with no *Penicillium verrucosum* and only a few *Aspergillus ochraceus* isolated. In all the field sampling found no OTA present in any of the grape musts.

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Having been in Silsoe for some time in one capacity or another, this may turn into an Oscar speech but I will do my best to keep it to one page.

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NOMENCLATURE

%	Percentage
>	More than/greater than
<	Less than
×	Times
Abs	Absorbance
a_w	Water Activity
CFU	Colony forming units
CYA	Czapek yeast agar
CZ	Czapek agar
DRBC	Dichloran Rose Bengal Chloramphenicol
°C	Celsius
DO	Demominación de Origen
EU	European Union
ERH	Equilibrium relative humidity
GJM	Grape juice media
ha	Hectare
HPLC	High performance liquid chromatography
IARC	International Agency for Research on Cancer
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LD_{50}	Lethal Dose, which given all at once, will cause 50 % death or 50%prevention of ochratoxin A production
LD_{90}	Lethal Dose, which given all at once, will cause 90 % death or 90% prevention of ochratoxin A production
M	Molar

MAFF	Ministry of Agriculture Fisheries and Food UK
no.	Number
OIV	The International Organisation of Vine and Wine
OTA	Ochratoxin A
P	Vapour pressure of water in solution or solid substrate Pa Pascals
Po	Vapour pressure of pure water at experimental temperature and pressure
rpm	Revolutions per minute
SGM	synthetic grape juice media
R	Ideal gas constant
RGJM	Red grape juice media
RO	Reverse osmosis water
SGM	Synthetic grape juice media
T	Absolute temperature
t_R	Retention time
V	Volume of one mole of water
WGJM	White grape juice media
WHO	World Health Organisation
w/v	Weight to volume
Ψ	Water potential

CHAPTER 1 LITERATURE REVIEW AND OBJECTIVES

1.1 GENERAL INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin which has increasingly become more important as new maximum levels have been set by the European Union (EU) for contamination of grapes, wine and other vine products (EU, 2005). The world-wide occurrence of OTA contamination of raw agricultural products has been known and studied for some time in a variety of commodities including cereal grain, coffee, cocoa and animal feedstuffs (Harwig *et al.*, 1983; Van Egmond & Speijers, 1984; Kuiper-Goodman & Scott, 1989). It was not till the mid 1990's that OTA was first recorded in wine, grape juice, and dried vine fruits (Majerus & Otteneder, 1996; Zimmerli & Dick, 1996; Pietri *et al.*, 2001; Soleas *et al.*, 2001; Battilani & Pietri, 2002; Abarca *et al.*, 2003). The fungi responsible for OTA production in cereals have been studied, especially *Penicillium verrucosum* (Thom) and *Aspergillus ochraceus* (Wilhelm), which have always been considered the main producers. However, much less is known about *Aspergillus carbonarius* ((Bainier)Thom) which is found in warmer climates where grapes are grown.

Ochratoxin A has carcinogenic properties, nephrotoxic effects and the ability to be teratogenic and immunosuppressive (Harwig *et al.*, 1983; Creppy, 1999). It has also been linked with Balkan endemic nephropathy (Pohland *et al.*, 1992). It was initially believed that cereals and animal products were the major sources of human exposure; but in 2001 Joint FAO/WHO Expert Committee on Food Additives (JECFA) reported that wine and grape juice were the second and third most important food categories responsible for human exposure to OTA. OTA was originally described as a metabolite of *A. ochraceus.* This species and *Penicillium verrucosum* were

considered to be the only OTA-producing species. *P. verrucosum* produces OTA in temperate climatic regions and is very rarely isolated from grape berries, while *A. carbonarius* is much more commonly isolated.

The first surveys carried out on wine reported a higher OTA concentration in red versus white and rosé wines (Zimmerli & Dick, 1996). A correlation with the latitude of the production region was also demonstrated: the further south the provenance, the more frequent the occurrence and the greater the concentration of OTA (Zimmerli & Dick, 1996; Otteneder & Majerus, 2000).

Figure 1.1 shows the importance to Europe of grape consumption and production. Not only are Europeans the largest consumers, but Europe is also the largest producer of grapes. In 2003, the total area of the world planted with vines was 8 million hectors (ha), with Europe having 53% of all vines (Organisation internationale de la vigne et du vin, (OIV) 2003). Europe is the world's largest producer and consumer of wine, drinking >60% of all the wine in the world and consuming 40% of the raisins. Asia consumes more table grapes (54%) than Europe (OIV, 2003). For this reason any decrease in OTA in grape products could result in a significant reduction in human consumption of OTA.

1.2 OCHRATOXIN A

The first report of the mycotoxin OTA was a one page article in Nature by (Van Der Merwe *et al.*, 1965) who isolated the compound from cultures of *A. ochraceus*. OTA is classed as a secondary metabolite and is a product of the primary metabolic process.

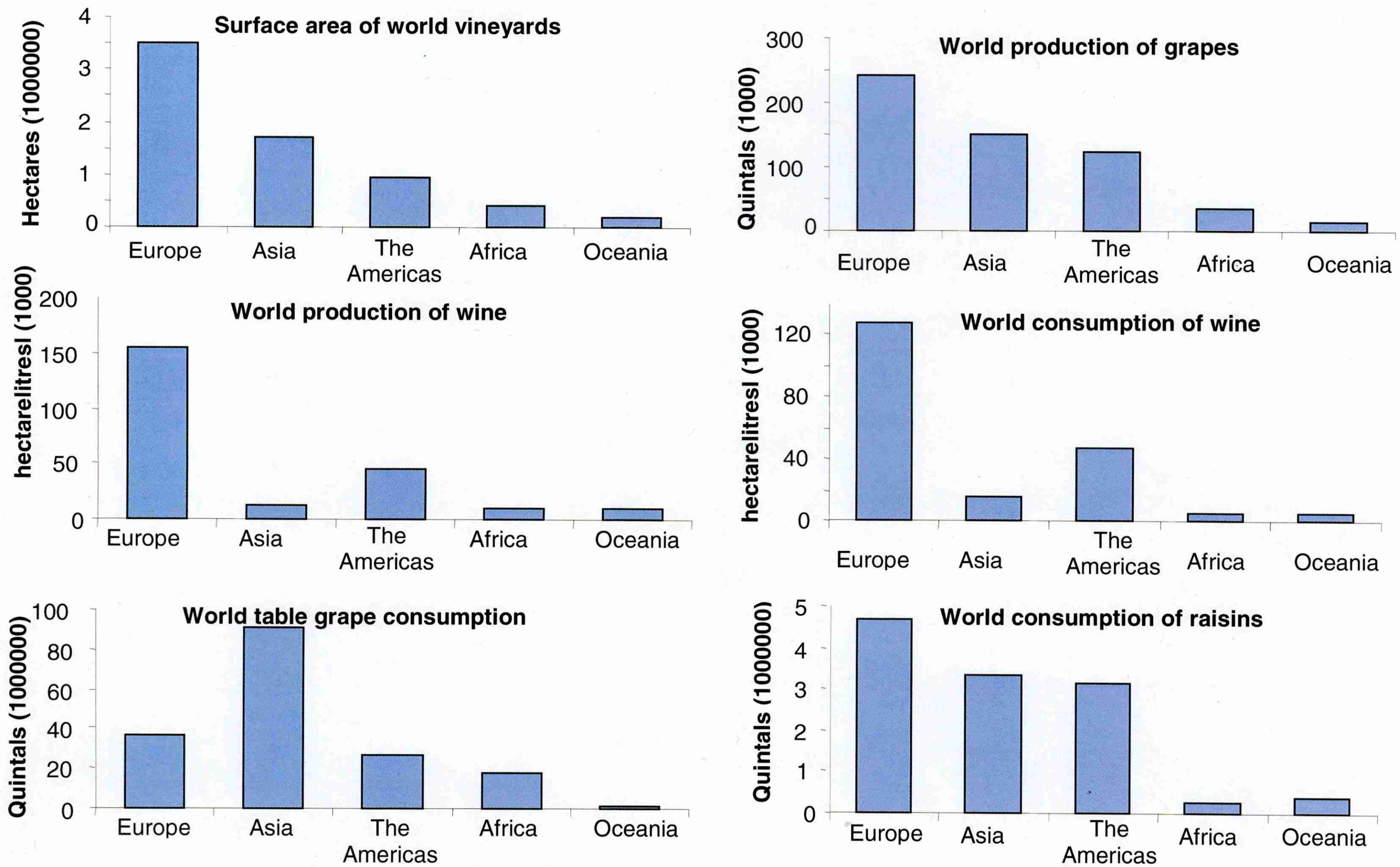


Figure 1.1 World grape, wine and raisin production and consumption by continent for 2003 (from OIV, 2005)

There are reports that secondary metabolites are secreted during a balanced growth phase and often associated with the approximate time of sporulation (Calvo *et al.*, 2002). The compound itself is fairly stable when kept out of direct light; it is a colourless, heat stable compound, with a blue or green fluorescence under UV light depending on a high or low pH, respectively. It is known to pass through industrial processing into consumer products such as wine, bread, pulses, and other food stuffs, without a large reduction in levels (Scott *et al.*, 1972; Krogh *et al.*, 1973; Zimmerli & Dick, 1996; Pietri *et al.*, 2001). The chemical structure of OTA is shown in Figure 1.2.

The half-life of OTA is long (840 hours) in humans after oral ingestion (Petzinger & Ziegler, 2000) and frequently found in blood suggesting continuous and widespread exposure to OTA (Jõrgensen & Rasmundsen, 1996; Peraica *et al.*, 1999). It has become increasingly important with the growing list of mycotoxic properties in humans and concern has thus increased about the daily intake of OTA by humans. In laboratory studies, it has proved to be carcinogenic to mice and rats (Bendele *et al.*, 1985; Kuiper-Goodman & Scott, 1989, NTP, 1989) and for this reason in 1993 the International Agency For Research on Cancer classed OTA as a category 2B carcinogen (possibly carcinogenic in humans).

It is also known to be a nephrotoxin in all mammals tested. The toxin has been linked to Balkan Endemic Nephropathy in Balkan countries (BEN) and Urothelial tumours (UT) (Pohland *et al.*, 1992; Kuiper-Goodman, 1995; Maaroufi *et al.*, 1995; Godin *et al.*, 1998; Wafa *et al.*, 1998). It has been suggested to be involved in some forms of testicular cancer (Schwartz 2002). It has been linked to Danish porcine nephropathy,

and was first reported in Danish pigs in 1972 (Krogh, 1972, 1976, 1977; Krogh *et al.*, 1977; Walker & Larsen, 2005).

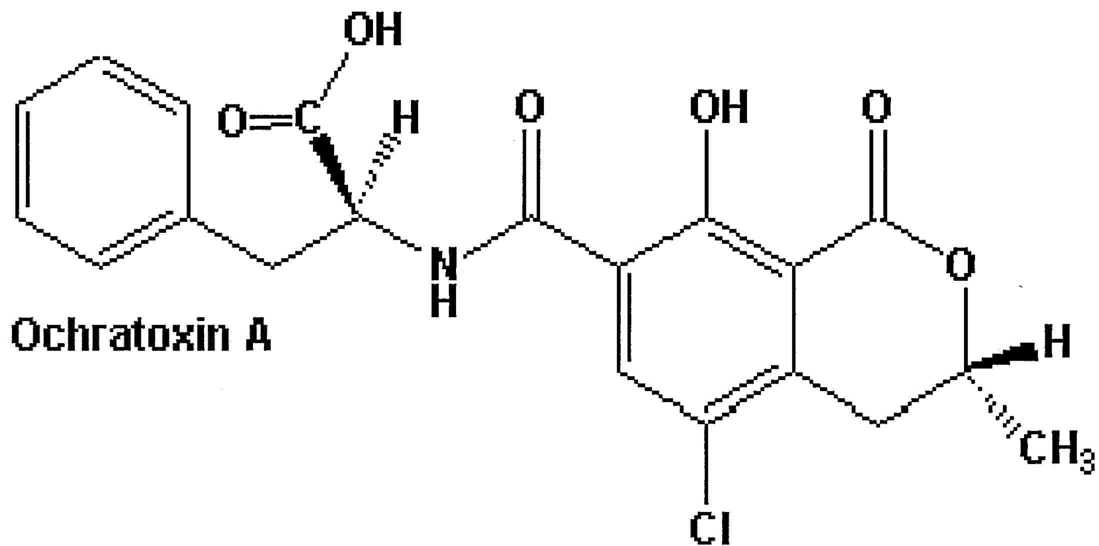


Figure 1.2 Chemical Structure of Ochratoxin A

It was first linked with teratogenic effects in rodents (Hayes *et al.*, 1974) then later followed by other mammals (Gilani *et al.*, 1978; Singh & Hood, 1985). It is also classed as being mutagenic to all mammals *in vitro* and to mice cells in *in vivo* tests (Creppy *et al.*, 1995; WHO, 1996; Walker & Larsen, 2005).

For these reasons it is imperative that concentrations reaching consumers are kept as low as possible. In 1998 the E.U Scientific Committee on Food initially concluded that it would be prudent to reduce exposure to OTA as low as possible (5ng kg^{-1} body weight day^{-1}) in line with other work (Őlsen *et al.*, 1993; Jõrgensen & Rasmundsen,

1996) This was amended in 2002 with stricter limits (EU, 2002). Table 1.1 shows the current EU legislation which was introduced on the 1st April 2005. This imposed stricter controls on a wider range of food products. It was also the first time that the EU has imposed legislation on vine fruit-based products (EU, 2005).

1.3 POTENTIAL MYCOTOXIGENIC AND COMMON VINEYARD FUNGI

There are many different fungi that are associated with grapes. Some are more common in the field, so-called phyllosphere fungi associated with the foliage and ripening berries, while others colonise post-harvest mainly during storage and transport of grapes. Not all mycotoxigenic fungi are able to produce mycotoxins on this nutrient substrate. It is not understood why some do and others do not. However, it is important to note that the presence of mycotoxigenic fungi does not necessarily mean a product is contaminated with the toxin. More importantly, the mycotoxins may persist long after vegetative growth has occurred and the moulds have died (Northolt, 1979).

1.3.1 Toxigenic fungi on grapes

There are numerous fungi that are known to produce toxins, but the ones associated with the human food chain can be broken down into four genera: *Alternaria, Aspergillus, Fusarium,* and *Penicillium* (Scott, 2004) all of which are commonly found on grapes (Bellí *et al.*, 2005).

Table 1.1 Summary of the European Commission regulation (EC) No 123/2005 of 26 January 2005 amending Regulation (EC) No 466/2001 on the permitted maximum levels of OTA in different products implemented on the 1st April 2005 (EU, 2005).

Products	Ochratoxin A: Maximum ($\mu g\ kg^{-1}$)
Cereals (including rice and buckwheat) and derived cereal products Raw cereal grains (including raw rice and buckwheat)	5.0
All products derived from cereals (including processed cereal products and cereal grains intended for direct human consumption)	3.0
Dried vine fruit (currants, raisins and sultanas)	10.0
Roasted coffee beans and ground roasted coffee with the exception of soluble coffee	5.0
Soluble coffee (instant coffee)	10.0
Wine (red, white and rosé) and other wine and/or grape must based beverages	2.0
Grape juice, grape juice ingredients in other beverages, including grape nectar and concentrated grape juice as reconstituted	2.0
Grape must and concentrated grape must as reconstituted, intended for direct human consumption	2.0
Baby foods and processed cereal-based foods for infants and young children	0.50
Dietary foods for special medical purposes intended specifically for infants	0.50
Green coffee, dried fruit other than dried vine fruit, beer, cocoa and cocoa products, liqueur wines, meat products, spices and liquorice.	Not set

Although grapes had been tested for mycotoxins before (Drawert & Barton, 1974), it was not until Zimmerli & Dick's study (1986) that OTA was detected in wines and grape for the first time. This was quickly followed by many different reports of OTA in wines and grape products (Majerus & Otteneder, 1996; MAFF, 1997; Ospital *et al.*, 1998; Burdaspal & Legarda, 1999; Lehtonen, 1999; Otteneder & Majerus, 2000; Hocking *et al.*, 2003; Bellí *et al.*, 2004a). Most authors found a correlation between OTA and the geography with a split between north and south, particularly around the Mediterranean basin. When OTA was first found in wine the two main species responsible for OTA were believe to be *P. verrucosum* and *A. ochraceus* (Ospital *et al.*, 1998) with *A. ochraceus* most likely responsible for OTA in grape products as it was more commonly found in tropical climates than *P. verrucosum*.

Although black *Aspergillus* species had been reported to be OTA producers (Ueno *et al.*, 1991; Horie, 1995; Teren *et al.*, 1996; Wicklow *et al.*, 1996; Abarca *et al.*, 1997; Teren *et al.*, 1997) it was not until reports of *A. carbonarius* strains producing OTA which were isolated from grape products (Frisvad, 1995; Heenan *et al.*, 1998; Cabañes *et al.*, 2002; Da Rosa *et al.*, 2002) that the attention changed from *A. ochraceus* to other species. Some other *Aspergillus* section *Nigri* species associated with grapes have been reported but are not found as frequently or do not produce as much OTA as *A. carbonarius* (Battilani *et al.*, 2003b; Bellí *et al.*, 2005; Medina *et al.*, 2005; Valero *et al.*, 2005).

1.3.2 Taxonomy of the *A.* section *Nigri*

The taxonomy of the *Aspergillus* section *Nigri* has changed greatly over the years but the basics have remained the same based on the dark colour of their conidial heads. Mosseray (1934) subdivided Professor Biourge's own collection of 63 strains into 35 black *Aspergilli* species including 25 new species. Raper and Fennell (1965) reduced this number to 12, and there were a few more changes before Kozakiewicz (1989) suggested 17 taxa in the *A. niger* group and distinguished two groups: echinulate and verrucose, depending on their conidial ornamentations. *A* section *Nigri* still remains very hard to identify to species level as shown by the different conidiophores of different *A* section *Nigri* (Figure 1.3). A more simplified identification to common taxa has also been suggested (Abarca *et al.*, 2004). *Aspergillus* section *Nigri* were identified as either uniseriates or biseriates as shown in Figure 1.4 from Raper & Fennell (1965), with *A. carbonarius* identified to species level (Table 1.2)

With advances in sequencing a new key and rearrangement has been suggested by Samson *et al.* (2004) with 15 taxonomic species. They also screened sclerotium producing species of *A* section *Nigri* finding only two OTA positive strains associated with grapes, i.e., *A. carbonarius* and *A. niger*, but not *A. japonicus*.

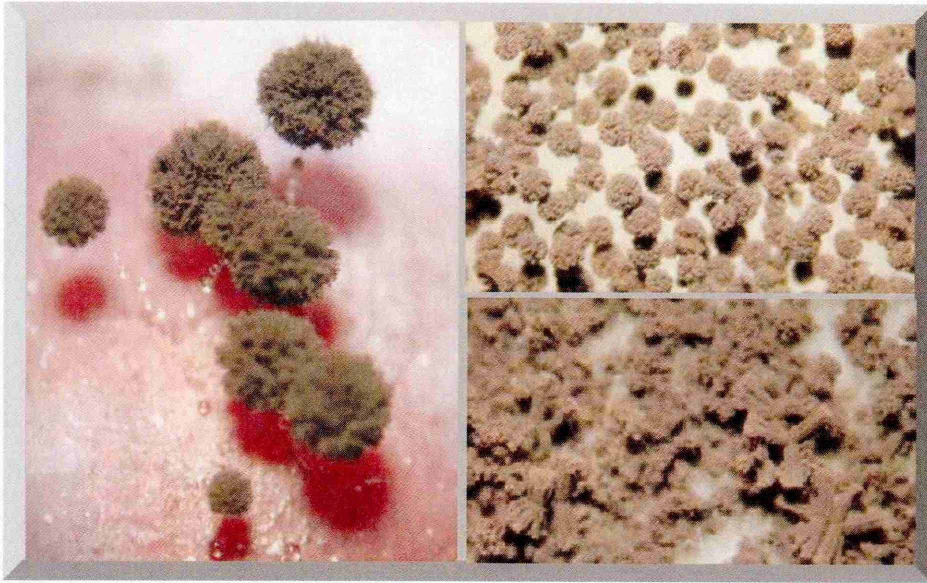


Figure 1.3 Conidiophores of different *A.* section *Nigri* under stereomicroscope.

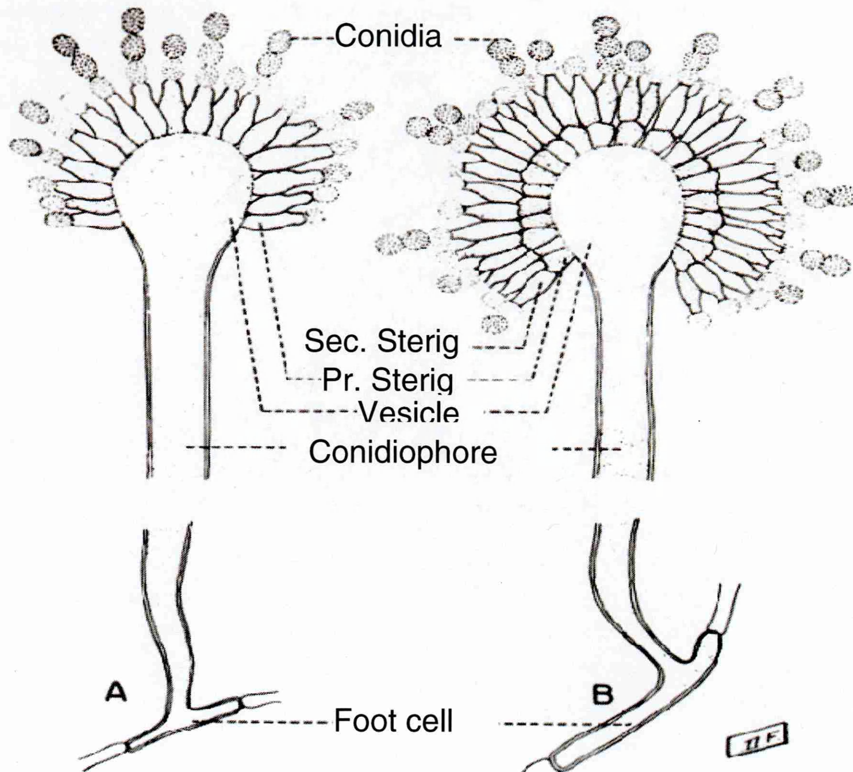


Figure 1.4 Conidial head showing, from left to right, the uniseriate and biseriate conditions of the sterigmata. (From Raper & Fennell, 1965).

Table 1.2 Key to identification of black aspergilli (Abarca *et al.*, 2004).

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

1.3.3 Main *A.* section *Nigri* and spoilage fungi found on grapes

Aspergillus carbonarius: *A. carbonarius* is possibly the easiest identified *Aspergillus* section *Nigri* species due to the spore size of up to 11µm and colour of the conidia (olive black to black). Klich & Pitt (1988) found it had not been reported frequently, whilst Kozakiewicz (1989) reported that it caused grape rot in India (Gupta, 1956).

Aspergillus ibericus: This is possibly a new *Aspergillus* species in the *Aspergillus* section *Nigri*, provisionally designated as *A. ibericus* (Cabañes *et al.*, 2004). Morphology is very similar to *A. carbonarius* with differences in the spore size, but all strains being OTA negative. The only reported *A. ibericus* isolates were in a survey conducted to assess mycotoxin-producing fungi on Portuguese grapes (Serra *et al.*, 2005).

Other black A*spergilli:* *Aspergillus* was classified into the Section *Nigri* by Gams *et al.* (1985), formerly '*A. niger* species group' by Raper and Fennell (1965). They have been isolated from a wide variety of food, are world-wide distributed and are considered as common fungi causing food spoilage and biodeterioration of other materials. Black aspergilli are commonly present in vineyards and have the ability to cause berry rot, know as *Aspergillus* rot or black mould (Snowdon, 1990).

Aspergillus ochraceus: *A. ochraceus* is associated with warmer and tropical climates similar to conditions for grapes, but is more commonly found on dried and stored cereal-based products (Kozakiewicz, 1989; Pitt & Hocking, 1997) .

Penicillium verrucosum**:** For nearly half a century was considered the most common OTA producer. Predominantly associated with mouldy grain, *P. verrucosum* is classified in subgenus *Penicillium*, section *Penicillium.* The major habitat of *P. verrucosum* is cereal crops in the cool and temperate climates of northern Europe and Canada (JEFCA, 2001). The inability of this fungus to grow above more than 30°C makes its presence in the tropics most unlikely. It is known to contaminate grapes in storage but is not common in the field (Pitt & Hocking, 1997).

Other contaminant fungi: *Botrytis cinerea* is known as the noble rot in certain wine grapes. It is a more serious problem on table grapes (Snowdon, 1990) and it develops on the stems and inside the berry erupting to the surface with grey conidia (Pitt & Hocking, 1997). It is predominantly a secondary pathogen in grapes. Pink and white yeasts are a major component of the natural flora of grapes but are not known to cause problems on healthy grapes, fermentative species are usually present in low numbers (Fleet & Heard, 1992). *Alternaria alternata* and *Cladosporium species* are also often commonly isolated from grapes (Snowdon, 1990). Pitt (1997) also reported that they caused rot and were commonly found on grapes.

1.4 EFFECT OF WATER AVAILABILITY, TEMPERATURE AND pH ON GROWTH AND MYCOTOXIN PRODUCTION

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, mycelial colonisation and ultimately mycotoxin production 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.* (1998a) examined the effect of water availability × temperature effects on growth of *A. niger* group strains isolated from cereal grain. More recently, Parra *et al.* (2004) and Parra 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) × temperature conditions on growth and sporulation. However, only a few studies have examined the effect of these abiotic variables alone and the interactions on growth and OTA production by the *A. section Nigri* group (Mitchell *et al.*, 2003; Bellí *et al.*, 2004b; Bellí *et al.*, 2004d; Mitchell *et al.*, 2004).

1.4.1 The concept of water availability and water activity

All life requires water to grow and fungi are no exception. Water is present in different percentages in all substrates, but it is the availability of the water which affects the

ability to grow. Having a large percentage of moisture content does not indicate the quantity of water that is readily available to an organism for metabolism (Aldred, 2000). There has been much published work about the fundamental requirement of water for microbial growth (Scott, 1957; Ayerst, 1969; Troller & Christian, 1978; Cooke & Whipps, 1993). Water availability determines if and how long it will take for spore germination, its metabolic rate, respiratory activity and rate of growth and possible toxin production. It will also affect the extent to which heat is released through respiration, modifications of temperature through spontaneous heating and altering metabolic rates. These factors influence the range of micro-organisms that can grow and sporulate, both anamorphic and teleomorphic (Lacey & Magan, 1991)

Water content of a substrate can be expressed as the percentage of moisture content, based on the ratio of the dry weight to the wet weight (expressed either on a wet weight or a dry weight basis). However, this does not indicate the amount of water available for fungal growth. This is because not all water is equally accessible to micro-organisms. Water is held via strong hydrogen bonding in a monolayer to the substrate and is known as water of constitution. Outside this layer, water is more weakly bound, decreasing with greater depth of molecules separating the outer molecules from the substrate constituents. Eventually, the water molecules become free from chemical binding but are held in micropores with dimensions at least equal to the water molecule. These micropores fill with increasing water content and low molecular weight compounds may dissolve to give additional osmotic effects (Lacey & Magan, 1991)

The water availability may be expressed in a number of different ways; Scott (1957) first introduced the concept of water activity (a_w). This is defined as the ratio of the vapour pressure of the water in a substrate to that of pure water at the same temperature and pressure (Ayerst, 1969).

$$a_w = p/p_o = ERH(\%)/100$$ (Labuza, 1974)

Where p = vapour pressure of water in solution or solid substrate

p_o = vapour pressure of pure water at experimental temperature and pressure

ERH (%) = equilibrium relative humidity at which a solution or solid substrate neither gains nor loses moisture to the atmosphere.

Both a_w and ERH are numerically the same, except a_w is expressed as a decimal fraction of one, whereas E.R.H. is expressed as a percentage. 1.0 represents the a_w of pure water. Water activity is always measured at a constant temperature, as this affects the relationship. The lower the a_w the less water is available to micro-organisms. Water activity is closely related to temperature, a given substrate and moisture content, and the a_w will increase with increasing temperature. This is primarily the consequence of the general increase in thermal motion (Multon, 1988)

1.4.2 The concept of water potential

Water availability may also be expressed as water potential (ψ) which is measured in pascals (Pa) and measures the potential free energy in a system (relative to a hypothetical pool of pure free water of specific mass (Papendick & Mulla, 1986). Pure

free water has a value of zero and water which is chemically or physically bound to the substrate has a lower water potential i.e. a negative value. Any microorganism must use energy in terms of effecting a physiological response to raise the thermodynamic potential of the water and make it available. ψ and a_w are associated by the following formulae:

$$\psi = (RT/V)\log_n a_w$$

Where R = the ideal gas constant

T = the absolute temperature

V = the volume of one mole of water.

In this study a_w will be used as it is more commonly used in the food industry whereas ψ is more commonly used in soil microbiology. There has been pressure to adopt ψ more widely to unify the expression of water availability (Lacey & Magan, 1991). One of the advantages of ψ over a_w is that a_w represents only one component of ψ and varies with temperature whereas ψ is expressed in the same units as osmotic potential of both substrate and cell and is independent of temperature. It can also be separated into its component osmotic, matric and turgor potentials to indicate their relative importance.

1.4.3 Water activity effects on growth

Fungal growth has been shown to occur over the a_w range of 1.0 to 0.6 for extreme xerophiles (Troller & Christian, 1978; Pitt & Hocking, 1997). If grown at sub-optimal a_w levels growth rates are reduced and lag times increased. Fungal growth occurs over a wider a_w range under optimal conditions likewise when a_w is optimal the temperature conditions for growth are the widest. As a_w changes from optimum for any given

fungus, so the range of environmental conditions it may grow over is reduced. Magan and Lacey (1988) found that pH, temperature and gas balance stress interacted with lowered a_w to markedly inhibit germination and growth of a range of spoilage fungi. There is no information on the effect of water activity on germination, germ tube extension and growth of *A .carbonarius in vitro* or on grape skins or grape flesh. This knowledge is critical for development of appropriate control measures to be devised to control this species pre-harvest.

1.4.4 Temperature

In the natural environment fungi are exposed to wide range of temperatures which vary daily and seasonally. The constant changing of temperature has an effect on germination, growth and mycotoxin production. *Aspergillus* section *Nigri* are classed as mesophiles due to the ability to grow at moderate temperatures but little is known about the upper and lower extremes for germination, growth or OTA production. Mesophiles are regarded to grow from about 10°C up to about 30-40°C (Carlile *et al.*, 2001; Deacon, 2005). Belli (2006) reported that temperatures in the shade in Spanish vineyards can vary from 12-32°C.

Living organisms are restricted in extreme temperatures by the break down of cellular compounds. To grow in extreme environment fungi must be able to survive reduced chemical reactions and an increase in the viscosity of water and possible protein denaturing (Robinson, 2001). Primary and secondary metabolism can also be affected by temperature in a variety of ways, most notably nutrient requirement can sometimes increase with increased temperatures (Carlile *et al.*, 2001).

It has been shown that temperature has an effect on the lag phase prior to growth and growth rates of a range of *Aspergillus* and *Penicillium* spp. (Ayerst, 1969; Magan & Lacey, 1984a; Marin *et al.*, 1998a). Many species in these two genera are classed as mesophiles and as the temperature becomes closer to the margins lag phases prior to growth increased and rates of mycelial extension decreased, until complete inhibition.

Temperature plays an important role in the ecological profile for fungal growth. Many studies have only examined germination and growth in relation to a_w at single temperatures (Snow, 1949; Pitt & Hocking, 1977). Both temperature and water relations need to be studied, as such interacting abiotic factors are important as they represent the fundamental two dimensional niche in which fungi can germinate, grow, and compete for available resources. There is very little knowledge of the effect of such interacting environmental factors on strains of *A. carbonarius* from different regions of Europe.

1.4.5 Hydrogen ion concentration

In general fungi can grow over a range of pH 4.0-8.5 or sometimes 3.0-9.0, with some *Aspergillus* and *Penicillium* known to be acid tolerant and grow down to pH 2.0 (Deacon, 2005). *Aspergillus carbonarius* has been shown to produce OTA over a range of pH from 2.00-10.00 on Czapek Yeast Agar (CYA) media (Esteban *et al.*, 2005). Whilst an *A. ochraceus* was recently shown to produce OTA over a narrower range of pH 5.5-8.5 (Muhlencoert *et al.*, 2004). OTA production by *A. carbonarius* has not been studied in relation to grapes and the low pH level of the juice.

1.5 FUNGAL INTERACTIONS

The vineyard is not an aseptic environment and fungi are not normally found homogeneously, but in mixed populations. Fungal species often grow in the same environment and as such share the same ecological niche, and may interact and influence other fungi due to excretion of exoenzymes and extracellular secondary metabolites (Magan *et al.*, 1984; Rayner *et al.*, 1995; Magan *et al.*, 1997; Marin *et al.*, 1997; Marin *et al.*, 1998e; Lee & Magan, 1999a 1999b; Marin *et al.*, 2001)

Magan & Lacey, (1984b) demonstrated that changes in the environment can significantly impact on fungal interactions and alter the competitiveness of individual species. Subsequent work with a range of spoilage mycotoxigenic fungi has supported this (Marin *et al.*, 1998e; Lee & Magan, 2000; Marin *et al.*, 2001). Changes in the environment or other stress factors such as fungicide applications can lead to one species having an advantage over a competitor as has been seen in field trials with mycotoxigenic *Fusarium* species and interactions with non-mycotoxigenic plant pathogens of wheat such as *Microdochium nivale* (Jennings *et al.*, 2000; Simpson *et al.*, 2001).

The interaction of spoilage fungi when studied *in vitro* can be scored (Magan & Lacey, 1984b) by observing the macro and microscopic interactions and giving individual interacting species numerical scores to represent categories of interaction type. The more dominant a species, the higher the score given. Interaction scores for each species can then be added together to obtain an Index of Dominance (I_D). This score can then be compared to see variations under different environmental conditions.

Interaction and competition have been shown to have a marked influence on OTA production (Ramakrishna *et al.*, 1993; Lee & Magan, 2000). The effects of interactions between *A. carbonarius* and other grape fungi have not been studied previously or the effects on OTA quantified, especially in relation to environmental factors.

1.6 EFFICACY OF FUNGICIDES AND OTHER CONTROL AGENTS AGAINST GROWTH AND OTA PRODUCTION BY *ASPERGILLUS CARBONARIUS*

1.6.1 Fungicides

Fungicides have been shown to control the growth of *A. carbonarius* on grapes (Tjamos *et al.*, 2004). Fungicides have also been shown to reduce OTA production *in vitro* by *A. ochraceus* isolates (Munimbazi *et al.*, 1997). In general, little is known about the interaction of fungicides on OTA production by *A. carbonarius.* A recent report by the grape and wine research & development corporation (2004) stated that while fungicide programmes are used to reduce the development of some bunch rots (e.g. *Botrytis* rot), to date, fungicide treatments have not been widely used to control *Aspergillus.* Bellí (2006) reported that fungicides produced mixed results with some fungicides inhibiting or reducing growth. There was some reduction in OTA, with a few fungicides also found to stimulate OTA production. 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.

1.6.2 Sulphur dioxide

Sulphur dioxide (SO_2) is one of the oldest food additives and has a long history as a disinfectant by the burning of elemental sulphur and the use of the resultant flames. After the development of inorganic chemistry SO_2 and its salts became commonly used as preservatives, particularly of food and beverages (Magan, 1993b). It is commonly used as a fungal inhibitory treatment of grapes and sometimes raisins. It is commonly used for table grape storage in order to prevent growth of *B. cinerea*. It is also used in the process of wine making and it is a necessity for the storage and preservation of white wines.

SO_2 can be highly toxic to micro-organisms as it has mutagenic effects, and thus inactivates mRNA and reacts with disulphide linkages in proteins, enzyme cofactors, aldehyde and ketone structures of five and six carbon sugars; it deaminates cytosine derivatives to uracil, and it has deleterious effects on the membrane (Babich & Stotzky, 1980). In contrast, small quantities of SO_2 may stimulate growth as the sulphur is an essential element for growth.

There is very little detailed information on the actual tolerance and sensitivity of OTA-producing fungi to SO_2. Previously, Magan (1993b) examined the effect of different concentrations of SO_2 in solution on the growth of *Penicillium* and *Aspergillus* spp. Furthermore, Majumber *et al.* (1973) reported the inhibition of both fungal growth and mycotoxin production. However, no studies have examined the effect of SO_2 on germination, germ tube extension, growth and OTA production by *A. carbonarius*.

1.6.3 Controlled atmospheres

Controlled atmosphere technology is well established for insect control especially as there is intense pressure to phase out methyl bromide as a fumigant. Changing the proportion of atmospheric gases in an environment can affect fungal development. The ratio of elevated CO_2 to reduced O_2 also has an influence on fungal development (Magan & Lacey, 1984c).

The effects of modified atmospheres on mycotoxigenic fungi has been studied but none have examined the black *Aspergilli* and OTA production. Paster *el al.* (1983) demonstrated that atmospheres containing 30% CO_2 or more completely inhibited OTA production by *A. ochraceus* regardless of the O_2 concentration, although growth was inhibited only with CO_2 concentrations > 60% and was stopped by 80% CO_2. Furthermore, T-2 toxin production was reduced by 80% or 50% CO_2 and 20% O_2 but the growth of *Fusarium sporotrichioides* was not affected by < 60%CO_2 (Paster *et al.*, 1986). Recently, Cairns-Fuller *et al.* (2005) showed that growth and OTA production by strains of *P. verrucosum* were only slightly affected by up to 50% $C0_2$, regardless of water availability, both *in vitro* and in wheat grain.

1.7 POPULATION DYNAMICS IN VINICULTURE

Little information exists on mycoflora and potential OTA-producing fungi in Spanish wine grapes. In this study the objective was to correlate the fungal populations isolated over a three year period in different vineyards with different cultivars. Attempts were

then made to try and correlate population dynamics with the prevailing climatic conditions to determine the key parameters influencing OTA contamination. Recent studies have suggested that black aspergilli, especially *A. carbonarius*, are the main OTA producing species on grapes (Cabañes *et al.*, 2002; Abarca *et al.*, 2003; Battilani *et al.*, 2003b; Bellí *et al.*, 2004c).

1.8 OBJECTIVES

This work was carried out as part of an EU funded project, Quality of Life Programme, Key Action 1 in Food Nutrition and Health; QLK1-CT-2001-01761; Wine Ochra Risk. Within this project the objectives of this work was designed to examine the following:

1. Population dynamics of fungi responsible for OTA production on grapes in the field.
2. Identification of the ecological parameters and host status governing growth and toxin production by the key OTA species/strains from different EU wine growing regions and table grapes from Israel.
3. The impact of a_w × temperature × pH effects on the germination, growth and OTA production profiles for *A.* section *Nigri* aggregate strains from 4 regions of Europe.
4. Determine the partitioning of OTA production in *A. carbonarius* and related species in biomass, spores and medium.

5. The effect of competition between *A. carbonarius* and other grape inhibiting phyllosphere fungi under different environmental factors on Index of Dominance and OTA production.
6. Efficacy of fungicides, SO_2 and controlled atmospheres on growth and OTA production under different environmental condition on germination, growth and OTA production.
7. Investigate the ability of alternative naturally occurring chemicals to control growth and OTA production.
8. Identify the most important factors which could be used in the development of models for the prediction of low and high risk climatic conditions on a regional basis.

Figure 1.5 Summarises the components of this work and interactions between them.

Phase I

Ecology of *Aspergillus carbonarius*

Water activity × *Temperature* × *pH*

- Germination – *in vitro*; grape skin, grape tissue
- Growth – grape based media
- OTA production – grape based media
- Partitioning of OTA in biomass, spores and medium
- Interactions –Index of Dominance
- Isopleth contour maps of growth/OTA production + interacting environmental factors for the first time

Phase II

Control of *Aspergillus carbonarius*

Fungicides SO_2 CO_2 *Other methods*

- Germination
- Growth
- OTA production
- LD_{50}, LD_{90} values

Phase III

Field population dynamics in vineyards

- Population dynamics of phyllosphere fungi on ripening berries
- Relative dominance of *A. carbonarius* relative to other species
- Correlation with climatic conditions
- Potential for OTA production in musts

Input into development of models to predict low/high risk years in southern Europe based on scientific data on *A. carbonarius* and regional GIS climatic information systems

Figure 1.5 Flow diagram of the experimental work carried out in this thesis.

CHAPTER 2
MATERIALS AND METHODS

2.1 FUNGAL SPECIES AND ISOLATES USED IN THIS STUDY

All the *A.* section *Nigri* species and isolates used in this work were isolated from vineyards from different European countries and are shown in Table 2.1. Other species of fungi used in this study are shown in Table 2.2.

Strains were courtesy of the Catholic University Piacenza, Faculty of Agriculture Italy. Departamento Engenharia Biologica, Universidade do Minho, Portugal, Department of Postharvest Science, ARO The Volcani Center, Israel. Agricultural University of Athens, Faculty of Crop Sciences and Production, Greece. Other species of fungi used in this study are shown in Table 2.2 and are held at Cranfield University Silsoe, Bedfordshire UK.

Fungal isolates were kept at -80°C and stored as a spore suspension in a 1% glucose (Fisher Loughborough UK) 90% reverse osmosis water (RO) and 0.9% NaCl (Fisher, Loughborough, UK) solution as a cryo-protectant. Purity of the cultures was checked by streak plating and microscopic analysis. Isolates were defrosted and then spread plated on synthetic grape juice media (SGM) plates and incubated at 20-25°C for 5-7 days before use.

2.2 BASIC MEDIA USED IN THIS STUDY

2.2.1 Media preparation

All agars were autoclaved at 121°C, 15 psi, for 15 minutes, cooled to approximately 50°C before pouring; the media had to be agitated prior to pouring into sterile 90mm

Petri plates to ensure an even distribution of the high agar content. All Petri plates were stored at 4°C in sealed polyethylene bags. Petri plates with the same a_w were enclosed together to ensure no fluctuation in a_w.

2.2.2 *In vitro* studies

(a) Malt + 10% NaCl agar

2% malt extract agar was prepared for preliminary growth rate experiments with the addition of 10% salt to modify the a_w to 0.95.

(b) Grape Juice Media

25% Grape Juice agar medium red or white gape juice media (RGJM, or WGJM) was prepared by mixing 25% (w/v) (supermarket long life white/red grape juice) and 2.5% agar (Oxoid, UK technical agar no.3) in RO water. A buffer was added to adjust the pH to 4.0 – 4.2 (see Appendix A). These media were then autoclaved.

(c) Synthetic grape juice media

Synthetic grape juice (SGM) was prepared as detailed in Appendix B. This represented grapes at mid-veraison and was more reproducible than GJM. This medium was adjusted with a buffer to pH 4.0 – 4.2 (Appendix A) and 2.5% agar was added. This was mixed thoroughly before autoclaving.

Table 2.1 *Aspergillus* species and strains used in the study with the country of origin, IMI number and the grouping they were placed in for this study. Strains provided by the Catholic University Piacenza, Faculty of Agriculture Italy; Departamento Engenharia Biologica, Universidade do Minho, Portugal; Department of Postharvest Science, ARO The Volcani Center, Israel; Agricultural University of Athens, Faculty of Crop Sciences and Production, Greece.

Country of Origin	Code	Species	Sub groupings	IMI number*
Italy	MPVP A204	*Aspergillus japonicus*	Uniseriate	388661
	MPVP A558	*Aspergillus ochraceus*	Ochraceus	387345
	MPVP A703	*Aspergillus ochraceus*	Ochraceus	387347
	MPVP A1102	*Aspergillus carbonarius*	Carbonarius	388653
	MPVP A212	*Aspergillus japonicus*	Uniseriate	388862
	MPVP A933	*Aspergillus carbonarius*	Carbonarius	388524
	MPVP A1099	*Aspergillus niger*	Biseriate	388550
	MPVP A1109	*Aspergillus niger*	Biseriate	388551
	MPVP A372	*Aspergillus carbonarius*	Carbonarius	386557
	MPVP A263	*Aspergillus niger*	Biseriate	
Portugal	01UAs127	*Aspergillus niger*	Biseriate	387208
	01UAs128	*Aspergillus japonicus*	Uniseriate	387209
	01UAs294	*Aspergillus carbonarius*	Carbonarius	387249
	01UAs203	*Aspergillus niger*	Biseriate	387227
	01UAs219	*Aspergillus carbonarius*	Carbonarius	387231
	01UAs263	*Aspergillus carbonarius*	Carbonarius	387248
Israel	1-4-1-9-10.8	*Aspergillus carbonarius*	Carbonarius	387416
	1-4-1-9-7.7	*Aspergillus carbonarius*	Carbonarius	388679
Greece	G 458	*Aspergillus carbonarius*	Carbonarius	
	G 444	*Aspergillus carbonarius*	Carbonarius	

* CABI Bioscience culture collection number (CABI Bioscience, Egham, Surrey, UK)

Table 2.2 Non *Aspergillus* species and strains used in the study, were they were isolated.

Origin	Cranfield University catalogue number	Species	Isolation
Spain	SVF01	*Epicoccum nigrum*	Grapes
Spain	SVF02	*Aureobasidium pullulans*	Grapes
Spain	SVF03	*Botrytis cinerea*	Grapes
Spain	SVF04	*Alternaria alternaria*	Grapes
Spain	SVF05	*Phoma* spp.	Grapes
Spain	SVF06	Pink Yeast	Grapes
Spain	SVF07	*Cladosporium* spp.	Grapes
UK	IBT2266	*Penicillium verrucosum*	Wheat

2.2.3 Field studies

(a) Dichloran Rose Bengal Chloramphenicol Media

Dichloran Rose Bengal Chloramphenicol (DRBC) was used to enumerate spoilage fungi from grapes (Appendix C). This medium was selected as the dichloran lowered the pH. It also has 50% of the traditional rose bengal chloramphenicol medium which inhibits bacteria and yeasts, and also fast growing mucoraceous fungi.

(b) Czapek Dox-based media

All *Aspergillus* and *Penicillium* species were subcultured from DRBC plates onto Czapek Dox medium (CZ) to obtain pure cultures for identification. This medium uses sucrose as its only source of carbon, and nitrate as the only nitrogen source. Most bacteria are inhibited on this medium as well as the *Mucorales* fungi (See Appendix H for preparation.

Czapek yeast medium was similar to the above, with the addition of yeast extract (0.5%). This was used for screening of fungi isolated in field studies for OTA production (See Appendix G for preparation).

2.3 GERMINATION OF *ASPERGILLUS* SECTION *NIGER IN VITRO,* ON GRAPE FLESH AND SKIN

The aim of this series of experiments was to determine the effects of a_w and temperature on germination and germ tube extension of *A.* section *Niger* on different

substrates. The SGM plates, grape flesh and grape skin were all incubated in environmentally controlled chambers with appropriate glycerol/water solutions to maintain a constant E.R.H. in the chambers (Dallyn & Fox, 1980) (Appendix D) under a range of temperature conditions to simulate field conditions.

(a) *In Vitro* studies

Germination and germ tube extension studies were carried out at 15, 25, 30, 35, and 40°C and at 0.85, 0.90, 0.95, and 0.99 $a_{w.}$ Samples were taken every 3 hours for a period of 36 hours. Spore germination was calculated as a percentage of 50 spores, germ tube extensions were the means of 10 measurements. All studies were carried out in triplicate and repeated at least once.

Agar treatments were spread plated with 200 µl spores of *A. carbonarius* (1×10^6 ml^{-1}). After incubation 10 × 10 mm sections were cut, stained with lactophenol blue and mounted on glass slides for examination.

(b) Grape skin

Holding the stork of a non-sterile grape it was dipped into a spore solution of *A. carbonarius* (1×10^6 ml^{-1}). Grapes were then incubated in environmentally controlled chambers. Grapes were carefully removed from the chambers, holding the stark and the base of the grape. A scalpel was used to cut the skin into squares of approximately 7 × 7 mm. Fine tweezers were used to carefully pull the skin away from the pulp of the grape. The fine grape skin was then mounted and stain with lactophenol blue, on a glass slide.

(c) Grape Flesh

Grapes were cut into approximately 1.5 mm thick cross sections. The grape flesh was then inoculated with a medium inoculation loop with a spore solution of *A. carbonarius* (1×10^6 ml^{-1}). Grapes were then incubated in environmental controlled chambers until sampled. The flesh treatments were stained as described earlier and mounted on glass slides until measurements were made.

2.4 MODIFICATION OF WATER ACTIVITY OF MEDIA FOR ECOLOGICAL STUDIES

The aim of this study was to modify the media to steady state interacting $a_w \times$ temperature conditions relevant to different natural environment conditions.

2.4.1 Modification of water activity of grape juice media

Water activity (a_w) was adjusted using either glucose or glycerol (Fisher, Loughborough, UK) to 0.99, 0.98, 0.95, 0.93, 0.90, 0.88 and 085 a_w, respectively. A moisture absorption isotherm was prepared for each medium. A known quantity of glucose or glycerol was added to the medium and the a_w determined using an Aqualab® WP4 device (Decagon Devices Inc, Washington, USA) connected to a PC using Hyperterminal software (Higraeve Inc, Michigan, USA). The a_w levels were kept constant by storing the same a_w treatments in airtight bags. All plates were checked

prior to use and at the end of the experiment to ensure a_w levels had not altered significantly (± 0.003 a_w).

2.5 PARTITIONING AND DISTRIBUTION OF OCHRATOXIN A IN SPORES, BIOMASS AND MEDIUM

Little is known about the production of OTA, where it is produced within the biomass. The aim of the study was to increase knowledge of where OTA was produced in *A.* section *Niger* and whether it is transported to the spores or predominantly secreted into the growth substrate.

2.5.1 Partitioning and distribution of ochratoxin A in spores, biomass and medium

The first aim was to examine partitioning of OTA between spores, mycelium and medium. This was carried out on SGM with sterile Cellophane discs (8.5 cm, P400, Cannings, Bristol, UK) as an overlay. Two a_w treatments were, used (0.99, 0.95), both modified with the non-ionic solute glycerol.

The plates were centrally inoculated with 1 µl of a spore suspension (1×10^6 spore ml^{-1}). The colonies were allowed to grow at 20 and 25°C for up to 20 days. In all cases about 20 Petri plates per treatment were used to enable destructive sampling to be carried out for quantification of biomass, spores and OTA content.

The entire mycelial colony was removed together with the cellophane layer. This was suspended in 10 ml of sterile water containing 0.1 ml Tween 80 (0.1%) to wet the spores (Ramos *et al.*, 1999). To obtain spores, the contents were centrifuged to obtain a spore pellet with the mycelial mat floating on the surface. The spore pellets, colonies and medium were weighed and then extracted for OTA. In all cases at least five replicates per treatment were used for OTA quantification of each isolate.

2.5.2 Distribution of ochratoxin A in *A. carbonarius*

Plates modified to 0.99 and 0.95 a_w were centrally inoculated with a spore suspension (1×10^6 spore ml^{-1}). The plates were incubated at 20°C for 10 days with plates of the same a_w enclosed in polythene bags to maintain a constant $a_{w.}$ A 2 cm diameter cork borer was used to extract the centre of the colony for OTA analyses. A 1 cm strip around the growing margin was extracted using a spatula 0.5 cm either side of the growing colony. The third extraction was taken from the edge of the plate, extracting only the agar with no mycelial growth. The extractions were analysed for OTA as described in section 2.12.

2.6 *IN VITRO* ECOLOGICAL STUDIES ON SPECIES AND ISOLATES OF *ASPERGILLUS* SECTION *NIGER*

The studies carried out were:

(a) Effect of temperature and time on growth and Ochratoxin A production

Studies were carried out on SGM at 10, 15, 25, 30, 35, and 40°C, for periods of up to 56 days. All treatments and replicates were inoculated centrally with a small loop of spore suspension (1×10^5 spore ml^{-1}).

Diametric growth rate was measured in two directions at right angles to each other. The radial extension rate was plotted against time and the linear regression calculated to give a growth rate in mm day^{-1} at each different a_w and temperature, for each replicate and treatment.

(b) Temporal study of the effects of water activity and temperature on Ochratoxin A production

Temporal studies were carried out to determine optimal time of production of OTA over the range 0.99-0.88 a_w at 15-40°C. Triplicate samples were removed every 7 days up to 56 days and frozen until analysed for OTA. This experiment was carried out on SGM, modified with either glucose or glycerol, and repeated with red grape juice medium modified to 0.99-0.88 a_w at 25°C with glycerol.

2.7 *IN VITRO* STUDIES ON SODIUM METABISULPHITE ($Na_2S_2O_3$), AND CONTROLLED ATMOSPHERES

2.7.1 Effect of a_w and $Na_2S_2O_3$ on, growth and ochratoxin A

(a) Medium preparation

The $Na_2S_2O_3$ was added to RGJM which had a base a_w of 0.99. This was then adjusted to 0.965 and 0.93 a_w. These were obtained by adding 18.73 and 50.35g $D^{(+)}$ glucose 100 g^{-1} (Fisher, Loughborough, UK), respectively. The pH of all treatments was modified to 4.2 using a buffer solution and checked using a pH meter Hanna H18424 (Hanna Instruments, Leighton Buzzard, UK). A 100ml solution of this buffer was made by mixing 55.90 ml of 0.1 M citric acid $C_6H_8O_7{\cdot}H_2O$ (AnalaR®,

Merck Pharmaceuticals, West Drayton, UK) and 44.10 of 0.2 M sodium phosphate Na_2HPO_4 (Sigma-Aldrich, Dorset, UK).

(b) Adjustment of concentration of sodium metabisulphite

Sodium metabisulphite ($Na_2S_2O_3$; BDH Chemicals Ltd, Poole, England) was used as the source for SO_2. Six concentrations were used: 0, 100, 250, 500, 750 and 1000 mg kg^{-1}. In order to obtain these concentrations a stock solution of 30,000 mg kg^{-1} was made by adding 30g of NaMBS 1l of distilled water. No stock solution was added to the control treatment.

(c) Inoculation and incubation

The Petri plates of each treatment were centrally inoculated with a 1 µl fine loop of spore suspension at (1×10^6 spores ml^{-1}). For germination studies 0.2 ml of the spore suspension was spread on the surface of the plates using a sterile glass spreader (Magan, 1988). Plates of the same a_w were enclosed in polyethylene bags. All experiments were carried out with 4 replicates per treatment and incubated at 25°C.

2.7.2 Effect of a_w and control atmospheres on spore germination, growth and ochratoxin A production

Increased levels of CO_2 has been used in industry for the prevention of fungal spoilage in the storage of grapes for many years. This was not originally intended specifically for control of *A. carbonarius* and little is known about what effects CO_2 has on growth

and OTA production. Three different atmospheres were examined as shown in Table 2.3, each replicate controlled atmosphere treatment chamber containing beakers of modified glycerol water to alter the a_w.

Table 2.3 Mix of gases used in control atmospheres studies with increased CO_2 levels.

	CO_2	O_2	N_2
control	0%	21%	79%
25%	25%	$<1\%$	74%
50%	50%	$<1\%$	49%

(a) *In vitro* studies

Three different a_w levels were examined (0.99, 0.97, 0.93). The plates were central inoculated with a spore suspension (1×10^6 ml^{-1}) and incubated at 25°C for 10 days. Growth rates and OTA production was carried out as detailed in section 2.6 and 2.12 respectively. Germination rates were examined after 24 and 48 hours with growth diameter of colonies examined after 5 and 10 days. OTA analysis was carried out after 10 days.

(b) Grapes

All grapes were washed in sterile RO and left to dry in sterile conditions on paper towels. When the bunches were dry they were dipped in water containing spores (1×10^6 ml^{-1}) of *A. carbonarius* (MPVP A 1102). When the bunches were dry they were placed in the CO_2 containers on top of test tube racks. This raised the bunches off the container allowing air flow around the bunches. CO_2 was then bubbled through a bottle of water either unmodified (0.999 a_w) or to 0.95 a_w with water/glycerol solutions depending on the treatment condition required. All bunches were incubated at 20°C for either 7 or 14 day. Two sub-samples were

removed at time zero for control groups, one group prior to washing and a 2nd after washing before inoculation.

A bunch of grapes was removed from each CO_2 chamber and placed in a sterile Stomacher 400 bag (Seward Ltd, Thetford, UK). Approximately 10 g grapes were used to determine the population changes in the grapes. The grapes were then homogenised for 5 minutes with 90 ml of sterile RO water. The stomached grapes were then serially diluted up to 10^{-7} and 100 µl spread plated onto Malt extract agar plates. These were incubated at 25°C for 5-7 days until populations enumerated.

2.8 OTHER ENVIRONMENTAL EFFECTS ON GROWTH AND OCHRATOXIN A PRODUCTION

Within the vineyard there are many different environmental conditions affecting the growth and OTA production of *A. carbonarius*. The aim of this study was to look at the effects of some other environmental conditions on growth and OTA production. Increased salt levels and variations in pH can occur. These parameters can be further altered by the application of fungicides.

2.8.1 Effects of pH on growth and OTA production on *A. carbonarius*

The preparation of the SGM was modified to double concentration and agar was omitted from the preparation, the pH was then adjusted to the required level (pH 4.0, 7.0) from the unmodified medium at pH of 2.7. The medium were autoclaved together with an equal amount of RO water with agar (2.5%). When the media had cooled to <90°C the RO water and media were mixed together, and allowed to cool to 50°C

before pouring. Seven day old colonies were used to prepared spore suspensions (1×10^6) from *A. carbonarius* colonies and centrally inoculated using 1 μl of spore suspension. Each treatment was carried out in triplicate with plates of the same a_w sealed in plastic bags and incubated at 20°C. The plates were incubated for 15 days and then analysed for OTA using the method detailed in section 2.12.

2.8.2 Effects of NaCl on growth and OTA production by *A. carbonarius*

The standard SGM media contained 0.015% NaCl. Additional media was prepared at 1% and 3%. Inoculation, growth measurement and OTA analysis were carried out as in section 2.8.1.

2.9 EFFICACY OF COMMON GRAPE FUNGICIDES AND CONTROL AGENTS ON GROWTH OF *ASPERGILLUS CARBONARIUS*

2.9.1 Effects of common grape fungicides on *A. carbonarius*

The aim of this experiment was to determine how common grape fungicides affect the growth and OTA production of *A. carbonarius* under different temperatures and a_w levels. The fungicides used and their percentage active ingredients are shown in Table 2.4. Synthetic grape juice media was used unmodified at 0.99 and at 0.95 a_w. Prior to pouring, after the media had cooled to approximately 60°C, the fungicides were incorporated into the media to obtain the concentrations shown in Table 2.4.

The molten medium was then poured into 90mm Petri dishes. A loop of 1 $\times 10^6$ conidial suspension from a seven day old *A. carbonarius* culture was used to inoculate the plates centrally. Plates of the same a_w were sealed in plastic bags and incubated at 20 and 30°C. The colonies were measured at regular intervals during the incubation period by taking two measurements at right angles to each other for calculations of the growth rates. The plates were then extracted for OTA production after 10 and 15 days. The experiment was carried out with three replicates per treatment.

2.9.2 Effects of other control agents on *A. carbonarius* on grapes

There has been a growing interest in naturally occurring agents that have fungicidal properties. In this study resveratrol and catechin were examined for control of *A. carbonarius* for any effects on growth or OTA production. Preparation of the media was the same as described in section 2.9.1 and resveratrol and catechin dose levels are also shown in Table 2.4.

Table 2.4 Active ingredient of common grape fungicide and control agents with dosage used in the field and study.

Active ingredients	weight/volume in fungicide	Examined dose of active ingredients (mg l^{-1})	Field Dose of fungicides in Spain
Cyprodinil/Fludioxonil	37.5/25% w/v	0.01, 0.03, 0.05, 0.10, 0.50, 1.00, 2.50	1.8 g l^{-1}
Carbendazim	50% w/v	0.10, 0.50, 1.00, 2.50	0.6 g l^{-1}
Copper Sulphate		5, 25, 50	2.4 g l^{-1}
Trifloxystrobin	50% w/v	0.10, 1.0, 2.5, 5.0, 7.5, 10.0	0.13 g l^{-1}
Catechin		50, 100, 150, 200	
Resverotrol		50, 100, 150, 200	

2.10 INTERSPECIFIC INTERACTIONS BETWEEN COMMON GRAPE FUNGI ON AGAR

The aims of this study were to:

a. Determine how *A. carbonarius* interacted with other species at 20 and 30°C and at 0.93, 0.95 and 0.99 a_w.
b. Investigate the effects of their interactions under the different environmental conditions on ochratoxin A production.

Seven day old colonies were used to prepare spore suspensions of (1 × 10^6) of *A. carbonarius, B. cinerea, Phoma sp,* pink yeast, white yeast, *A.* section *Nigri* biseriate (01UA s203) and uniseriate (MPVP A 204). Due to problems harvesting spores from *E. nigrum, and A. alternata* mycelium from the growing edge of colonies was included in the spore suspension. Synthetic grape juice media plates were then inoculated using a 1 μl loop with *A. carbonarius* and one of the other species inoculated approximately

4 cm apart. Controls were inoculated centrally using the 1 μl loop. Each treatment was replicated three times and plates of the same a_w were sealed in plastic bags and incubated at 25 or 30°C. The plates were incubated for 15 days and analysed for ochratoxin A using the method detailed in section 2.12.

Colony diameter was measured by taking two measurements at right angles to each other during the incubation period to calculate a growth rate by linear regression. The colonies were checked regularly for interactions by macroscopic and microscopic analysis. The interactions were then scored with each interaction given a score based on mutual intermingling (1-1), mutual antagonism on contact (2-2), mutual antagonism at a distance (3-3), dominance of one species on contact (4-0) and dominance at a distance (5-0). In the case of the dominant interactions the higher score was always awarded to the more competitive fungus (Magan & Lacey, 1984b). For example, if *A. carbonarius* was dominant over *B. cinerea* upon contact this would result in a 4 and 0 respectively being awarded to the two fungal species. The scores for each species were totalled to give an overall Index of Dominancy (I_D) value as a measure of competitiveness.

2.11 FIELD WORK

The aim of the field work was to identify the most common mycoflora on wine grapes, study their evolution during grape ripening and to identify the main ochratoxigenic species present and their ability to produce OTA.

2.11.1 Vineyards and grapes

Four sampling areas were chosen in Spain in collaboration with the wine industry. Ten vineyards were sampled in each region, representing a cross section of the country, including international, national as well as common regional varieties (Table 2.5).

The four samples areas were made up of at least one Demominación de Origen (DO) or Demominación de Origen e Calificada (DOCa) in each, the DO and DOCa production and agricultural methods were tightly controlled and management was done following the criteria of the DO or DOCa (Figure 2.1).

2.11.2 Selection of vines

Three sampling dates were chosen (1 month after setting, Veraisòn, and harvest time). Ten vines were chosen from each vineyard, along a diagonal of the vineyard as shown in Figure 2.2. On the first sampling date, vines were identified and marked. On the subsequent sampling dates the adjacent vine was selected. Grapes bunches were chosen at random. Bunches could only be changed if they were extremely damaged or unsuitable for sampling i.e. insufficient berries.

2.11.3 Bunch handling

Paper bags were placed around the bunches tc minimize possible contamination from sample handling, they were then cut, allowing the bunches to enter the bags without handling. The bags were sealed and kept cool until return to the laboratory when they were stored at 4°C until the next day when analysed.

Table 2.5 Grape variety and colour by sampling region and Demominación de Origen,

Latitude		41.37 N	41.22 N/ 41.23 N	42.30 N	39.34 N /39.30 N
Longitude		0.38 E	1.41 E /1.10 E	2.18 W	1.10 W /1.06 W
Variety	**Colour**	**Costers del Segre**	**Penedés/ C. Barberà**	**Rioja**	**Utiel-Requena**
Chardonnay	White	3	2	-	-
Macabeo	White	-	-	2	1
Sauvignon blanc	White	-	2	-	2
Bobal	Red	-	-	-	3
Cabernet Franc	Red	1	-	-	1
Cabernet Sauvignon	Red	2	2	-	1
Garnacha	Red	-	2	1	1
Graciano	Red	-	-	1	-
Merlot	Red	2	-	-	-
Tempranillo	Red	2	2	6	1
Total:		10	10	10	10

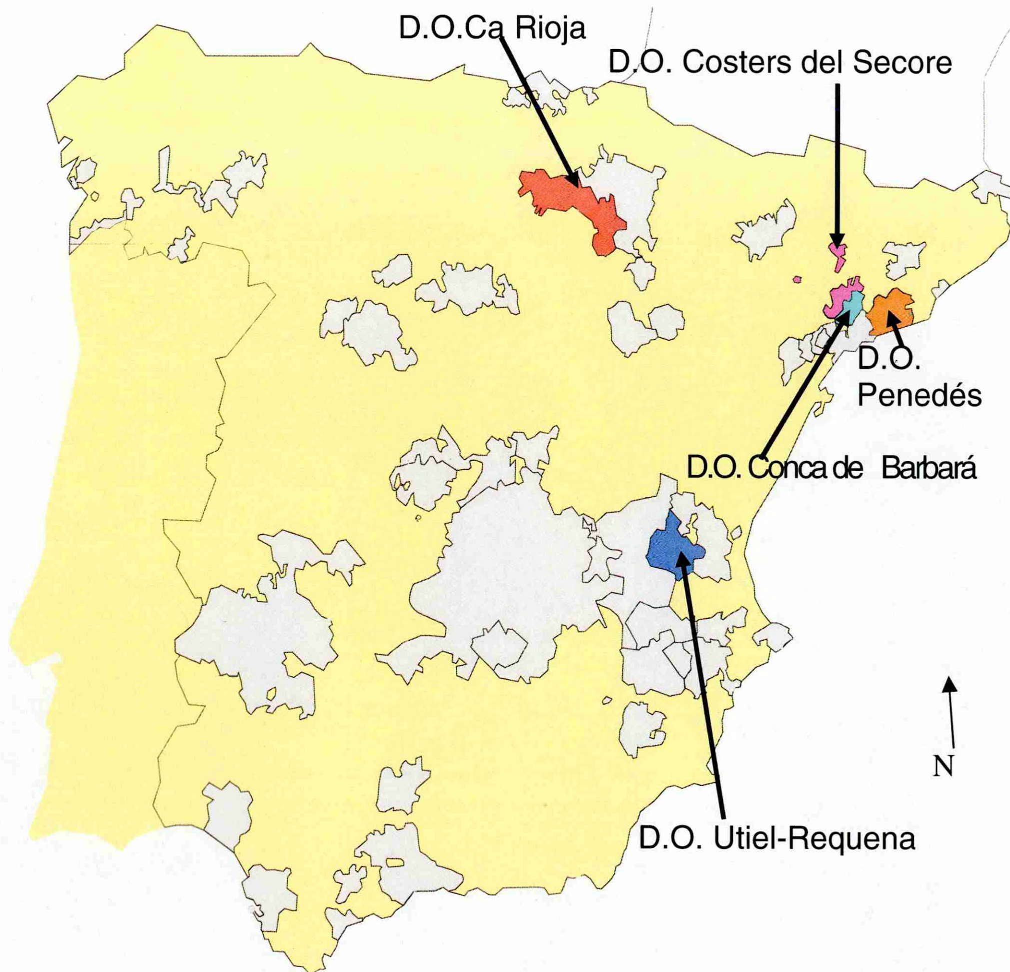


Figure 2.1 Map of the wine Demominación de Origen of Spain with the five used during the sampling studies highlighted.

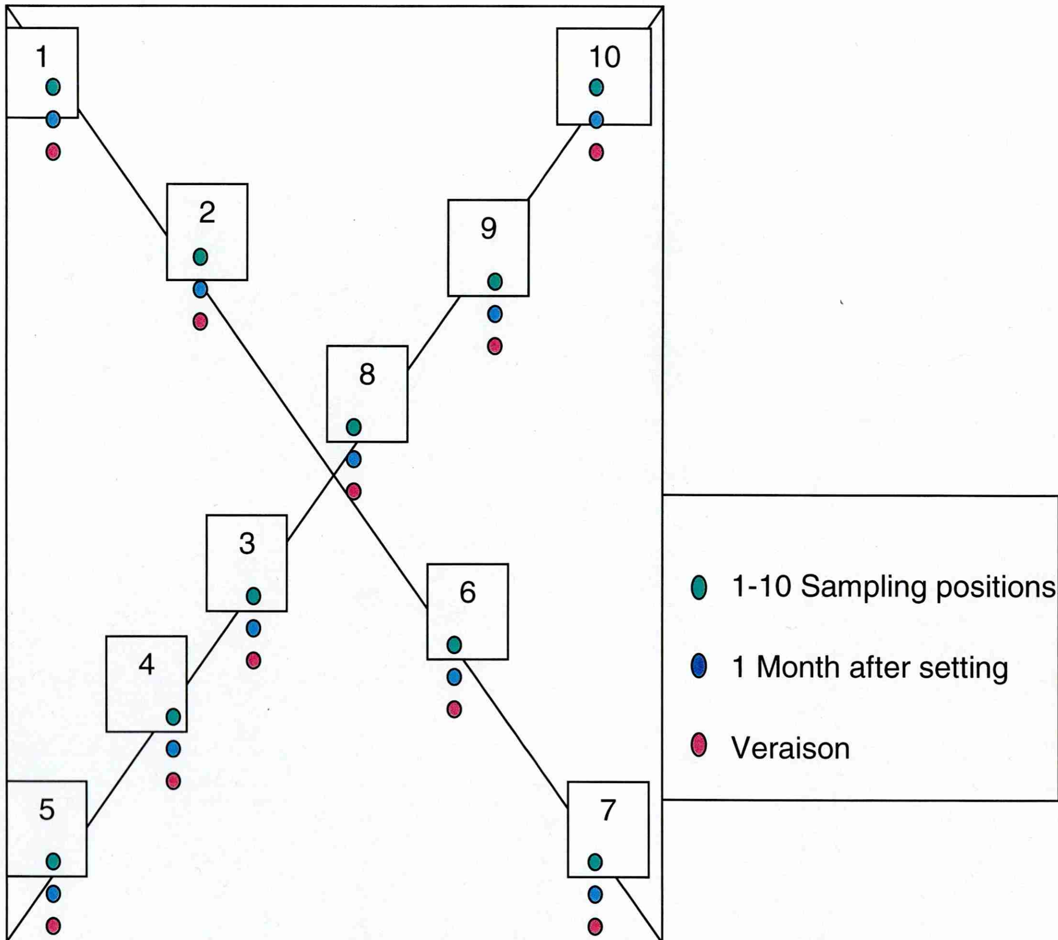


Figure 2.2 The ten locations with the three selected vines for sampling. The primary vines are marked in green (one month after setting), on the subsequent sampling dates the adjacent vine was sampled blue (Veraison), and red (harvest time).

2.11.4 Incubation and isolation of fungi from berries

In the laboratory five berries were randomly chosen from each bunch. These were transferred under sterile conditions to Petri dishes containing DRBC medium. Berries that were too large to be placed directly into Petri dishes were cut and placed (cut side up) on the DRBC medium. They were then incubated at 25°C for 5-7 days.

All *Aspergillus* and *Penicillium* spp. that could be identified on the DRBC medium were isolated and transferred under sterile conditions to CZ and CYA plates. *Aspergillus* and *Penicillium* spp. transferred to CYA were analyzed for OTA production and onto CZ for easier identification. Spores were taken from the DRBC media and inoculated as three point inoculations onto CZ and CYA media plates.

2.12 OCHRATOXIN A ANALYSIS

2.12.1 *In vitro* analyses of Ochratoxin A from media

The method used was adapted from Bragulat *et al.* (2001). The HPLC system used consisted of a Millipore Waters 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 (150 x 4.6mm, 5μm) (Pheonomenex, Cheshire, UK), with a guard column of the same material used to extend the column life and reduce drift. Run time for samples was 10 minutes. The flow rate of the mobile phase (57% acetonitrile, 41% water and 2% acetic acid) was 1 ml min^{-1}. Analyses of the results was carried out on a computer running Kroma system

2000 operating system (Bio-tek Instruments, Milan, Italy). An example of a chromatogram and standard calibration curve are shown in Appendix E

2.12.2 Extraction and analyses method

Three agar plugs were removed from the plate using a cork borer (4.5mm diameter). These samples were placed into a 2 ml microtube (Eppendorf, Cambridge, UK) and weighed. A 1ml of HPLC grade methanol (Fisher, Loughborough, UK) was added and the samples shaken and incubated at room temperature for 60 minutes. The extracts were filtered (Millex® HV 13mm, Millipore, Watford, UK) directly into amber HPLC vials (Jaytee Biosciences LTD, Kent, UK) and stored at 4°C until HPLC analysis was performed. This method was employed for the first year of study. In the second year the method was adapted and used in all further studies. Six plugs were used to ensure that a cross section of the whole growth area was examined (Figure 2.3). The samples were added to 28 ml Universal bottles, and 5 ml of methanol added. The Millex® HV 13mm, Millipore filters were changed to Fisher filter paper number QL 10, (Fisher, Loughborough, UK Celite® 545 (Sigma-Aldrich, Dorset, UK) (was added at approximately 0.25g to the filter paper) to improve filtration.

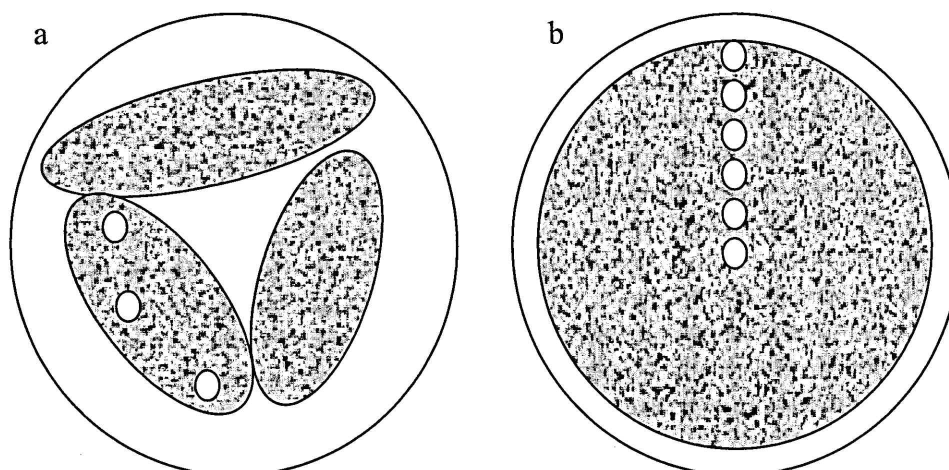


Figure 2.3 Agar plates used in analysis of OTA by HPLC. (a) CYA plate with three plugs cut from the agar covering a cross section of growth. (b) Adapted method used from the second year with 6 plugs cut from the plate covering a cross section of the growth area.

2.12.3 Must Preparation

Must was made from all berries collected at harvest time and all Cabernet Sauvignon collected at veraison (extra berries were collected at veraison to ensure sufficient berries to produce the must). A sub-sample of the collected berries were used to identify the fungi, with the remaining berries crushed by hand to produce must. The must was sieved into sterile bottles (250 ml) and stored at 4°C.

The OTA extraction was based on a method recommended by the OIV (Bezzo *et al.*, 2002) using an immunoaffinity column (Zimmerli & Dick, 1996; Tricard *et al.*, 1999). A 125 ml sample of the must was centrifuged at 3830 g for 15 minutes. The supernatant was filtered (0.45μm Sartonics, Spain). The pH was adjusted using 4M

NaOH (Sigma-Aldrich, Spain) to pH 7.0. The must (100ml) was passed through the immunoaffinity columns (R-Biopharm Rhone UK) at a flow rate of 2-3 ml min^{-1}. The column was washed with 20ml of RO water at a flow rate of 5ml min^{-1}. A syringe was slowly pushed down onto the column to ensure removal of all water. The OTA was eluted from the columns by passing under gravity with 2ml of methanol/acetic acid (98%:2%) (Sigma-Aldrich Spain). Air was then passed through the column to collect the last few drops. The eluate was evaporated under vacuum at 40°C. The precipitated OTA powder was then dissolved in 2ml of mobile phase, (57% acetonitrile; 41% water; 2% acetic acid) and stored at 4°C until analysed.

2.12.4 OTA standards

A stock solution of 50 mg kg^{-1} solution of OTA (Sigma-Aldrich, Dorset, UK) was prepared. After making the stock solution the concentration was checked before calibration standards for the HPLC were made. The HPLC standards were then made freshly by diluting the stock solution to the following concentrations of 12, 9, 6, and 3 mg kg^{-1}. The standards were used for the HPLC calibration curve, an example is shown in Appendix E.

2.13 STATISTICAL HANDLING OF DATA

Data input, data handling/manipulation, linear regression, and graph plotting was carried out using Microsoft Excel 2003 (Microsoft Co.). Other statistical tests were performed using STATISTICA version 7.1 (StatSoft, Inc. 2005). For ANOVA and LSD significance was determined at the 95% confidence limits.

CHAPTER 3
RESULTS

3.1 INITIAL ECOLOGICAL STUDIES

For knowledge of growth and ochratoxin A (OTA) production by *Aspergillus carbonarius*, *Aspergillus niger* aggregate and *Aspergillus ochraceus* the key impacts of interacting environmental parameters needed to be understood. This information is important for developing control strategies including modelling and risk prevention of OTA contamination of grapes. Initially several media were examined to find a realistic and practical nutritional substrate which gave consistent results and which could be replicated easily.

3.1.1 Initial experiments on malt extract agar and malt + 10% salt modified media

Initial experiments were carried out on a MEA + 10% NaCl to examine the growth of six Italian strains of *A. carbonarius*. This medium proved to be unsuccessful as growth was very restricted. Growth rates over 28 days were < 0.7 mm day^{-1} and subsequently (days 28-56) no visual measurements could be made. Sporulation was poor in these cultures, with the majority of the strains only producing mycelial colonies. Only two of the six strains sporulated in the 56 day incubation period, but even this was very restricted, whereas all the other strains produced no spores even after 56 days (Plate 3.1).

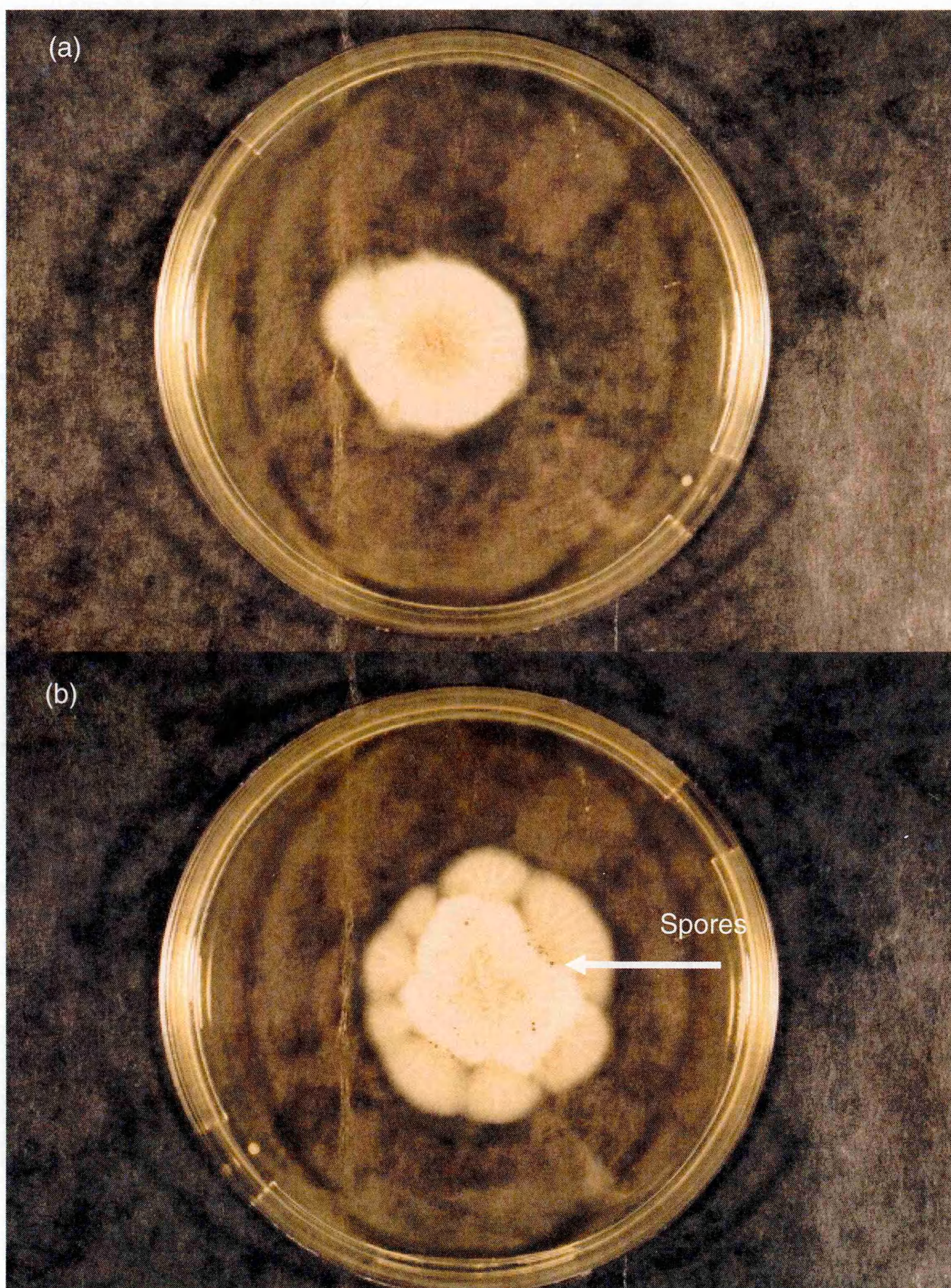


Plate 3.1 *A. niger* grown on MEA + 10% salt agar (a) limited growth after 56 days with no production of spores (b) sporulation after 56 days.

3.1.2 Grape juice-based media

Six strains of *A.* section *Niger* and a strain of *Penicillium verrucosum* were grown on a range of different concentrations of red/white grape juice media (RGJM/WGJM) at between 0-50% concentrations to find the optimal concentration for use in ecological experiments. An example of the results for two strains of *A. niger* aggregate and one of *P. verrucosum* are shown in Figure 3.1. It was found that at low concentrations, (<20% RGJM and WGJM), growth was limited, with at least one of the three replicates at each a_w treatment showing no growth. However, with higher concentrations, (>30% of RGJM and WGJM), sporulation occurred rapidly at the inoculation point but was not even over the rest of the colony (Plate 3.2). Although there appeared to be little difference between RGJM and WGJM there was a statistically significant difference in growth rates between RGMJ or WGJM with the exception of *P. verrucosum* (IBST22626) (Table 3.1). Further analyses of the statistics using a Box Whisker plot, showed that there are similarities in growth rates at concentrations of between 20-40% but this was not uniform across all strains on both RGMJ or WGJM but concentrations at <20% had the most significant effect on growth rates (Figure 3.2). Another factor in choosing a grape juice concentration was sporulation. On higher concentrations (30-50%) sporulation was visibly reduced compared to that on media containing <30% grape juice (Plate 3.2). For low concentrations of grape juice <10% there were problems with consistency between plates and thus was not used. For this reason a concentration of 25% RGJM and WGJM was used in some studies, as this concentration gave both good even growth and spore production across the whole plate.

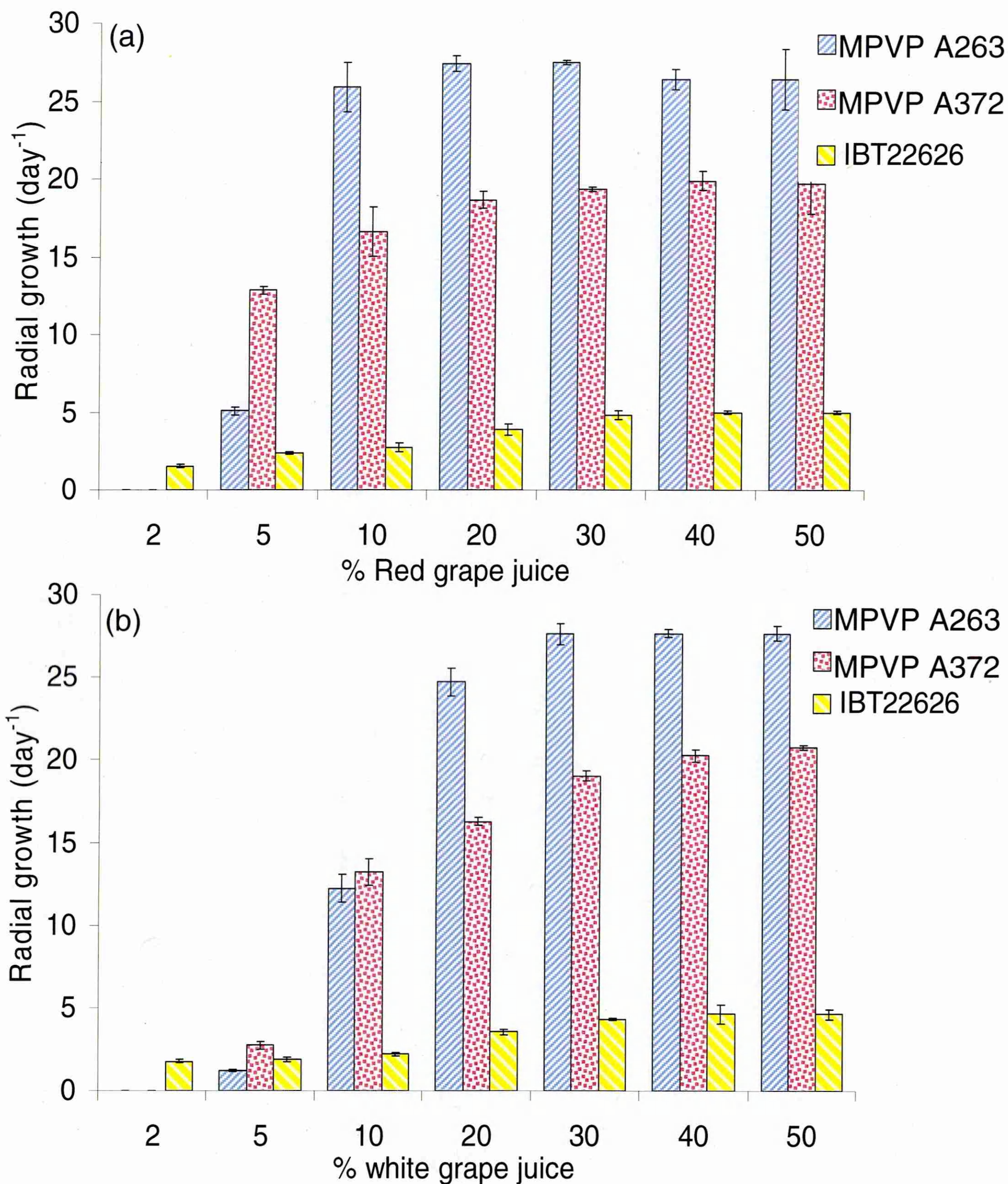


Figure 3.1 An examples of radial growth of two *A. niger* aggregate species (MPVP A 263, MPVP A 372) and one *P. verrucosum* (IBT22626) grown on (a) red or (b) white grape juice media at 25°C over a range of grape juice concentrations (2% - 50%). Bars indicate standard error of means.

Table 3.1 ANOVA of growth rate of *A. niger* aggregate species (MPVP A 263, MPVP A 372) and *P. verrucosum* (IBT22626) grown on two different substrates red or white grape juice media (sub) over a range of 2-50% , at 25°C * Indicates factors which were significantly different ($p<0.05$).

Source	Strain	DF	SS	MS	F	P
Sub	MPVP A 372	1	47.154	47.154	156.91	>0.001*
% grape Juice	MPVP A 372	6	2097.548	349.591	1163.27	>0.001*
Sub × % grape Juice	MPVP A 372	6	134.182	22.364	74.41	>0.001*
Substrate	IBT22626	1	1.5654	1.5654	8.821	0.006*
% concentration Juice	IBT22626	6	66.5258	11.0876	62.480	>0.001*
Sub × % grape Juice	IBT22626	6	0.4354	0.0726	0.409	0.867
Sub	MPVP A 629	1	72.42	72.42	37.375	>0.001*
% concentration Juice	MPVP A 629	6	4983.90	830.65	428.667	>0.001*
Sub × % grape Juice	MPVP A 629	6	241.21	40.20	20.747	>0.001*

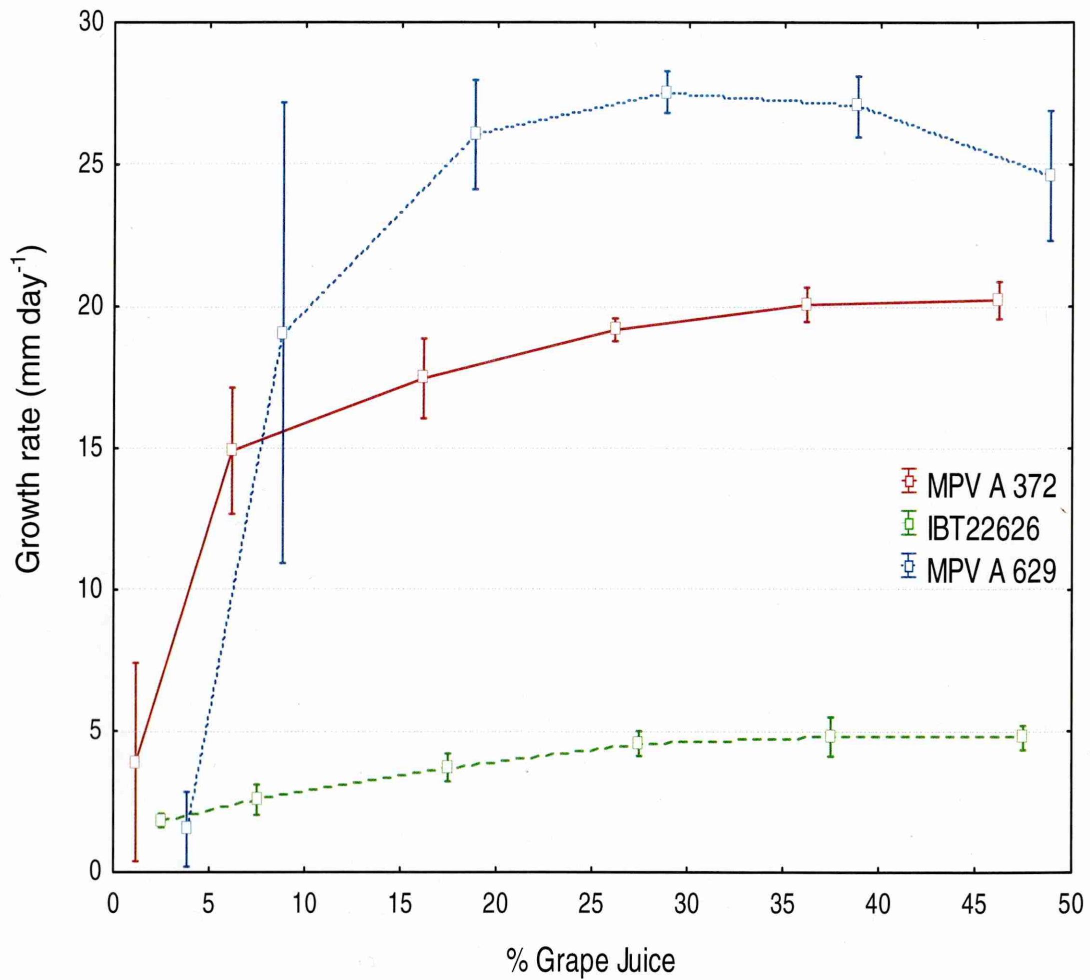


Figure 3.2 A Box Whisker statistical analysis of growth rate of two *A. niger* aggregate species (MPVP A 263, MPVP A 372) and one *P. verrucosum* (IBT22626) grown on red or white grape juice media at 25°C over a range of grape juice concentrations (2% - 50%). Analyst set to median; Box: 25%-75%; Whisker: non-outlier range.

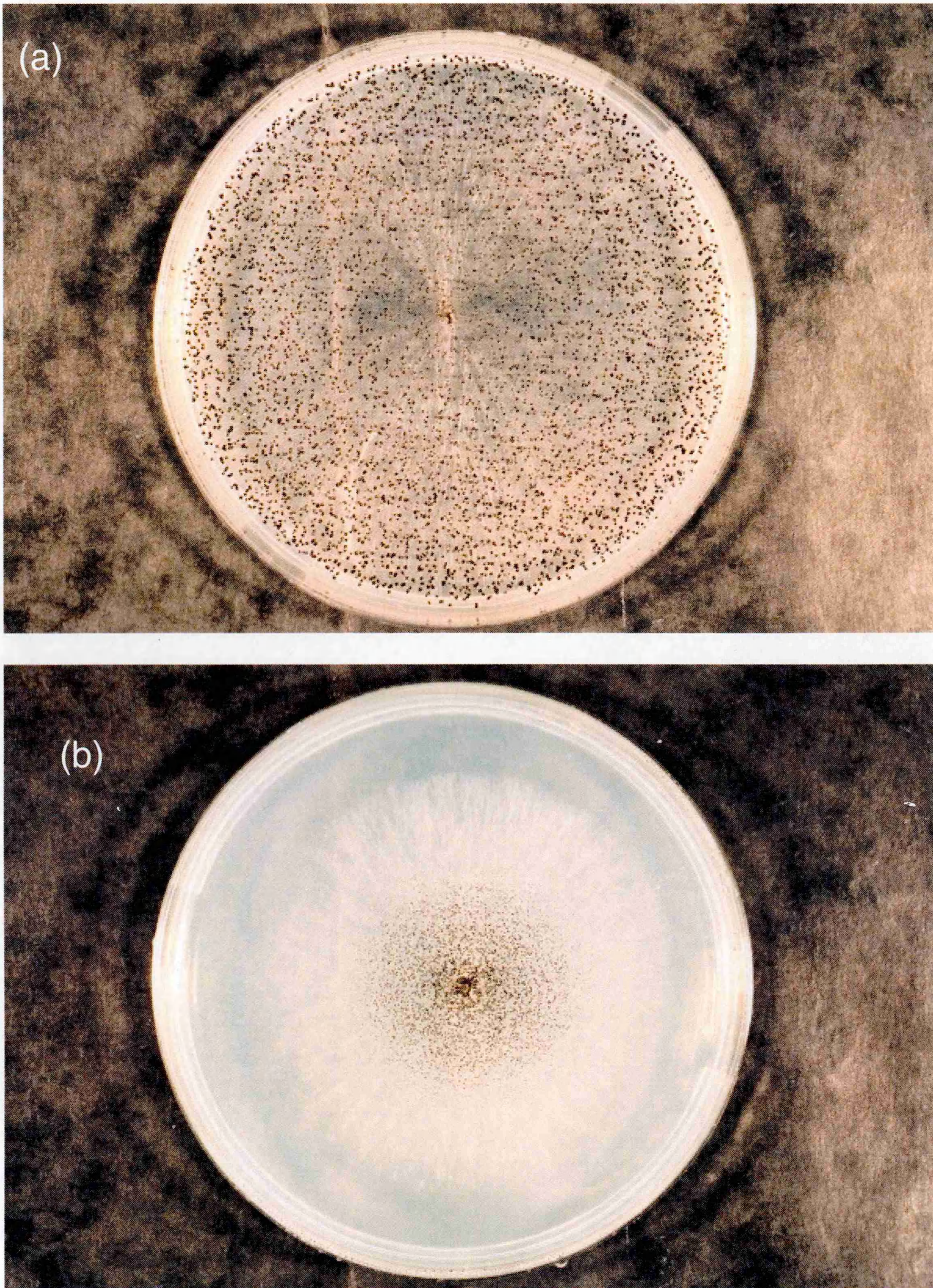


Plate 3.2 (a) *A. niger* aggregate showing profuse spore production over the whole plate after 30 days On WGJM at 25°C, (b) fast mycelial growth but limited spore production on WGJM at 25°C after 8 days.

3.2 WATER ACTIVITY MODIFICATION OF MEDIA

Isotherms were constructed for the concentrations of glucose or glycerol required to reduce the water activity (a_w) of the media for all *in vitro* studies. Figure 3.3 shows the amount of glucose that was used to modify the synthetic grape juice media (SGM). The a_w of the unmodified medium was 0.99 a_w and this was used as the control treatment. Glycerol was used to modify RGJM, WGJM, and SGM (Figure 3.4). Media were modified in the range 0.99- 0.85 a_w with both solutes.

3.3 WATER AND TEMPERATURE EFFECTS ON GERMINATION OF *ASPERGILLUS CARBONARIUS*

Germination and germ tube extension studies were initially carried out on SGM to give information on time scales and the range of a_w levels and temperatures over which *A. carbonarius* would germinate. These measurements were then compared with that on grape skin and grape flesh.

3.3.1 *In vitro* temperature and water activity effects on germination and germ tube extensions on synthetic grape juice medium

Not all spores germinated under all conditions examined, a 50% germination level was reached under nearly all condition. The time taken for germination to <30% was showed limited variation between optimum conditions, and did not allow for full analyses of differences which could be seen with a higher percentage germination rate. Therefore time taken to reach 50% germination was thus compared.

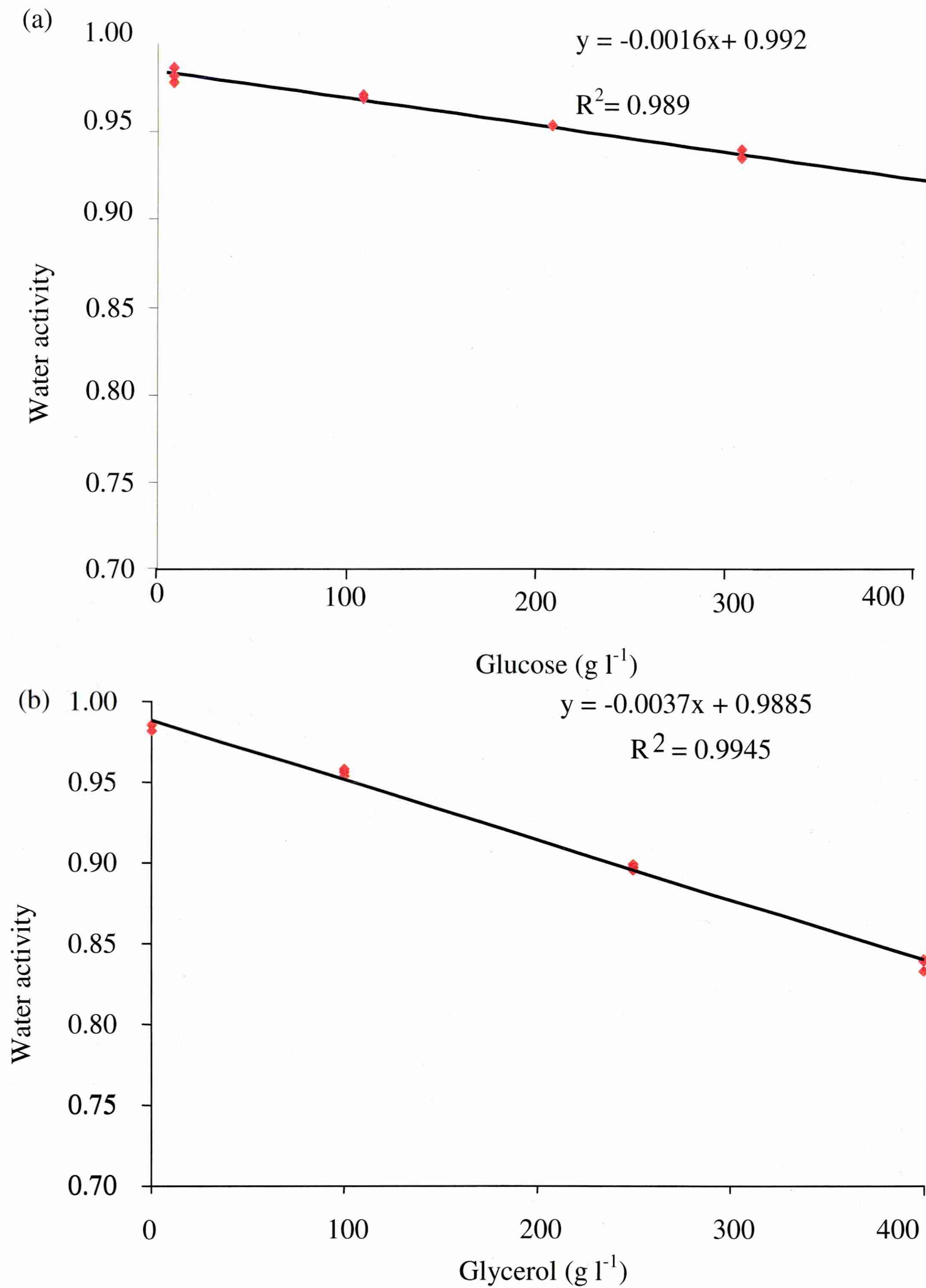
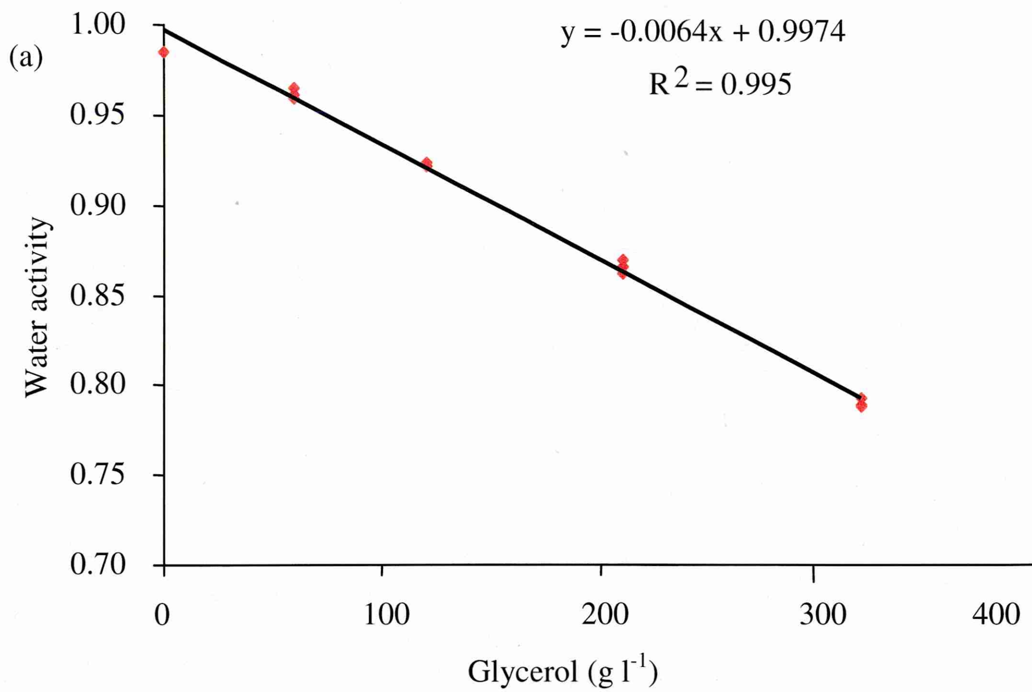


Figure 3.3 Effect of added (a) glucose or (b) glycerol on the water activity per 1000 ml of synthetic grape juice media. Readings were taken in triplicate and a linear regression calculated.



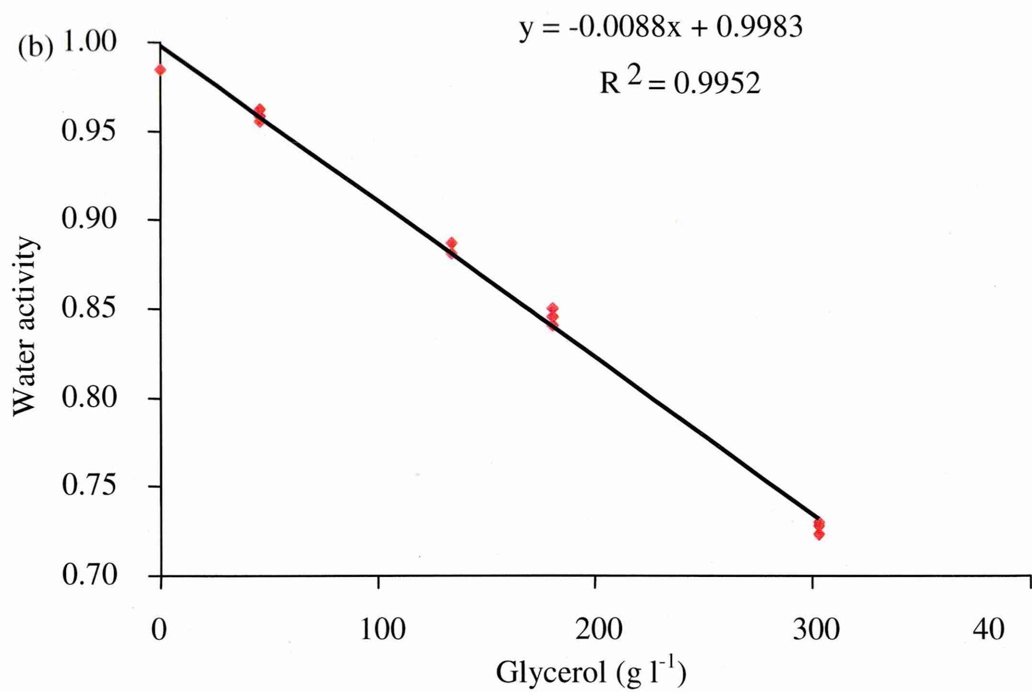


Figure 3.4 Effect of added glycerol water activity per 1000 ml of white (a) and red (b) grape juice media. Readings were taken in triplicate and a linear regression calculated.

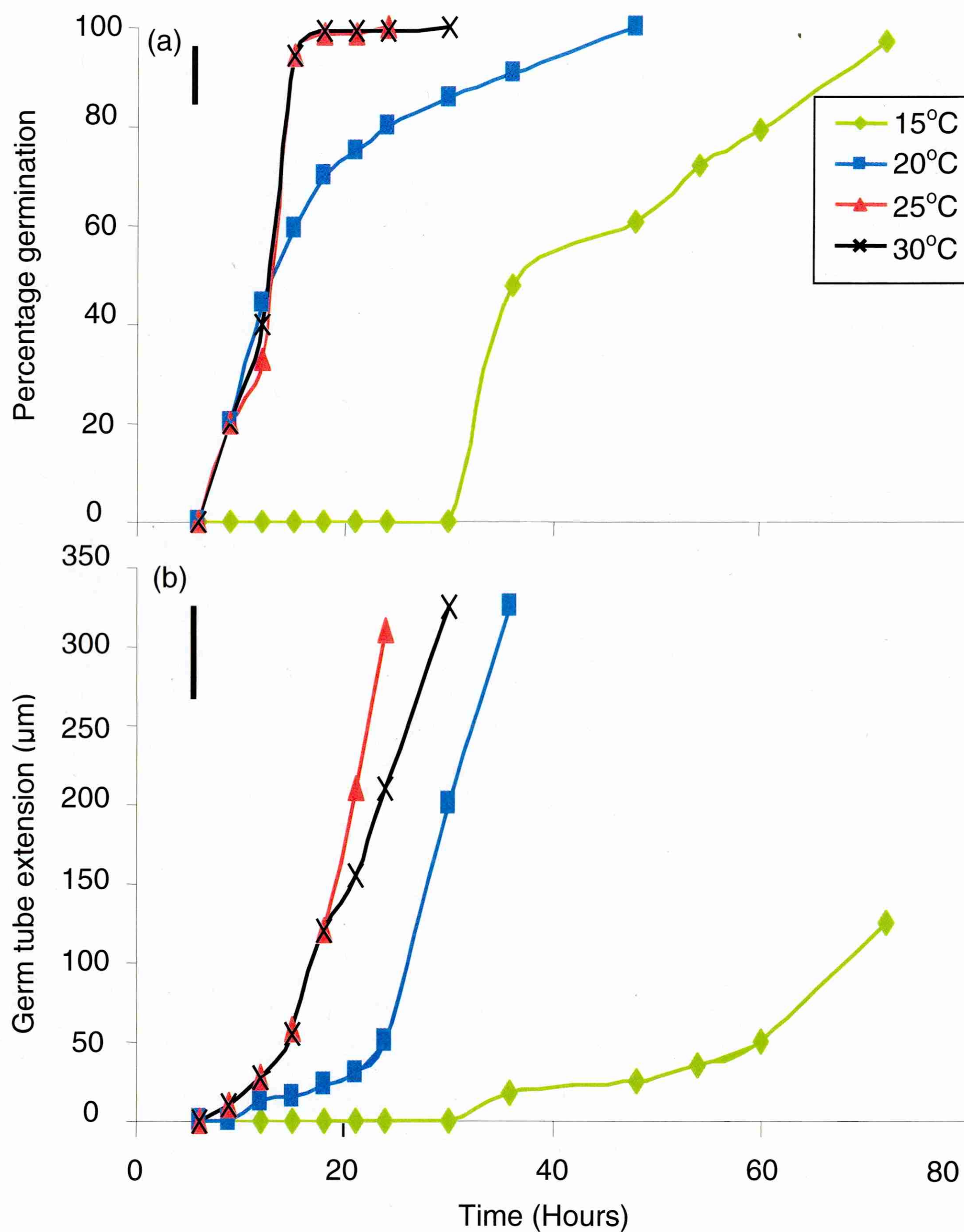


Figure 3.5 Effect of temperature on (a) germination and (b) germ tube extension of an isolate of *A. carbonarius* (MPVP A 1102) on a synthetic grape medium. Bars indicate LSD (P=0.05).

Figure 3.7 'shows the temporal effect of temperature on germination and germ tube extension at a steady state a_w of 0.99. This shows that germination and initial establishment at 25-30°C is very rapid under freely available water conditions. However, at 15°C germination was significantly slower with 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.

The effect of water availability on germination and germ tube extension was examined and Figure 3.6 shows the impact of this abiotic factor at 25°C. Germination was rapid at between 0.99-0.90 a_w with almost 100% of spores successfully germinating within 24 hours. At 0.88 a_w, there was a lag phase of about 18 hrs before germination occurred, with almost all spores germinating after about 60 hrs. The germ tube extension reflected this with 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 two strains from Italy and Portugal.

3.3.2 Comparisons of temperature and water activity effects or germination and germ tub extension on synthetic grape juice medium, grape flesh and undamaged grape skin

Spore germination on grape skin (Figure 3.7a), was optimum at 30°C and 0.99 a_w with germination reaching 50% in <9 hrs, at 25°C and at 0.95 a_w in <12 hrs. Outside of these two conditions germination was restricted on grape skin requiring >18 hrs to reach 50% germination. There appears to be two main conditions required for

germination on grape skin. The first is a very high a_w 0.997 a_w or equilibrium relative humidity (ERH) and the secondly, at least 30°C. Germination did occur outside of these conditions but was restricted with little germination occurring after 36 hours at <20°C. At temperatures >30°C on grape skin germination did occur, but required >24 hrs to reach 30-50% germination.

On grape flesh (Figure 3.7b), the optimum temperature from germination was 35°C with 50% germination reached in <4.5 hrs. Again a_w had an influence on germination but to a lesser effect than on grape skin. Germination reached 50% over all temperatures and a_w levels examined in <12 hrs. Growth at the lowest temperature (15°C) was restricted and required >30 hours.

Germination *in vitro* on SGM (Figure 3.7c), had similar trends to that on grape skin and grape flesh. Optimum germination temperatures were 30-35°C and at a_w levels in the range of 0.99-95 a_w. Germination rates were more consistent than on grape skin with a gradual increase in time taken to reach 50% as temperature or a_w was increased or decreased from the optimum.

Figure 3.8 shows the profiles for the lag phase (hrs) prior to germination. This reflects the germination results and confirms that the lag times were shortest on grape flesh followed by that on SGM and longest on grape skin. The lag phases also showed a broader optimum temperature and a_w range than was seen in the time taken to reach 50% germination.

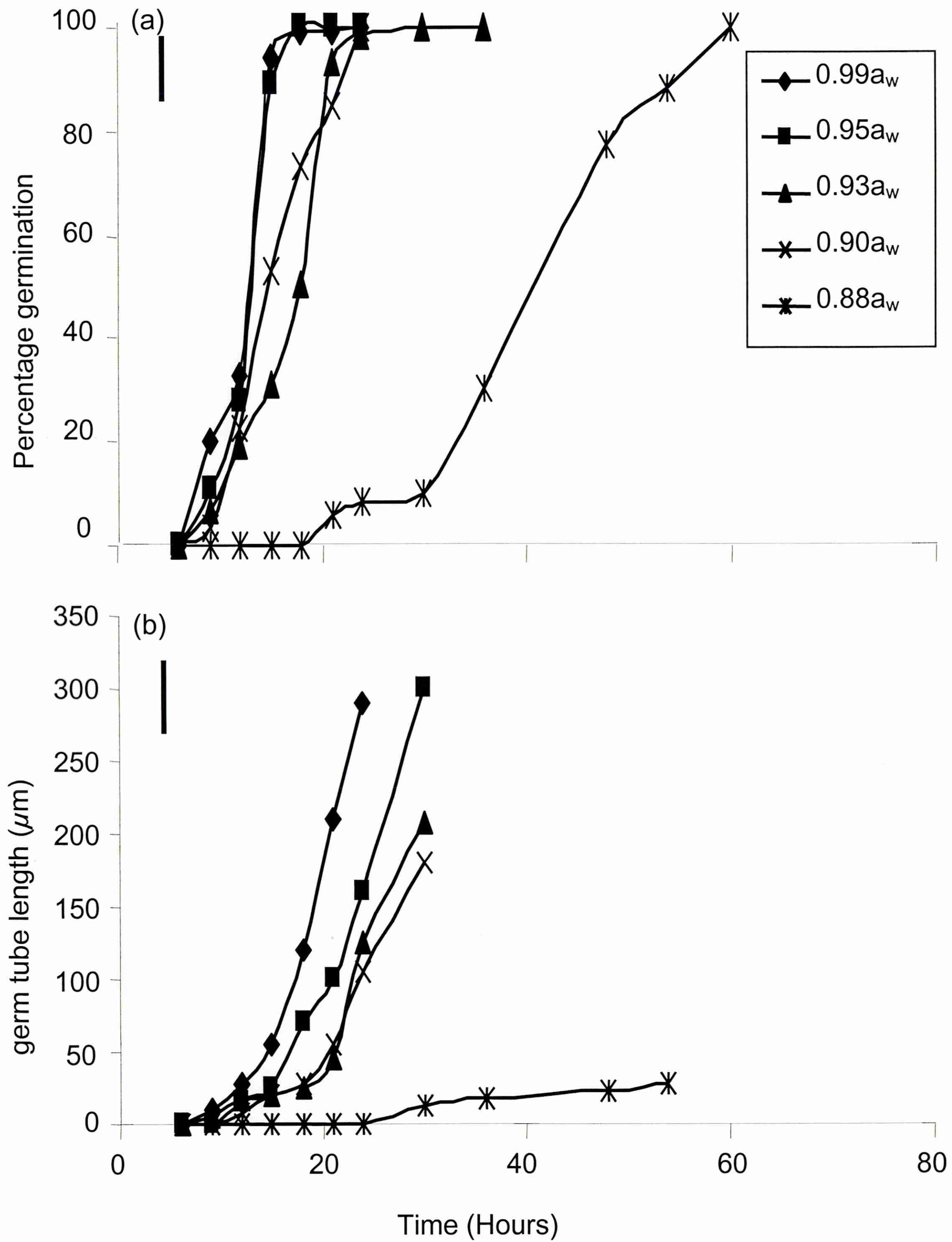


Figure 3.6 Effect of different water activity conditions (a_w) on (a) germination and (b) germ tube extension of an isolate of *A. carbonarius* (MPVP A 1102) at 25°C on a synthetic grape juice medium. Bars indicate LSD (P=0.05).

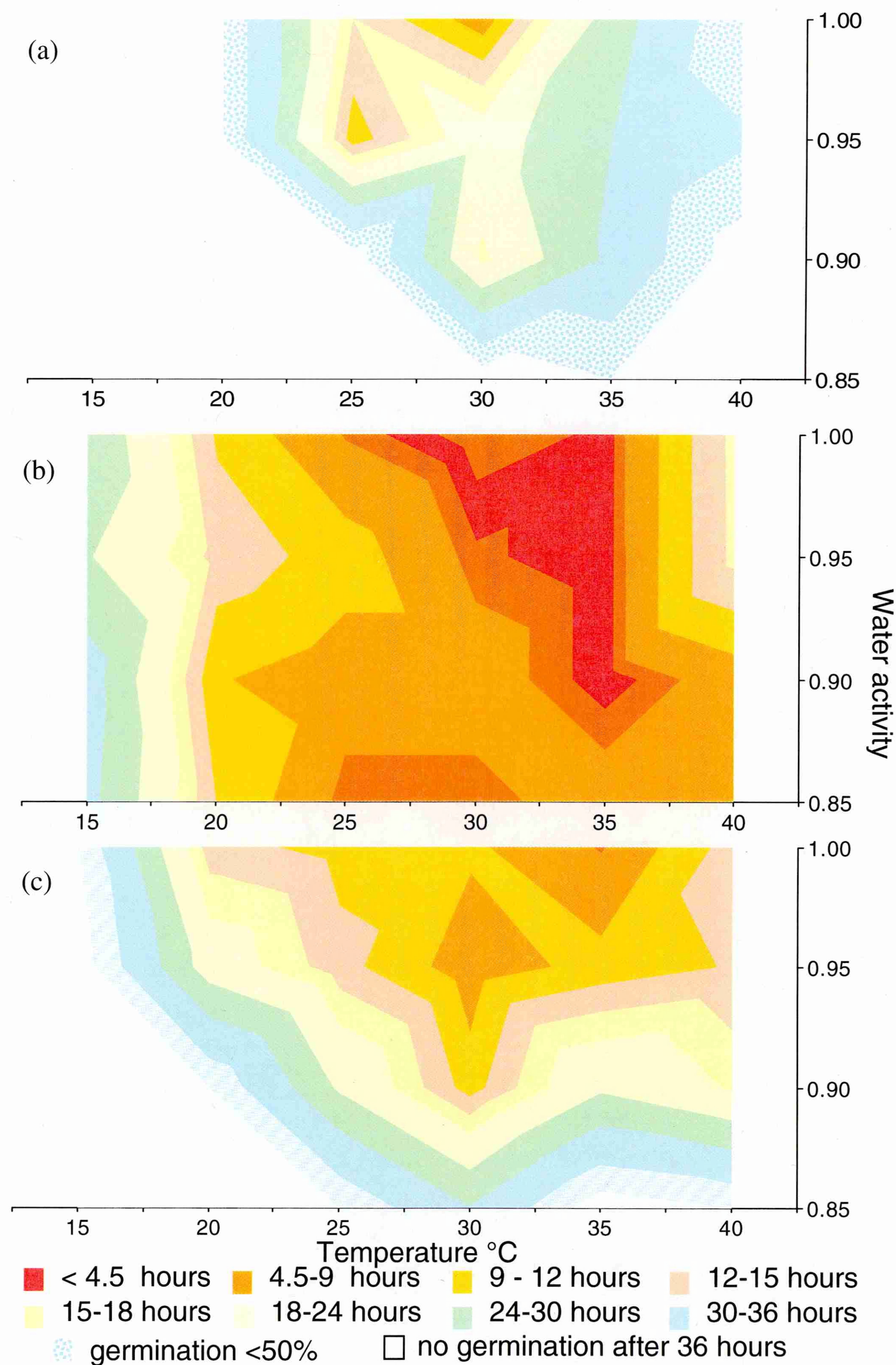


Figure 3.7. Time taken to 50% spore germination by *A. carbonarius* (MPVP A 1102) on (a) grape skin (b) grape flesh (c) synthetic grape juice media over a range of temperatures (10-40°C) and water activity levels (0.99-0.85).

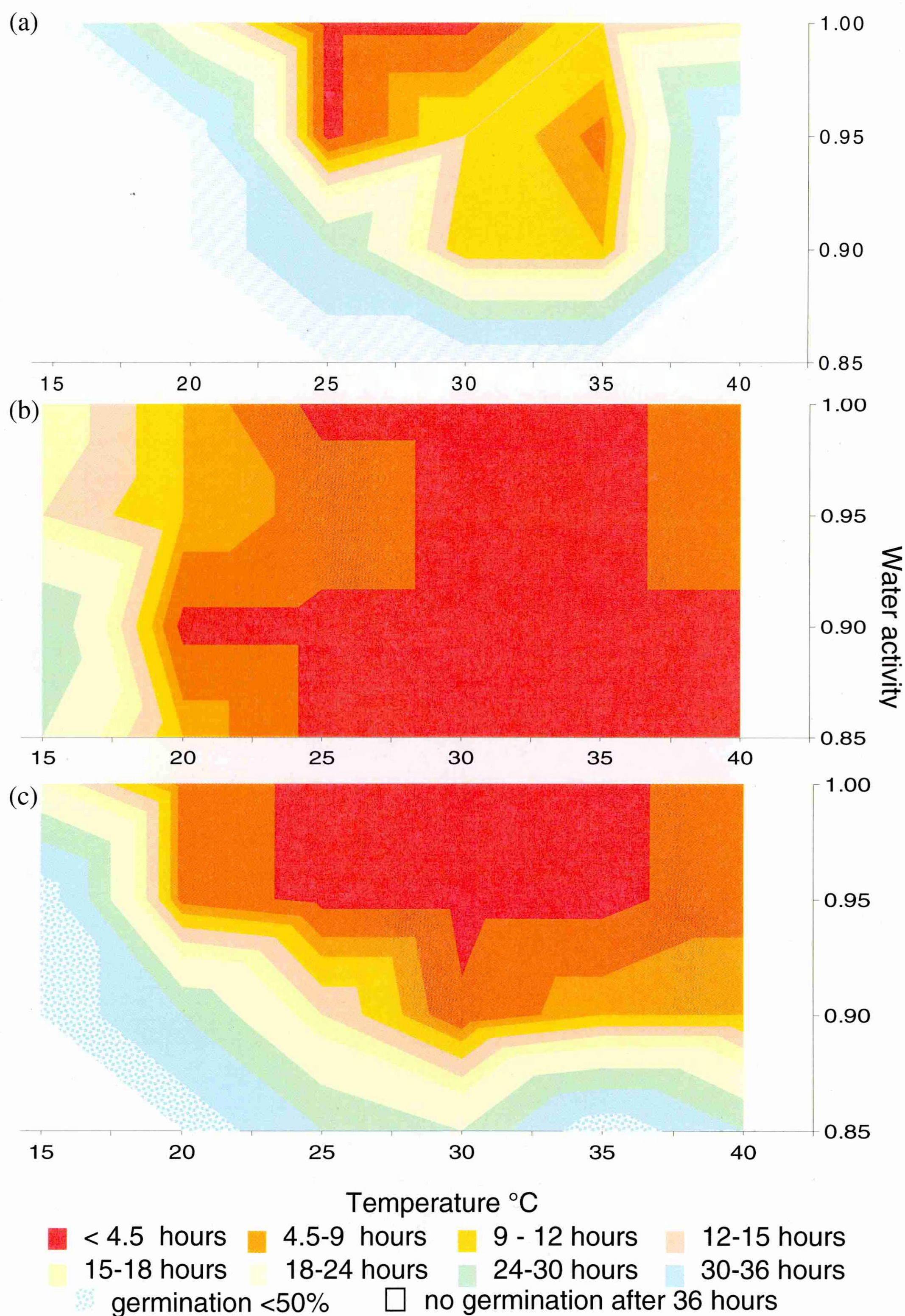


Figure 3.8 Lag phases prior to spore germination (Hours) by *A. carbonarius* (MPVP A 1102) on (a) grape skin (b) grape flesh (c) synthetic grape juice media over the temperature range (10-40°C) and at different water activity levels (0.99-0.85).

3.3.3 Comparison of the times taken for germ tube extension to reach 100 μm

Germ tube extensions were measured over time on grape skin, grape flesh and SGM and compared. The speed of growth varied greatly and it was decided to carry out comparisons on the time taken to reach 100 μm. Figure 3.9 shows a comparison of germination and germ tube extensions with noticeable better germination and germ tube extensions on grape flesh.

Figure 3.10 compares the time to reach 100 μm in relation to grape skin, grape flesh and media. Optimum germ tube extension growth was at 25-30°C but a_w appeared to be the most important factor on grape skin. Germ tube extensions were also quite rapid at 0.90 a_w producing the hour glass effect on the surface plot. Although the results were taken in triplicate looking at the growth at 0.95 a_w and 30°C, and 0.90 a_w and 25-35°C it would suggest this result may be an anomaly. If this is considered an outlier, growth on skin was only observed at 0.997 a_w and is severely restricted at lower a_w levels.

Germ tube extension on grape flesh was most rapid at 35°C as was seen for rates of germination. At 25-35°C and 0.99-0.95 a_w germ tubes all reached 100 μm. Temperature had the biggest impact on germ tube extension; at 15°C germ tube extension only reached 100 μm in >36 hours.

The speed of germ tube extension on SGM followed the same pattern as germination, with optimum temperatures of 30-35°C and water availability of 0.99-0.95 a_w. Germ tube extensions decrease as the temperature and a_w conditions became more peripheral. At low a_w (0.90-0.85 a_w) and 20-25°C germ tube extensions were not observed although germ tubes < 15 μm were observed.

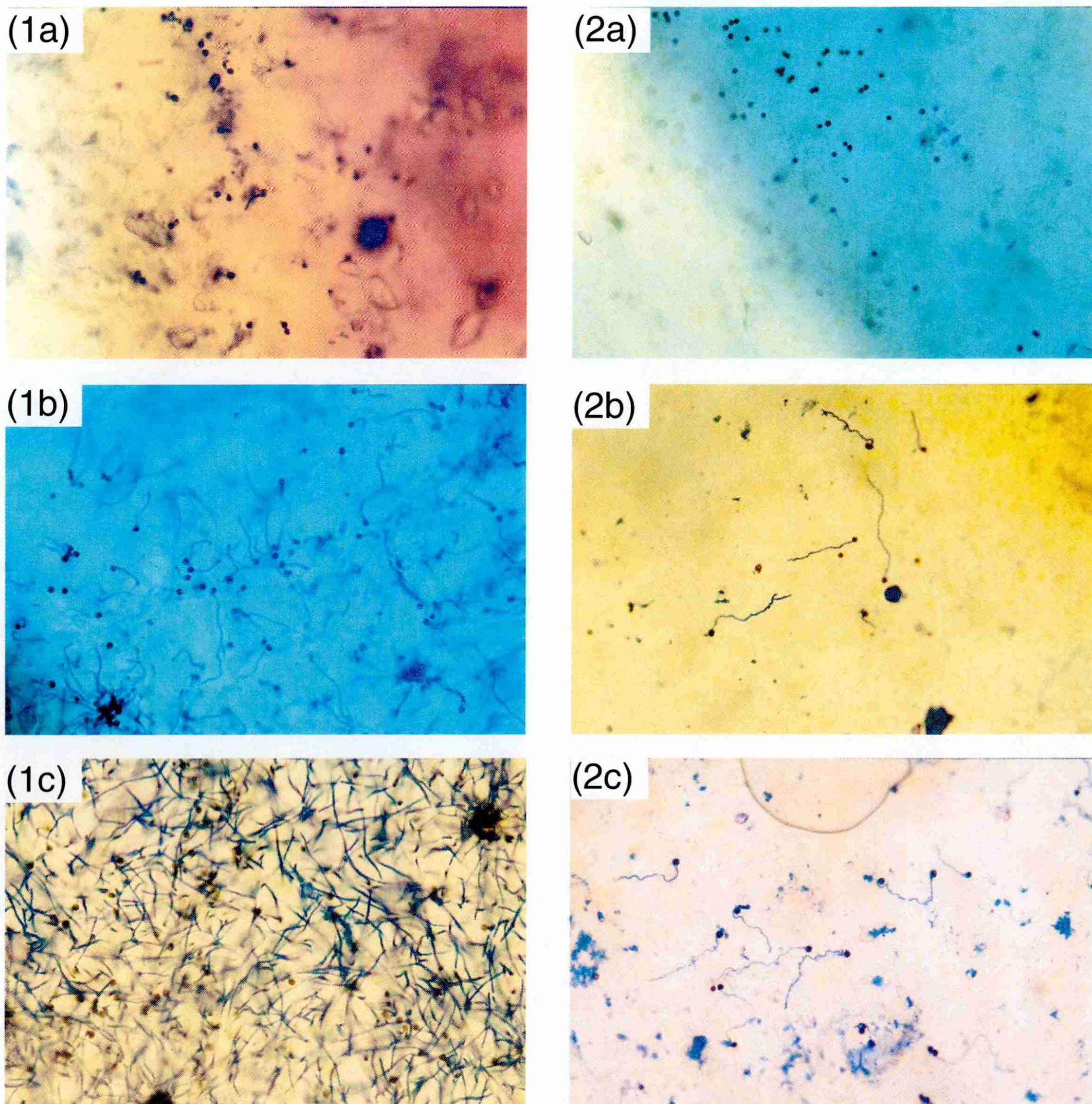


Figure 3.9 *A. carbonarius* (MPVP A 1102) spore germination on (1) grape flesh and (2) grape skin after (a) 6 hours, (b) 12 hours and (c) 18 hours at 25°C 100% equilibrium relative humidity.

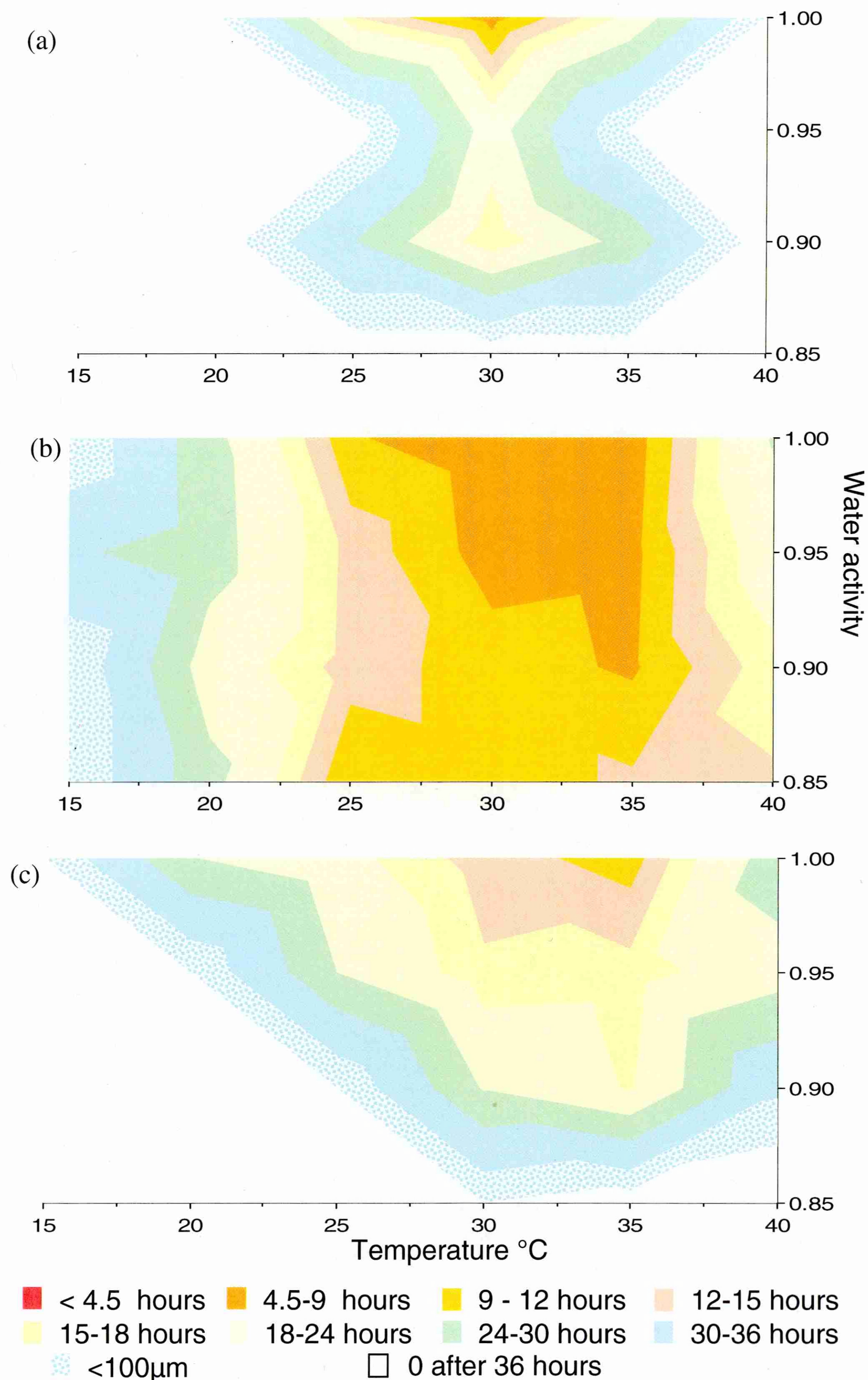


Figure 3.10 Time taken for germ tube extensions to reach 100 µm by *A. carbonarius* (MPVP A 1102) on (a) grape skin (b) grape flesh (c) synthetic grape juice media over a range of temperatures (10-40°C) and water activity levels (0.99-0.88).

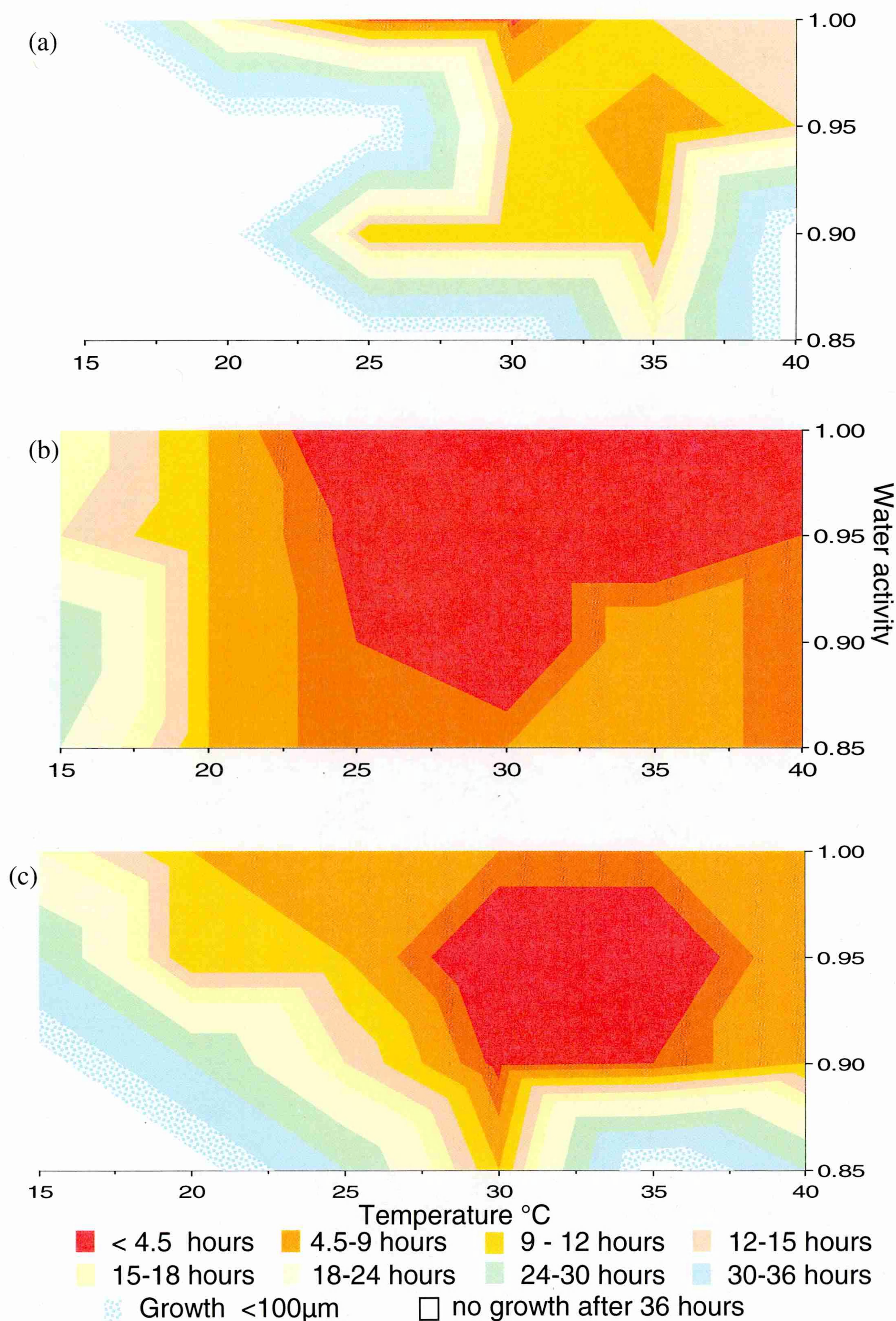


Figure 3.11 Lag phase for germ tube extensions (Hours) by spores of *A. carbonarius* (MPVP A 1102) on (a) grape skin (b) grape flesh (c) grape juice media over a temperature range (10-40°C) and at different water activity levels (0.99-0.88).

3.3.4 Temporal germination and germ tube extension

The pattern of germination on grape skin (Figure 3.12a) was different from that on SGM or grape flesh (Figure 3.12b). There was an initial lag phase, followed by an exponential phase, exactly the same as was seen in SGM and grape flesh. While on both the other substrates germination continued exponentially until 97-100% of spores had germinated. On grape skin there appeared to be a stationary phase, after the exponential phase. Germination rates at 30-35°C increased throughout the 36 hours but after the initial exponential phase germination rates were greatly reduced. At 25°C there is a death phase after 18 hours were germination rates decreased over the next 18 hours. At lower a_w levels the lag phase was extended and the exponential phase was longer but limited outside of optimum conditions.

Figure 3.13 compares the temporal germ tube extension on grape skin, with that on agar medium. There appeared to be a similar lag, exponential and stationery phase. In contrast, on SGM (Figure 3.13b) after the lag there was an exponential phase followed by the latter phase indefinitely. A one way ANOVA of germ tube extensions between 12-36 hours shows that there was a significant difference in germ tube extensions (Table 3.2). When a Fisher analysis of homogenised groups were carried out on the germ tubes extensions with significant differences, similarities were found (Table 3.3). 20°C fells into 2 significant groups 12-18 hours and then 24-36 hours. At 25°C there were three significant groups, of which only germ tube extension at 30 hours were significantly different from all other measurement times, the rest of the germ tube extensions fell into two groups with a large amount of overlap between the two groups. At 35°C there was the most significantly varied with four different groups. Sample

times after 12, 15 and 24 hours were all different with 18, 30 and 36 hours falling in the same group. 24 hours is not in the same group as 18 or 30 hours because there was a reduction in growth compared to germ tube extensions at these times. If one takes the reduction in growth into account there was no significant increase in germ tube extension from 18-36 hours.

The homogenised groups show there were differences in germ tube extensions, but the differences were small. Many of the germ tube extensions fell into statistically similar groups which would not be the case if the germ tube extensions were growing exponentially as on SGM and grape flesh.

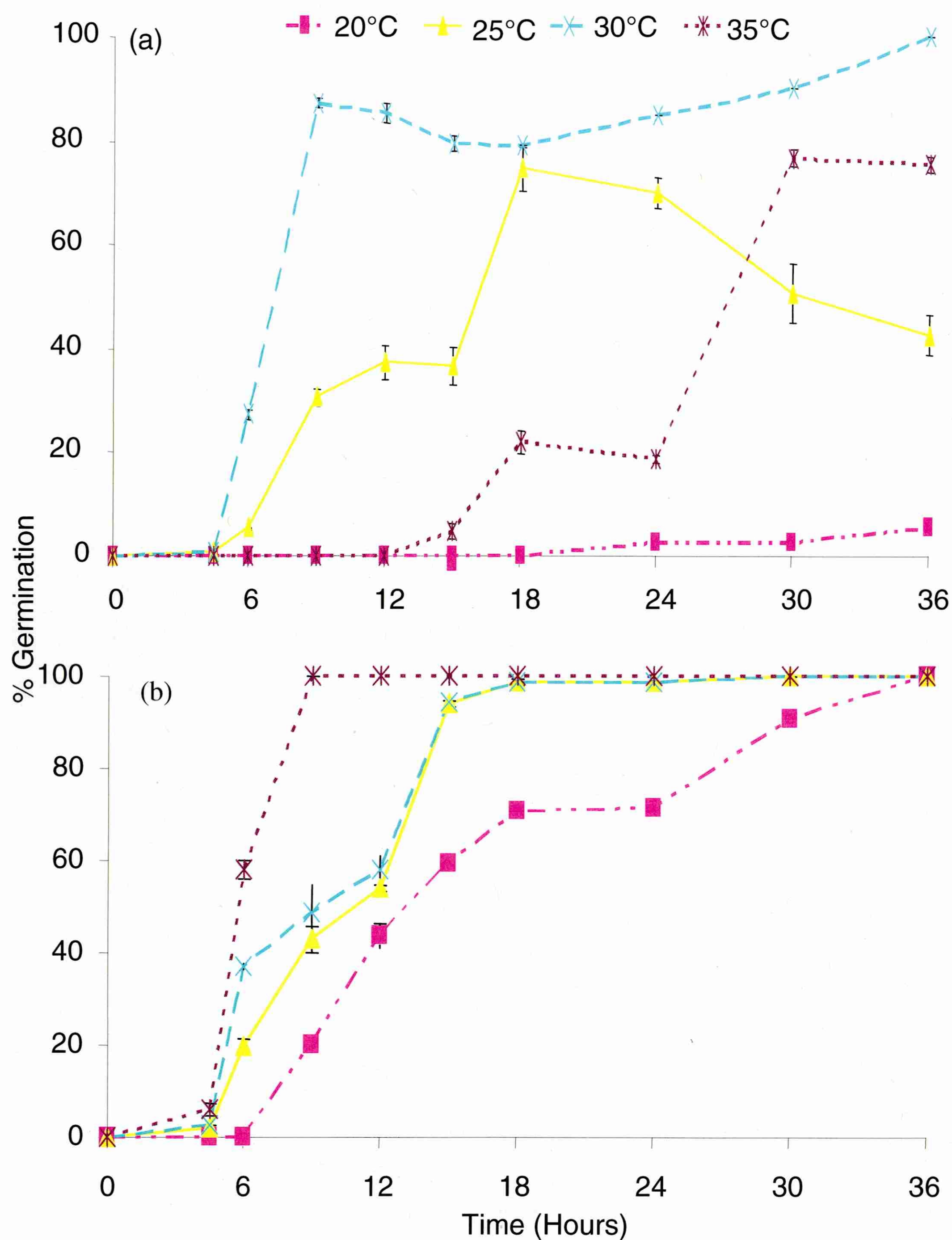


Figure 3.12 Comparison of temporal germination by *A. carbonarius* (MPVP A 1102) on (a) grape skins and (b) synthetic grape juice media over a temperature range (25-35°C) and 0.99 a_w. Bars indicate standard error of the mean.

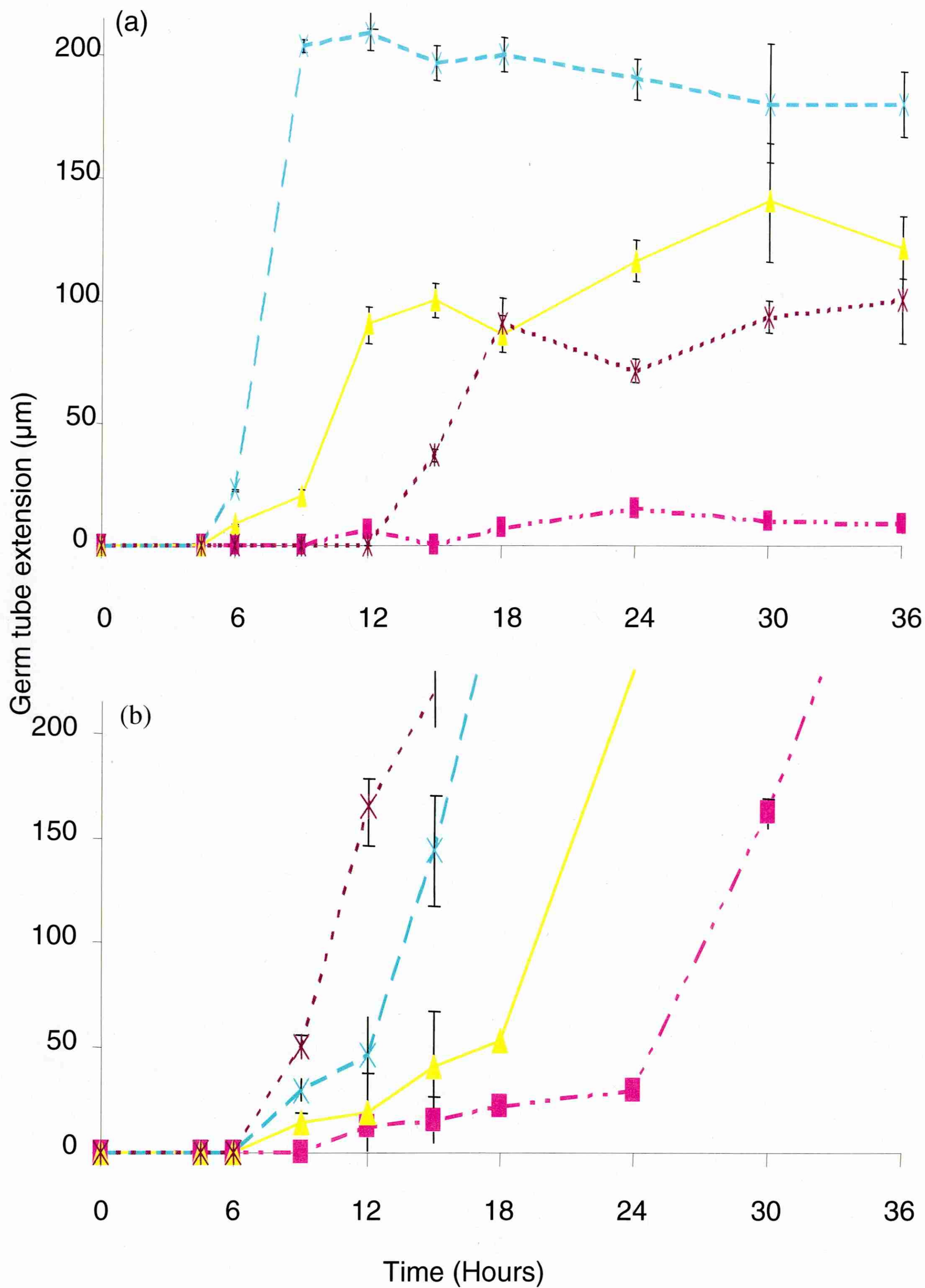


Figure 3.13 Temporal germ tube extensions for *A. carbonarius* (MPVP A 1102) on (a) grape skin, (b) synthetic grape juice media over a temperature range (25-35°C) at 0.99 a_w. Bars indicate standard error of the mean.

Table 3.2 One way ANOVA of germ tube extensions of *A. carbonarius* (MPVP A 1102) at 20-35°C from 12-36 hours on grape skin at 0.99 a_w. * Indicates factors which were significantly different ($p<0.05$).

Temperature	DF	SS	MS	F	P
20°C	5	9804.44	1960.89	49.022	<0.001*
25°C	5	178254	35651	7.5214	<0.001*
30°C	5	20253	4051	0.4993	0.777
35°C	5	231672.9	46334.6	43.4291	<0.001*

Table 3.3 Fisher least significant difference homogenised groups (95% confidence) of germ tube extension (µm) on grape skin at 20-25°C.

Temperature	MS	DF	Time (hours)	Mean	Grouping			
20°C	40.000	174	12	10.00000	****			
		174	15	10.83333	****			
		174	18	12.50000	****			
		174	30	25.00000		****		
		174	24	26.16667		****		
		174	36	26.16667		****		
25°C	4739.9	174	18	86.3333	****			
		174	12	90.1667	****	****		
		174	15	100.0000	****	****		
		174	24	111.6667	****	****		
		174	36	121.5000		****		
		174	30	180.0000			****	
35°C	1066.9	174	12	0.0000		****		
		174	15	36.6667			****	
		174	24	71.5000				****
		174	18	90.0000	****			
		174	30	93.3333	****			
		174	36	100.0000	****			

3.4 WATER AND TEMPERATURE EFFECTS ON GROWTH OF *ASPERGILLUS* SECTION *NIGRI*

Growth rates were calculated by using linear regression taken from the mycelial extension rates plotted against time (Figure 3.14). The effect of temperature and a_w significantly changed the growth rate as well as the lag phase prior to growth. Lag phases increased with decreasing temperatures (Figure 3.15a). Figure 3.15 b shows the effect of temperature on growth at different steady state a_w levels for an isolate of *A. carbonarius*. Optimum growth occurred at 30-35°C between 0.99-0.98 a_w. In comparison growth at 15°C was about 10% that at 30-35°C.

Figure 3.16 compares the effect of glucose/glycerol modification of growth over the whole a_w range at 15, 25 and 35°C for one isolate of *A. carbonarius*. This shows that there are some differences, but not a large variation with the optimum range for both glucose /glycerol modified media being 35°C and at 0.99- 0.98 a_w. A comparison was carried out for all isolates on glucose/glycerol and all showed similar results. Statistical analyses of growth rates showed no significant difference except at 15°C and <0.93 a_w (Table 3.4). Comparisons were made between of growth *A niger* aggregate uniseriate and biseriate on grape based medium Table 3.5. They showed that similar growth patterns occurred under the treatment conditions tested. Growth was limited at 15°C, with no growth at 0.90 a_w. At 25 and 35°C there were generally good growth of all strains at all a_w levels tested. For both uniseriate and biseriate strains of *A. niger* aggregate there was no similarities between strains or country of origin. There were both fast and slow growing strains. Some grew at > 16 mm day^{-1} and some at

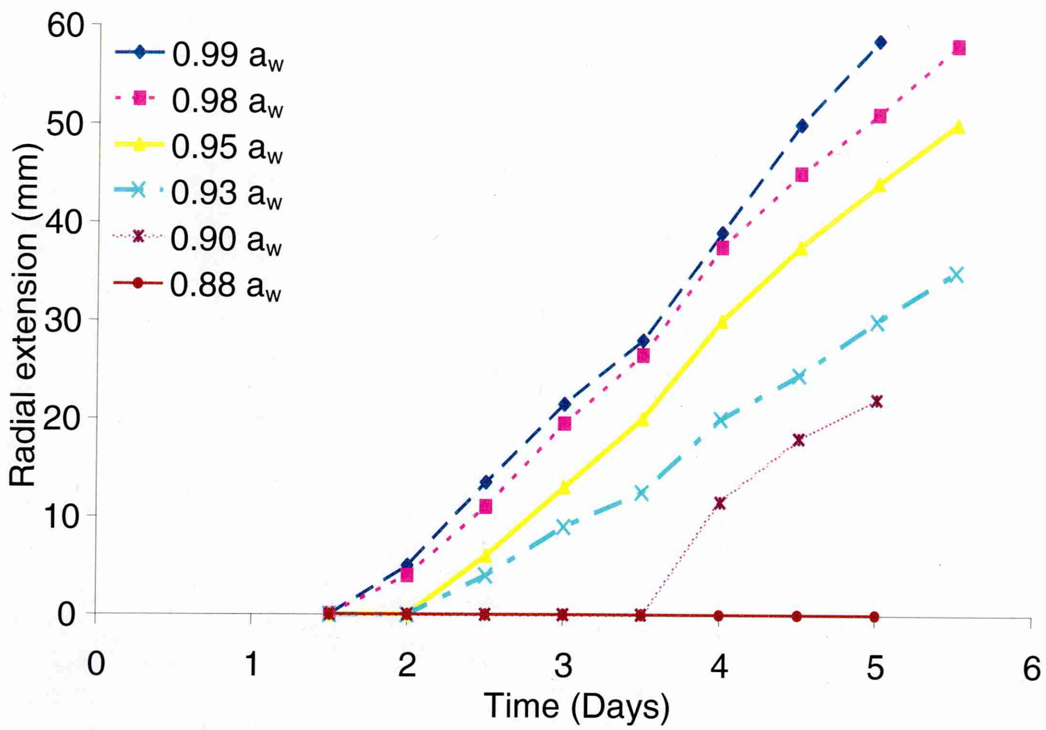


Figure 3.14 The effects of time and water activity on radial extension of mycelium of *A. carbonarius* (01UA s294) at 25°C.

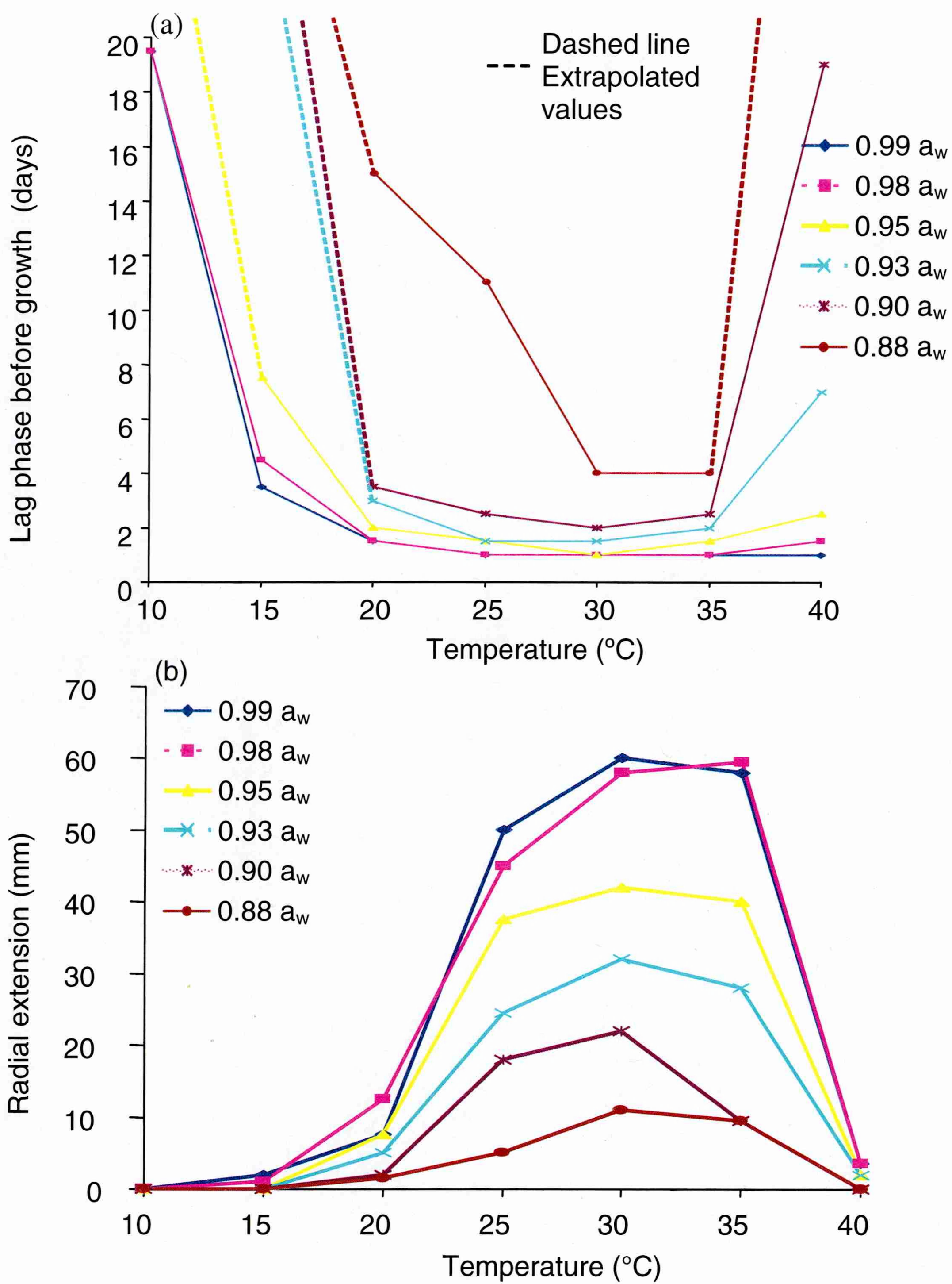


Figure 3.15 The effect of water activity and temperature on *A. carbonarius* (01UAs294). (a) Lag phases (days) prior to growth and (b) radial extension of growth after 5 days.

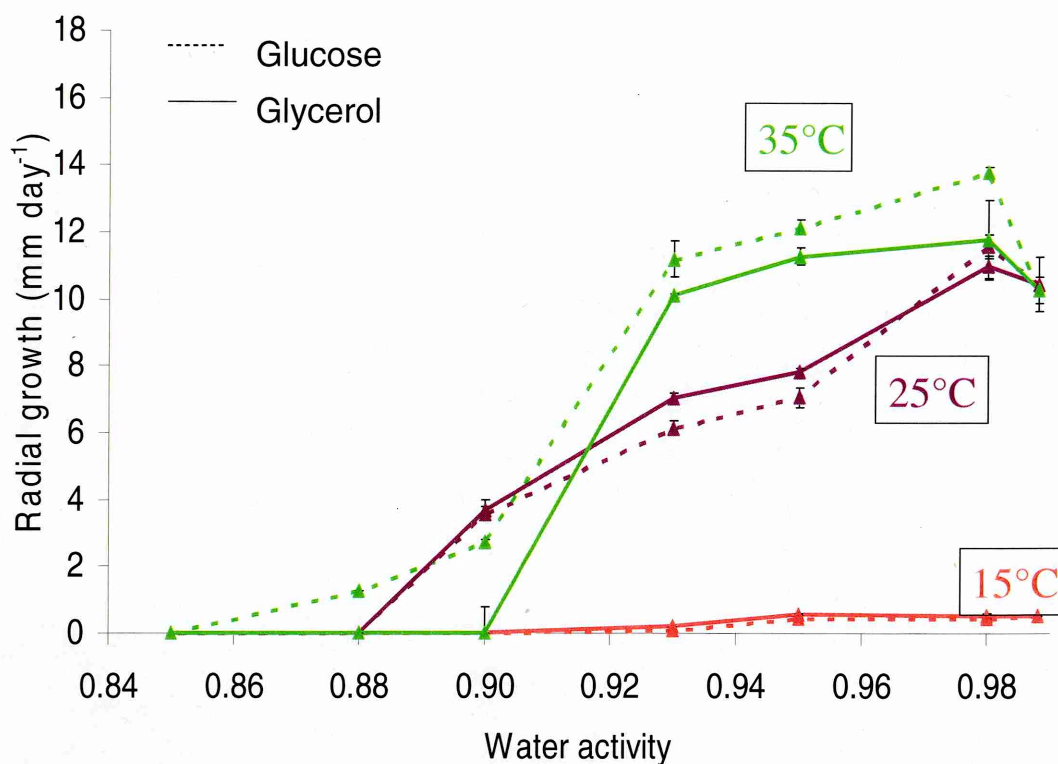


Figure 3.16 Growth of *A. carbonarius* (01UA s294), a representative isolate comparing growth on glucose or glycerol modified media at different temperatures and water activity levels. Bars indicate standard error of the mean.

Table 3.4 ANOVA of growth rate of *A. carbonarius* (01UA s294) grown on either glucose or glycerol (substrate) modified synthetic grape juice media at 15-35°C at 0.99-0.85 a_w.* Indicates factors which was significantly different ($p<0.05$).

Source	DF	SS	MS	F	P
Substrate 15°C	1	0.000500	0.000500	0.230	0.636
Water activity 15°C	5	1.933622	0.386724	178.025	>0.001*
Substrate × Water activity 15°C	5	0.004840	0.000968	0.446	0.812
Substrate 25°C	1	0.168	0.168	0.367	0.551
Water activity 25°C	5	539.475	107.895	234.959	>0.001*
Substrate × Water activity 25°C	5	1.259	0.252	0.548	0.738
Substrate 35°C	1	1.147	1.147	2.893	0.102
Water activity 35°C	5	776.842	155.368	391.973	>0.001*
Substrate × Water activity 35°C	5	3.056	0.611	1.542	0.214

<13 mm day^{-1} on SGM alone. However at 0.88 a_w some strains of *A. niger* could still grow at about 20% of optimum rates, suggesting limits of 0.85-0.83 a_w as the minimum a_w for growth.

Comparisons with an OTA producing isolate of *A. ochraceus* was also examined (Table 3.6). *Aspergillus ochraceus* grew at rates 80% slower than *A. niger* aggregate under the same conditions. At 15°C and below, growth of *A. ochraceus* was less severely restricted than other species. *A. ochraceus* were affected by temperature as much as species in the *A. niger* aggregate.

Figure 3.17 shows three examples of the surface response profiles for growth of strains of *A. carbonarius* from Italy, Israel and Greece respectively. This confirms that optimum growth was at higher temperatures (25–35°C) and intermediate a_w levels (0.97–0.99). Some strains had a marginal decrease in growth rate on SGM alone compared to 0.98 a_w. At lower a_w levels there was restricted growth with no growth recorded at 0.88 a_w, and 15°C. At 15°C growth was generally very slow (<0.20mm day^{-1}) at 0.98 a_w with no growth at 0.88-0.90 a_w. However, at 35°C growth rates varied considerably, even between strains from the same countries.

Comparisons were made of growth of strains from each of the four countries examined at different a_w levels at 25°C (Figure 3.18). There was no consistent pattern of growth for strains from different countries or between strains from the same country. At 0.98 a_w the fastest growth rates were just over double that of the slowest strains. Statistical

comparison showed that for only a few strains within a country were there statistically significant differences in growth rates (Table 3.7).

Figure 3.19 shows the surface plots of representative isolates of (a) *A. carbonarius*, (b) *A. ochraceus* (c) *A. niger* aggregate uniseriate, and (d) *A. niger* aggregate biseriate strains over the temperature range 10-40°C at 0.99-0.85 a_w *A. carbonarius* has the slowest growth rate and is most restricted by temperature and a_w range. At the optimum temperature (30-35°C) *A. carbonarius* can grow at the lower end of the a_w levels, close to 0.85 a_w. At optimum a_w the range of temperatures over which *A. carbonarius* can grow was increased and occurred at 15-40°C. However, at marginal temperatures and a_w levels growth rates were often <4mm day^{-1}.

The *A. niger* aggregate uniseriate strain (Figure 3.19b) had very similar growth conditions to *A. carbonarius,* with growth rate, optimum growth temperature and a_w conditions all similar. The main difference was the temperature range over which growth occurred at lowered a_w levels. In this case the optimum temperature range for growth was wider (20-35°C) than those for *A. carbonarius.*

A. niger aggregate biseriate strain (Figure 3.19c) had the highest optimum a_w range of 0.99-98 of all strain types examined. Optimum temperature was also at 30-35°C. These strains also had the fastest growth of any of the strains at optimum conditions with growth rates of >16mm day^{-1} and even at marginal a_w levels >6mm day^{-1} growth occurred. Most of the other strains only had limited growth at 40°C, whereas *A. niger* aggregate biseriate was still near optimum.

Table 3.5 Growth rates of *A. niger* aggregate uniseriate (01UA s128) and biseriate (MPVP A 212) strains on synthetic grape juice media at 10-40°C at 0.99-0.88 a_w

		Temperature						
	Water activity (a_w)	10°C	15°C	20°C	25°C	30°C	35°C	40°C
	0.99 a_w	0.6	0.8	6.3	11.6	16.0	16.7	13.7
Uniseriate	0.95 a_w	0.3	1.0	5.7	11.4	15.0	15.4	13.3
	0.90 a_w	0.0	0.5	4.5	9.3	11.3	11.2	6.7
	0.99 a_w	0.0	0.7	8.3	11.0	12.4	7.3	2.0
Biseriate	0.95 a_w	0.0	0.9	7.3	11.9	13.5	9.8	0.8
	0.90 a_w	0.0	1.1	7.0	10.1	12.3	9.0	0.4

Table 3.6 Growth rates of *A. ochraceus* (MPVP A 703, MPVP A 558) on synthetic grape juice media at 10-40°C at 0.99-0.88 a_w

		Temperature						
	Water activity (a_w)	10°C	15°C	20°C	25°C	30°C	35°C	40°C
	0.99 a_w	0.5	1.3	5.0	5.9	7.8	1.5	0.0
MPVP A 703	0.95 a_w	0.5	1.6	5.1	7.3	9.8	1.0	0.0
	0.90 a_w	0.0	0.8	3.8	4.5	5.7	1.2	0.0
	0.99 a_w	0.0	1.3	N/A	5.9	N/A	1.7	0.0
MPVA A 558	0.95 a_w	0.0	1.5	N/A	7.1	N/A	1.1	0.0
	0.90 a_w	0.0	0.8	N/A	4.0	N/A	0.9	0.0

N/A= no data

Table 3.7 Statistical comparisons of mean growth rates of strains of *A. carbonarius* in relation to temperature × water availability for all strains over a range of water activities (0.99-0.88) and a temperature range (10-35°C) on glycerol modified synthetic grape juice media. *Indicates factors which was significantly different ($p<0.05$). M, overall mean of single strain.

Country	strain	Italy		Portugal		Israel		Greece	
		MPVP A 933	MPVP A 1102	01UA s219	01UA s263	1-4-1-9-10.8	1-4-1-9-10.8	G 458	G 444
		(M= 2.3000)	(M=3.3597)	(M=1.9100)	(M=3.8155)	(M=1.8671)	(M=3.8172)	(M=3.8172)	(M=3.3552)
Italy	MPVP A 933		0.286	0.669	0.098	0.948	0.120	0.110	0.288
	MPVP A 1102	0.286		0.132	0.635	0.134	0.645	0.645	0.996
Portugal	01UA s219	0.669	0.132		0.031*	0.963	0.037*	0.037*	0.133
	01UA s263	0.097	0.635	0.031*		0.034*	0.998	0.998	0.631
Israel	1-4-1-9-10.8	0.647	0.134	0.962	0.034*		0.040*	0.040*	0.134
	1-4-1-9-10.8	0.110	0.645	0.037*	0.998	0.040*		1	0.642
Greece	G 458	0.110	0.645	0.037*	0.998	0.040	1		0.642
	G 444	0.288	0.996	0.133	0.631	0.135	0.641	0.641	

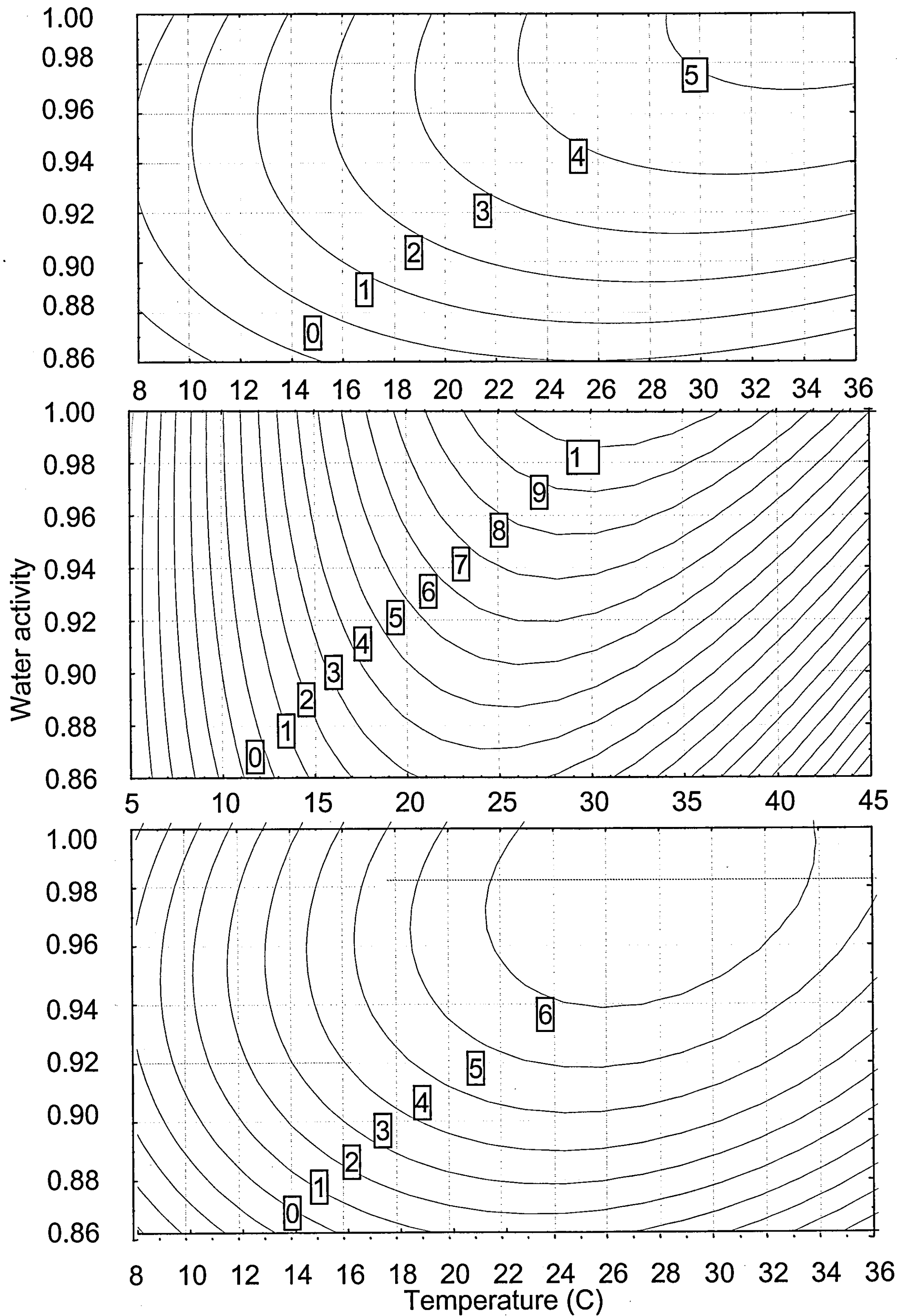


Figure 3.17 Surface response curve for three strains of *A. carbonarius* from Italy (MPVP A 933) Portugal (01UA s219) and Israel (1-4-1-9-10.8) in relation to water activity and temperature. Numbers on isopleths are growth rates (mm day^{-1}).

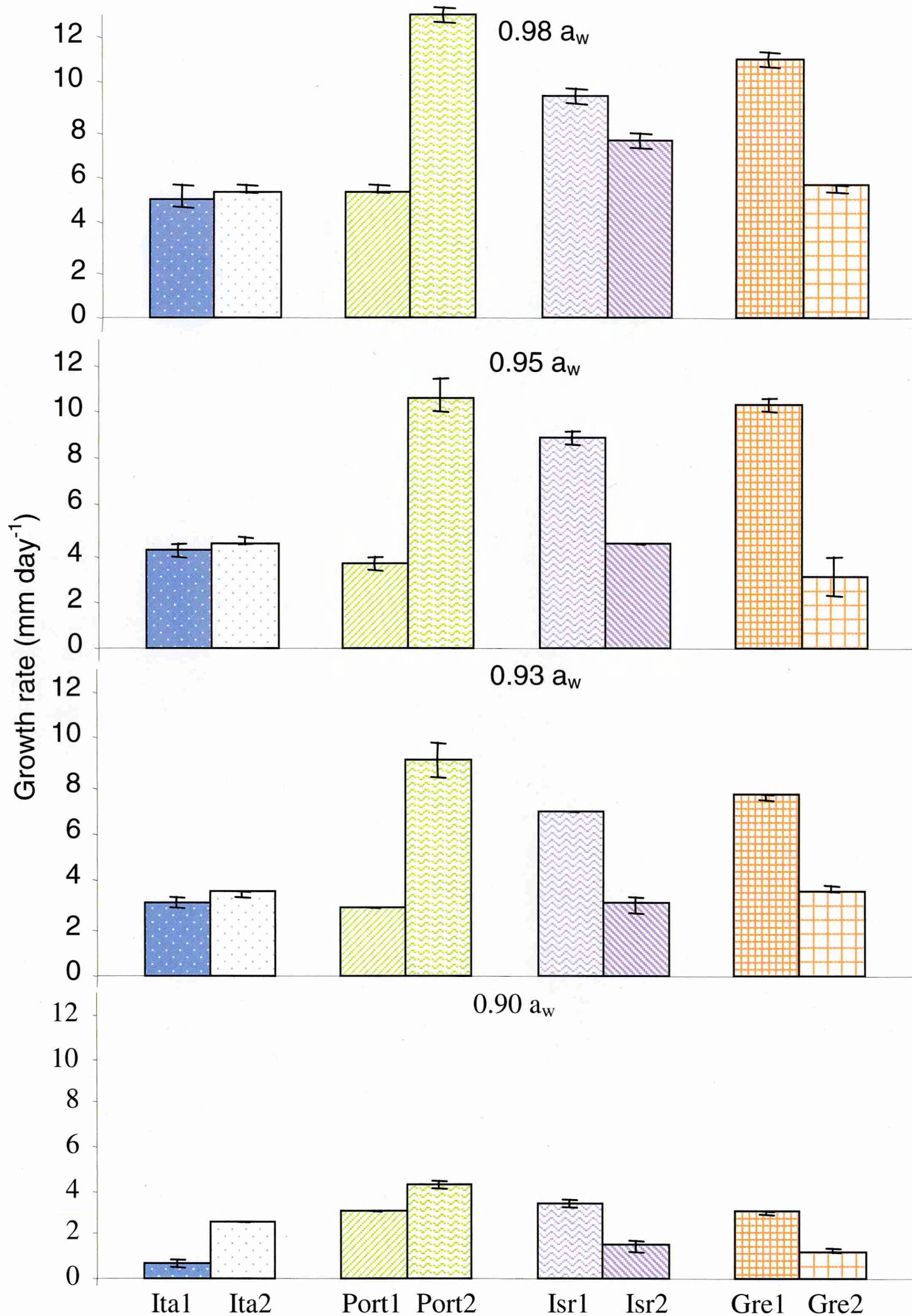


Figure 3.18 Comparison of growth rates of two *A. carbonarius* isolates from four European countries at 25°C at 0.98-90 a_w Italy, Ita1 (MPVP A 933) Ita2 (MPVP A 1102) Portugal, Port1 (01UA s219) Port2 (01UA s263) Israel, Isr1 (1-4-1-9-10.8) Isr2 (1-4-1-9-7.7) and Greece Gre1 (G 458) Gre2 (G 444). Bars indicate standard errors of the mean.

A. ochraceus (Figure 3.19 d) was least affected by a_w with the optimum ranging from 0.98-0.90 a_w levels. The optimum temperature range was similar to the other strains (25-30°C) but slightly lower. *Aspergillus ochraceus* was more susceptible to high temperatures with growth being inhibited at 40°C.

3.5 OCHRATOXIN A EXTRACTION AND ANALYSIS

Several sample weights were tested to find the best amount of culture for ochratoxin A extraction Figure 3.20 showed that there was no statistical difference found between weights or generic syringe filters or Milipore Milex filters. A 0.3g of sample in 1 ml of methanol did produce the lowest standard error, and was used for the rest of the studies. Figure 3.21 shows the comparisons of ochratoxin A extractions using two different filter methods and a range of times (1-24 hrs) using a generic syringe filter with a paper filter over a 24 hour time period. Statistical analyses showed that there was no difference between the two. There was also no difference between 1 hour and 24 hours extraction in methanol. Table 3.8 shows the one way ANOVA of OTA with the different extraction methods of cultures and extraction times. There was no statistically significant difference between them.

3.5.1 Effect of time and water activity on OTA A production by strains of *A. niger* aggregate

A temporal study was carried out to determine the maximum production time of OTA in relation to environmental factors. Examples of these results for *A. carbonarius* (01UA s219) are shown in Figure 3.22. The maximum amount of OTA when grown at 25°C occurred between 7 and 35 days. Below <0.90 a_w no OTA production occurred.

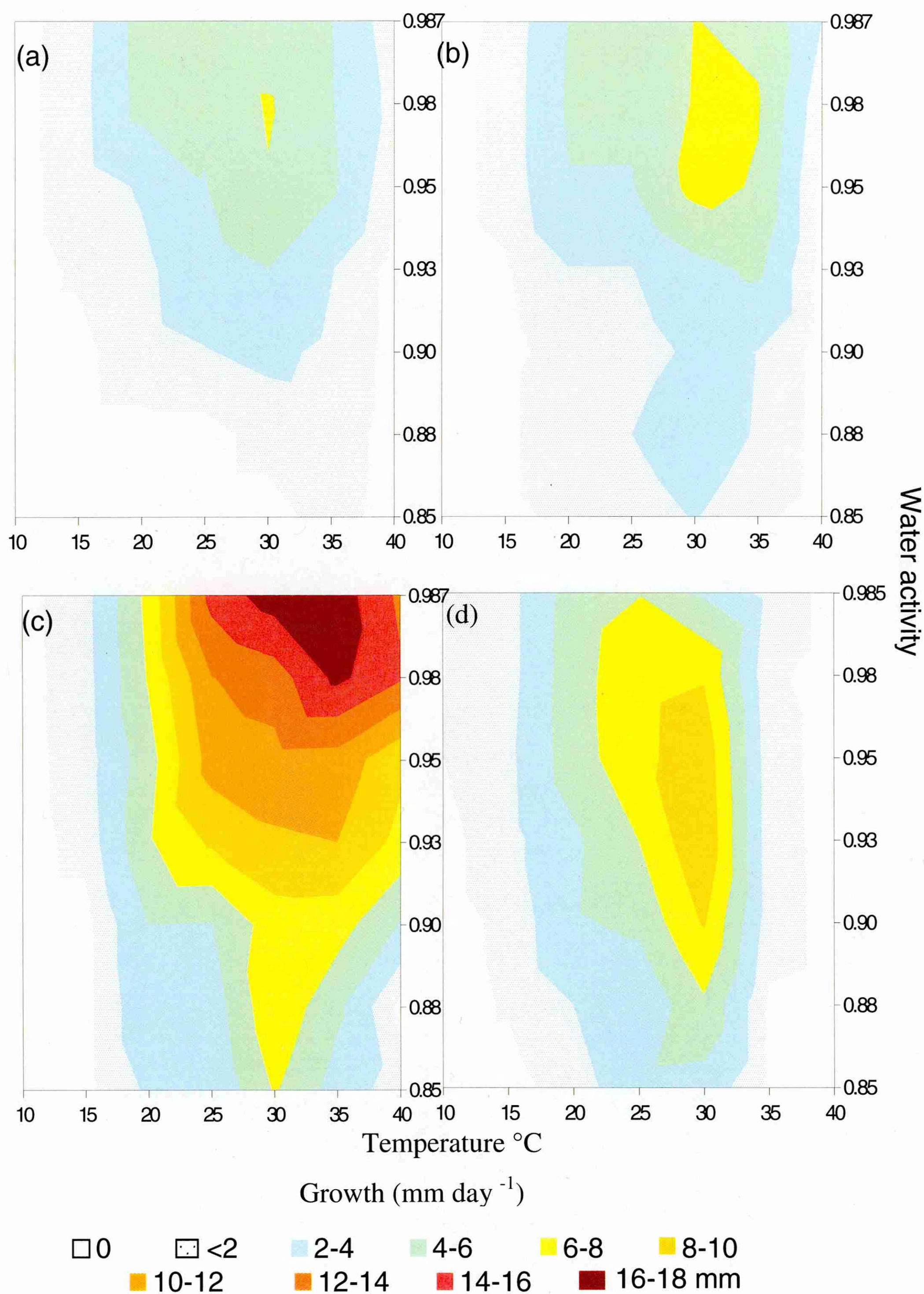


Figure 3.19 Comparison of growth rates over 10-40°C at 0.99-0.85 a_w (a) *A. carbonarius* MPVP A 1102 (b) uniseriate MPVP A 204 (c) biseriate MPVP A 203 (d) *A. ochraceus* MPVP A 703, strains on synthetic grape juice media. The colours represent different growth rates (mm day^{-1}).

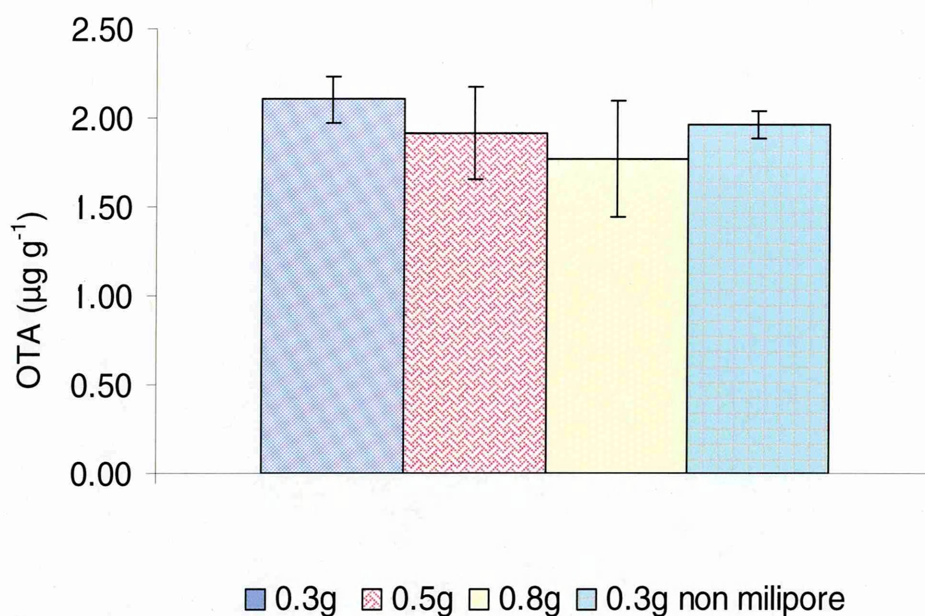


Figure 3.20 A comparison of ochratoxin A extraction using 3 different sample weights and two different filters (Millipore Milex and a generic syringe filter). Bars indicate standard error of the means.

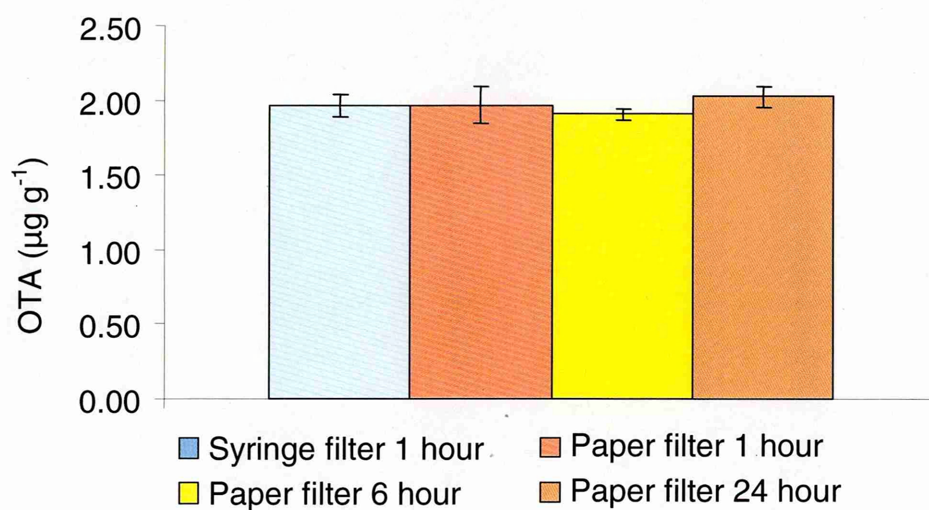


Figure 3.21 A comparison of ochratoxin A extraction using two different filter methods and a range of extraction times 1-24 hours. Bars indicate standard error of means.

Table 3.8 One Way ANOVA of Ochratoxin A using 3 different extractions methods (Filter, Millipore Milex, generic syringe filter, and paper filtration). Weights of 0.3, 0.5 and 0.8g of extracted sample and 3 different times 1, 6, and 24 hours, each sample done in triplicate.

Source	DF	SS	MS	F	P
Filter	2	0.00282	0.00141	0.0220	0.978
Extraction weight	2	0.08143	0.04071	0.1645	0.852
Extraction time	2	0.02059	0.01030	0.479	0.641

There were fluctuation in the amounts of OTA production at different a_w levels. There was a large lag phase point to point at 0.93-0.90 a_w.

The effect of temperature on OTA production by *A carbonarius* strain (01UAs 219) is shown in Figure 3.23 at 0.98 and 0.93 a_w More toxin was produced at 25°C than at 20°C, with OTA production only occurring after 14 days. At 20°C, 0.93 a_w OTA production was, 50% of that at 0.98 a_w at both temperatures examined.

Comparison of OTA production by all strains in relation to three a_w levels at a steady-state temperature (15°C) after 10 days incubation are shown in Figure 3.24. Significant differences between amounts of OTA produced by an individual strain, and between strains from the same country or different countries were observed. Statistical analysis showed that the overall, the mean OTA production by seven of the eight strains over all treatment conditions were relatively similar. Table 3.9 shows the statistical comparison for all eight strains and the statistically significant effect of single factors of a_w, temperature, and two-way interactions of a_w × temperature.

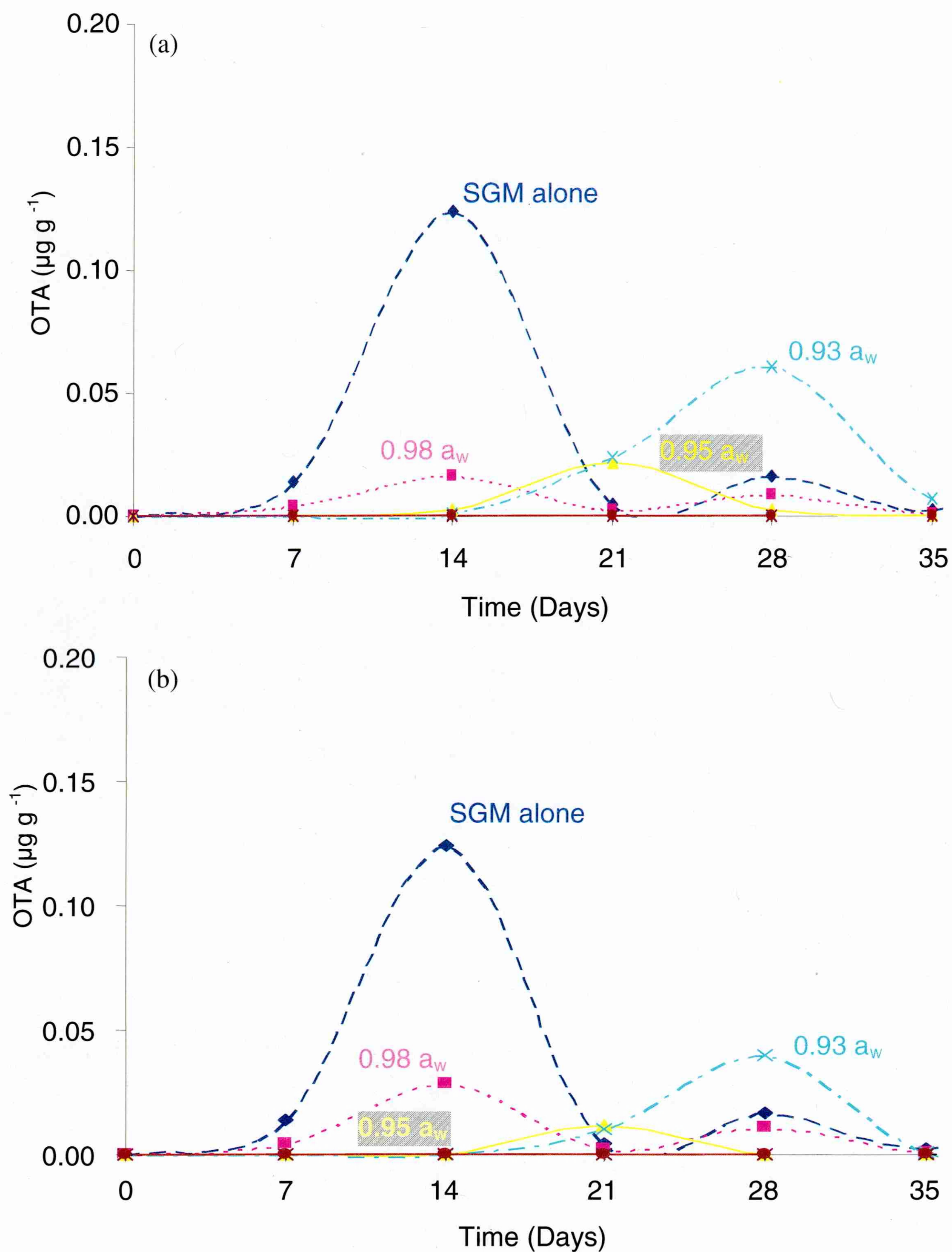


Figure 3.22 Ochratoxin A production by *A. carbonarius* (01UA s219) on synthetic grape juice media (a) modified with glycerol (b) modified with glucose over a period of 35 days at 25°C results are means of three replicates per treatment.

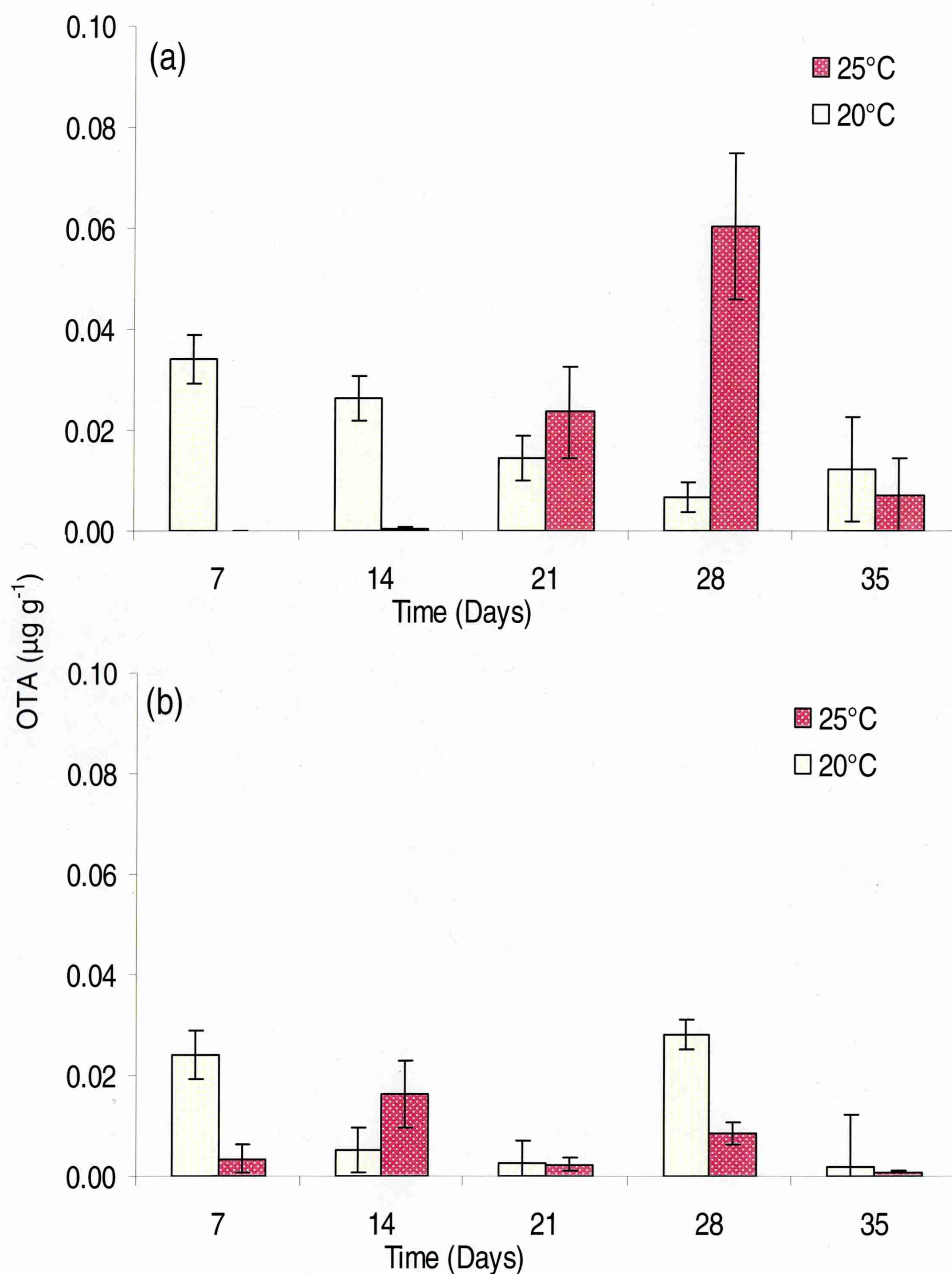


Figure 3.23 Ochratoxin A production by *A. carbonarius* (01UA s219) grown on synthetic grape juice at (a) 0.98 a_w and (b) 0.93 a_w and 20-25°C over a period of 35 days Results are means of three replicates per treatment. Bars indicate standard error of means.

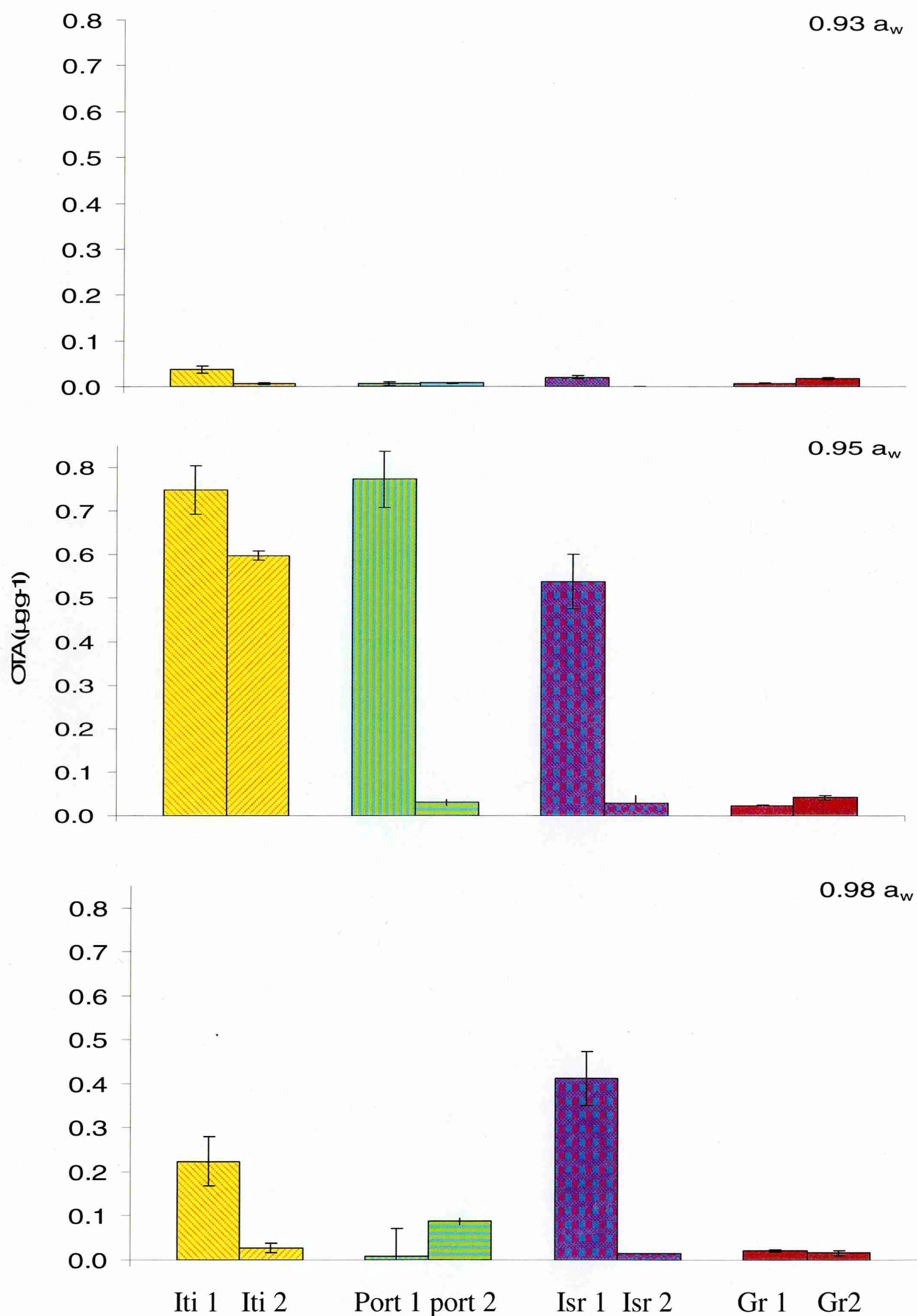


Figure 3.24 Ochratoxin A production of two *A. carbonarius* from Italy (Ita 1, MPVP A 933, Ita 2, MPVP A 1102) Portugal (Port 1, 01UAs219, Port 2 01UAs263) Israel (Isr 1, 1-4-1-9-10.8, Isr 2, 1-4-1-9-7.7) and Greece (Gr1, G458, Gr2, G444) at 15°C and water activities of (0.93-0.98. Bars indicate standard error of means.

Table 3.9 ANOVA Ochratoxin A production of two *A. carbonarius* from Italy, Portugal, Israel and Greece at 15°C and water activities (0.93-0.98) Italy (Ita 1, MPVP A 933, Ita 2, MPVP A 1102) Portugal (Port 1, 01UAs219, Port 2 01UAs263) Israel (Isr 1, 1-4-1-9-10.8, Isr 2, 1-4-1-9-7.7) and Greece (Gr1, G458, Gr2, G444) * Indicates factors which was significantly different ($p<0.05$).

Source	DF	SS	MS	F	P
Iti 1	2	1.017763	1.017763	300.9803	<0.001*
Iti 2	2	0.396042	0.396042	2592.902	<0.001*
Port 1	2	0.617591	0.617591	150.3792	<0.001*
Port 2	2	0.015604	0.015604	115.4382	<0.001*
Isr 1	2	0.940720	0.940720	234.2343	<0.001*
Isr 2	2	0.001944	0.001944	5.099011	0.065
Gr 1	2	0.002685	0.002685	11.01949	0.016*
Gr 2	2	0.005577	0.005577	62.21167	<0.001*

3.6 EFFECTS ON GROWTH AND OCHRATOXIN A PRODUCTION BY OTHER ENVIRONMENTAL FACTORS

Other factors other than just temperature and a_w play a role in the microclimate conditions that are found within a bunch of grapes. Juice from damaged grapes and watering can modify the pH and would need to be also considered. Vine regions are spread throughout the world and several are close to the coast and thus subjected to possible deposition of salt on plant and grape surfaces.

3.6.1 Effects of pH on growth and ochratoxin A production

Figure 3.35a shows effect of different pH levels on growth. Optimum was at pH 4.0 followed by pH 7.0. At pH 2.6 which was the unadjusted SGM, the growth rate was just measurable. In contrast OTA production was the opposite of the growth rates with pH 4 treatments having the lowest amounts, and pH 2.6 having the highest OTA production. Although growth rates at the lowest a_w were only just measurable, OTA was still detected (for statistical analyses of growth rates see Appendix N).

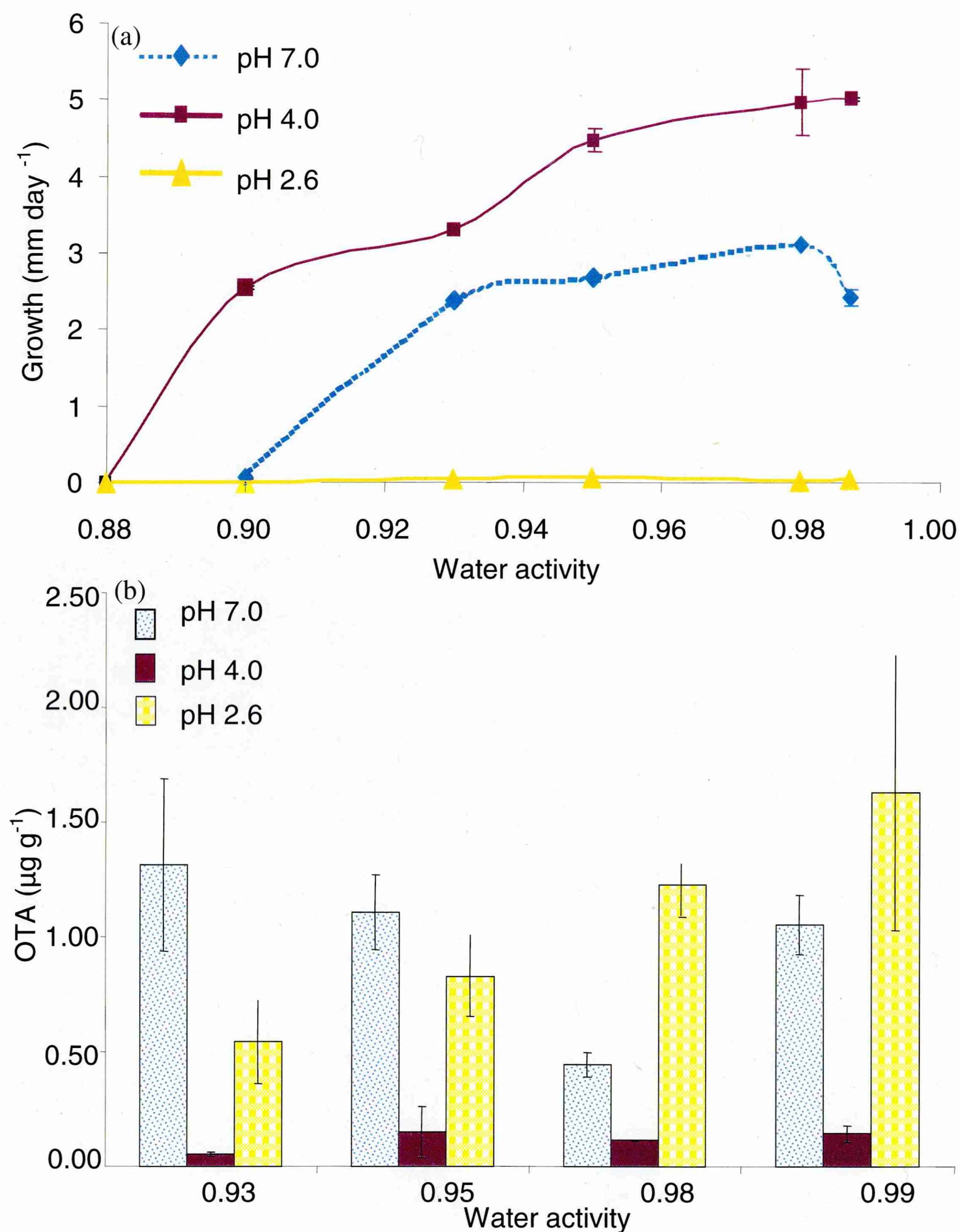


Figure 3.25 Growth rates (a) and ochratoxin A production (b) by *A. carbonarius* (MPVP A 1102) on three different pH 2.6-7.0 on modified synthetic grape juice media 0.93 0.99 water activities and 20°C.

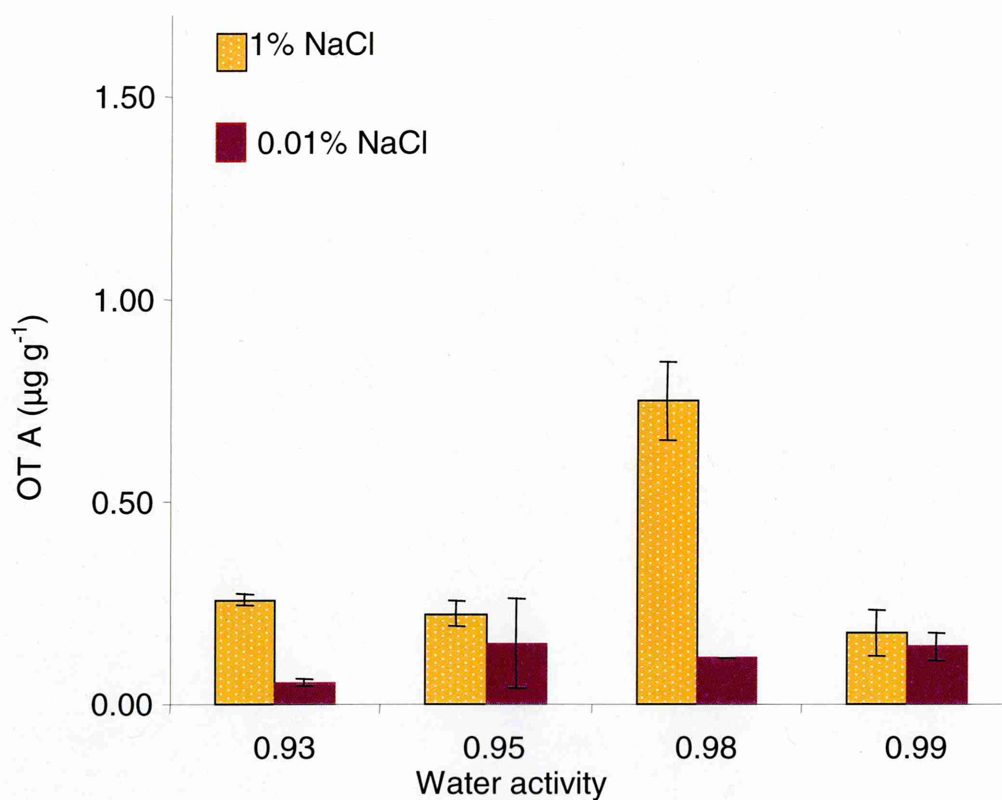


Figure 3.26 Ochratoxin A production by *A. carbonarius* (Italy MPVP A 1102) on different concentrations of NaCl 0.1-1.0% modified synthetic grape juice media at activities 0.93 0.99 water activity and 20°C.

3.7 PARTITIONING AND DISTRIBUTION OF OCHRATOXIN A IN SPORES, BIOMASS AND MEDIUM

Ochratoxin A is a secondary metabolite but little is known about how it is produced or why. A better understanding is needed on where the toxin is found, and whether it is transported in the mycelium to spores, or whether it is secreted into the substrate.

3.7.1 Partitioning of ochratoxin A into mycelium, agar and spores

All the *A. carbonarius* strains examined produced more OTA in the spores, than was found in either the mycelium or secreted into the substratum (Figure 3.27). There was a variation between the strains, strain 458 (Greece) had 94% of all detected OTA in the spores while strain 10.8 (Israel) had only 49%, the lowest of all the *A. carbonarius* examined. The OTA in the substratum remained consistent between 3-6% of the OTA. Strain 458 was the only one not to have any OTA present in the mycelium, with the other three strains having 25-50%.

3.7.2 Distribution of ochratoxin A within the colony

More than 75% of OTA in all the strains examined was found in the centre of the colony. Only a small percentage of all OTA was found in the SGM similarly the growing margin but A. *ochraceus* had more than double the amount of any of the other strains. *Aspergillus ochraceus* and *A. niger* aggregate unbiseriate had double the amount of OTA distributed in the growing edge of the colony compared to the other strains examined (Figure 3.29).

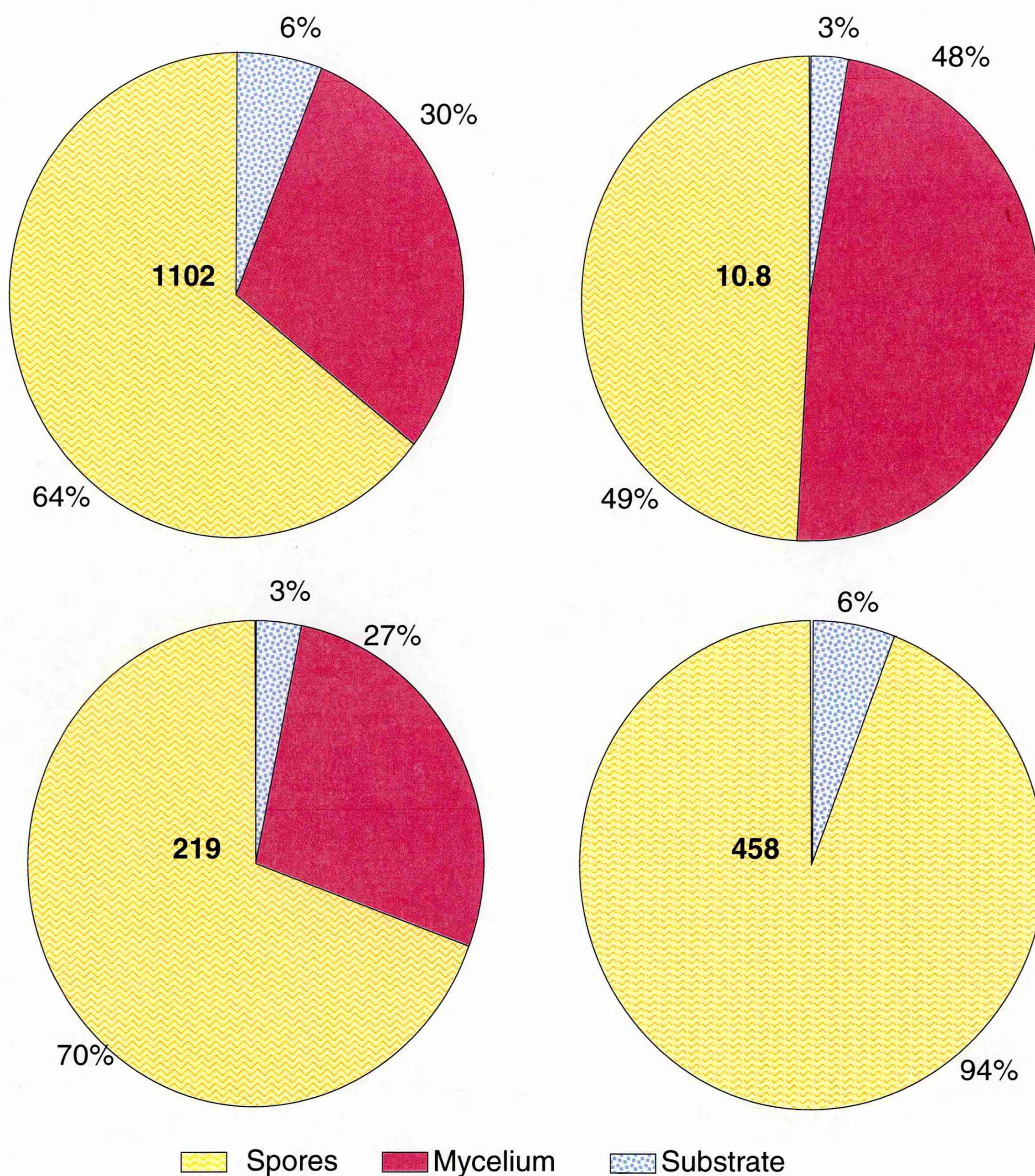


Figure 3.27 Partitioning of ochratoxin A production as a percentage distributed between the dry weight of *A. carbonarius* spores, mycelium and substratum, grown on synthetic grape juice medium at 20°C, 0.95 water activity for 20 days. Strains examined: Italy MPVP A 1102 (1102), Israel 1-4-1-9 10.8 (10.8) Portugal 01VA S 219 (219), Greece 458 (458).

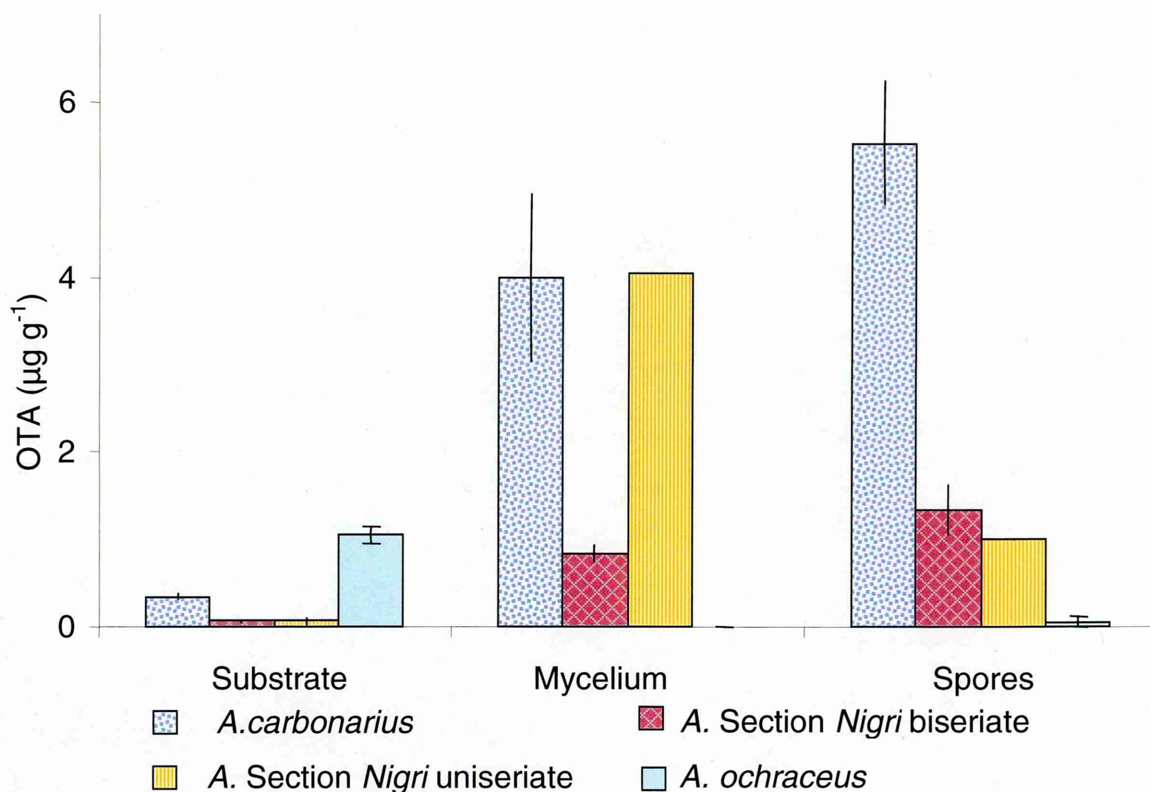


Figure 3.28 Partitioning of ochratoxin A production as a percentage distributed between the dry weight of spores, mycelium and substratum, grown on synthetic grape juice medium at 20°C, 0.95 water activity for 20 days. Mean of *A. carbonarius* from four European countries Italy MPVP A 1102, Israel 1-4-1-9 10.8, Portugal 01VA S 219, Greece 458 (458), *A. niger* aggregate biseriate (MPVP A 203), *A. niger* aggregate unbiseriate (MPVP A 204), A. *ochraceus* (MPVP A 703).

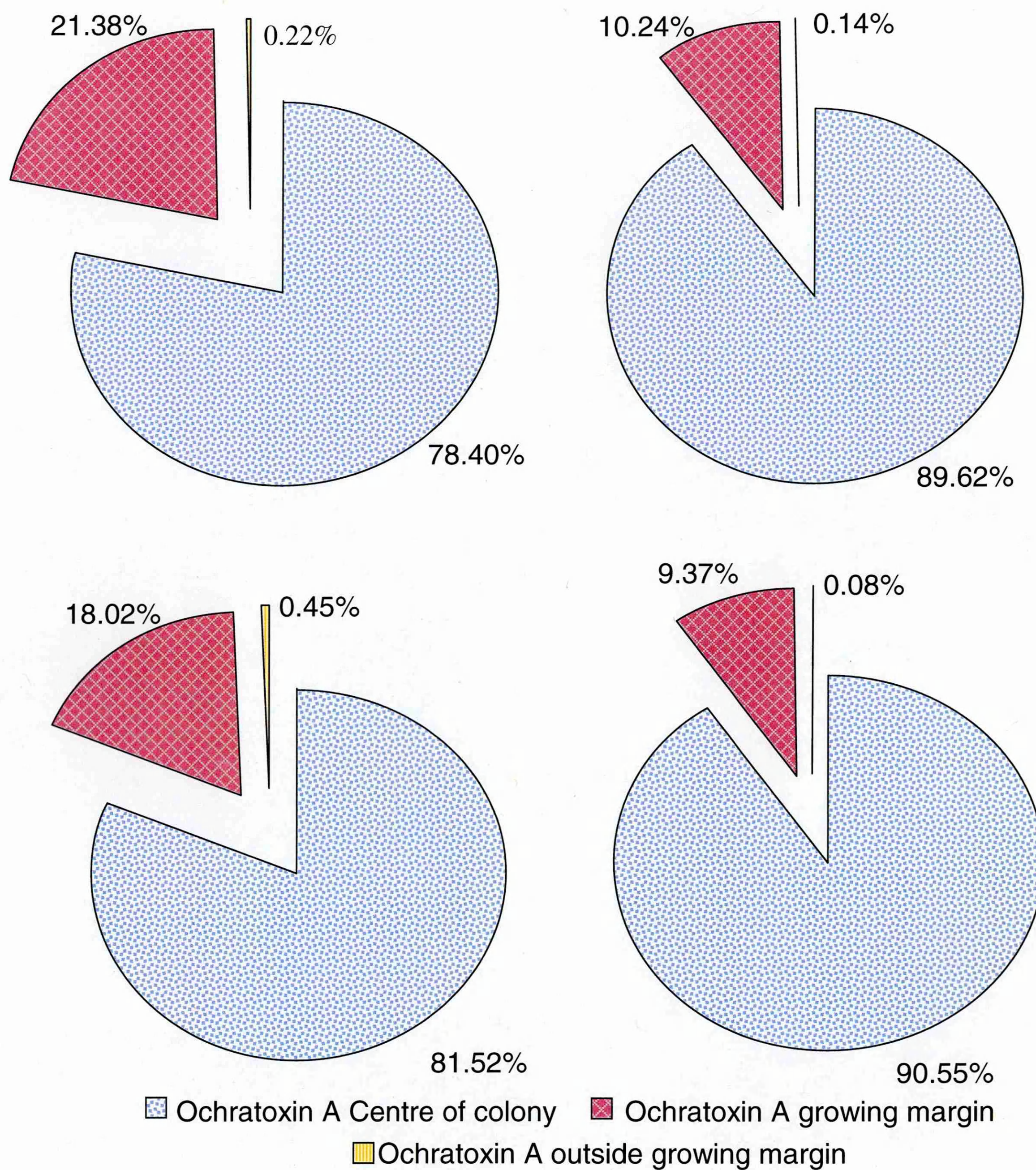


Figure 3.29 distribution of ochratoxin A production as a percentage from the centre of the colony, growing margin and out side the growing margin, grown on synthetic grape juice medium at 20°C, at 0.95 water activity for 20 days. Results are for 3 replicates from strain MPVP A 1102 (1102), *A. niger* aggregate biseriate MPVP A 203 (203), *A niger* aggregate unseriate MPVP A 204 (204), *A. ochraceus* MPVP A 703 (703).

3.8 EFFECTS OF FUNGICIDES AND OTHER CONTROL AGENTS

The majority of fungicides used on grapes had no information for the efficiency against *A. carbonarius* because it has not been considered a significant economic species. *Aspergillus carbonarius* is only associated with grapes as a secondary pathogen. For this reason studies were conducted on a limited range of fungicides under different temperatures and a_w levels.

3.8.1 Effects of fungicides on growth and ochratoxin A production

Copper sulphate has been commonly used on grapes for the past 120 years and grapes can often be seen covered in the blue colour from spraying. In this study it was the fungicide used in the highest and largest concentration range. The growth rates at all concentrations had little effect on growth of *A. carbonarius* with no significance between treatments (Table 3.10). Some concentrations did appear to result in a slight stimulation in growth as shown in Figure 3.30, but no differences were found in efficacy on growth. Growth was very even across the range of concentration, but this was not found in OTA production. Stimulation in OTA occurred at all concentrations. The higher a_w showed at least a doubling in OTA production whilst at 0.95 a_w a significant increase in OTA occurred at 20°C. At 30°C, OTA were similar to that in the control but under no concentrations was there a reduction in OTA production.

Trifloxystrobin, a strobilurin fungicide is used to control a range of foliar fungi by inhibiting spore germination. Ttrifloxystrobin incorporated into SGM (0-5 mg l^{-1}) had no significant effect on growth of *A. carbonarius*. Strain MPVP A 1102 was grown

over a larger range of concentrations of Trifloxystrobin (0-10 mg l^{-1}) but this still had no effect on growth inhibition (Table 3.10). Ochratoxin A production was not uniform across the Trifloxystrobin concentrations. The higher a_w did show a slight stimulation in OTA production but was very similar to the control levels (Figure 3.33).

Carbendazim has been used for the control of *Botrytis* and *Fusarium* spp. on grapes for over 30 years. The effect on growth of both *A. carbonarius* strains examined are in Appendix J. The growth rate decreased with increasing concentrations of Carbendazim, with no growth at the highest concentrations (2.5 mg l^{-1}), with strain 01VAS 219 (Figure 3.32). All concentrations of Carbendazim resulted in an increase in OTA production when compared to the control. Most of the fungicide treatments resulted in at least double the OTA amounts compared to control, only at 30°C and 0.95 a_w was OTA levels similar to the control levels.

Switch is another fungicide used in viniculture and the active ingredients are a combination of Cyprodinil and Fludioxonil, with the former, 50% greater than Fludioxonil, in the formulation. It had the greatest effect on *A. carbonarius* growth statistically (Table 3.10). The study was first carried out at 0.1-2.5 mg l^{-1}, but after 15 days growth was only observed on the lowest concentrations with both strains. The study was repeated at lower concentrations with only one strain (MPVP A 1102). OTA production increased as fungicide concentration was increased (Figure 3.35). At concentrations where growth was 5-20% of the controls, OTA levels were notably higher than controls. At 0.1-0.5 mg l^{-1} Switch was effective at completely inhibiting growth and OTA production.

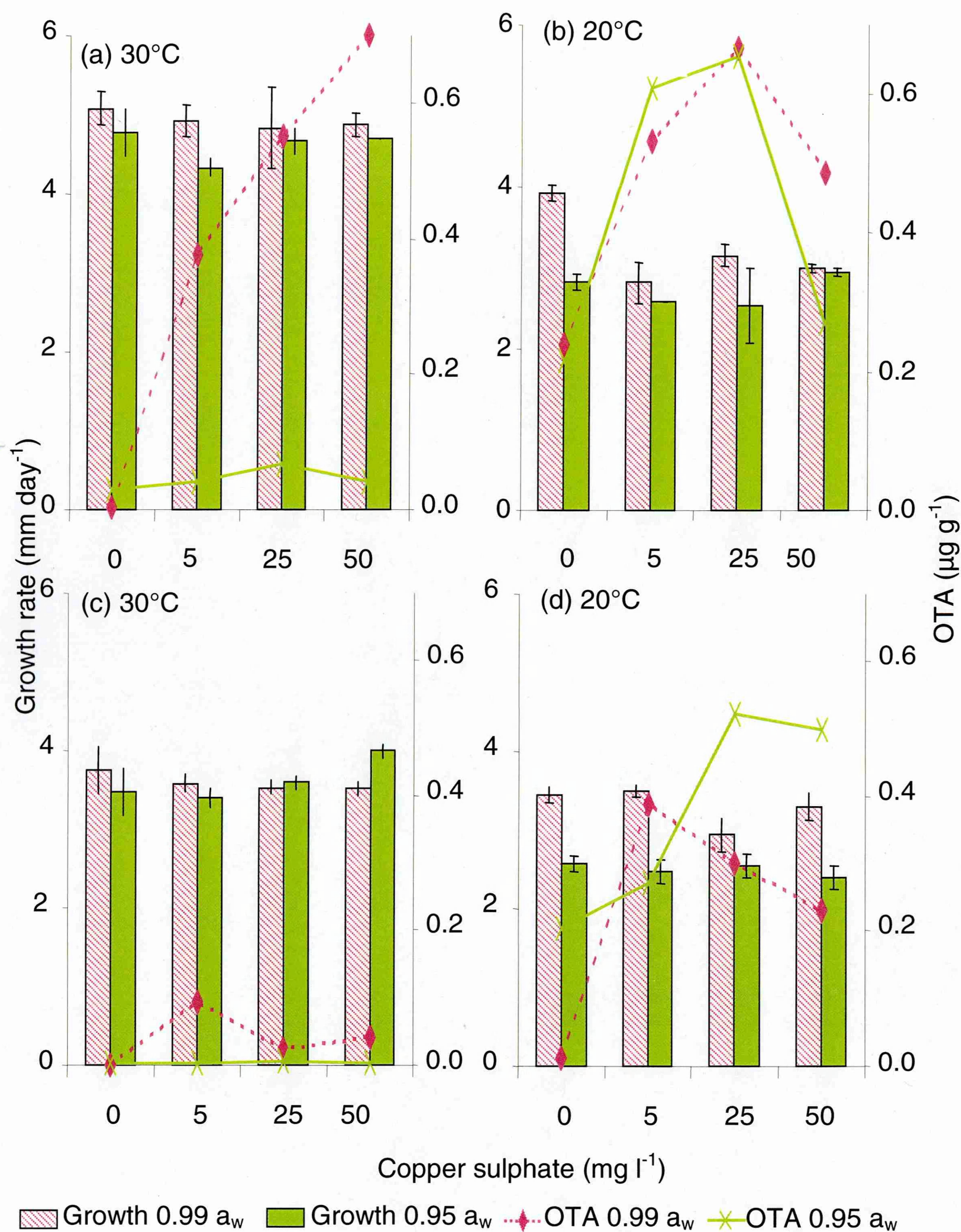


Figure 3.30 Growth and ochratoxin A production by *A. carbonarius* strain Italy MPVP A 1102 (a,b) Portugal 01VAS 219 (c,d) grown on two different glycerol modified media to 0.99 and 0.95 a_w at 30°C and 20°C in relation to a range of copper sulphate concentrations (0 - 50 mg l^{-1}). Bars indicate standard errors of the mean.

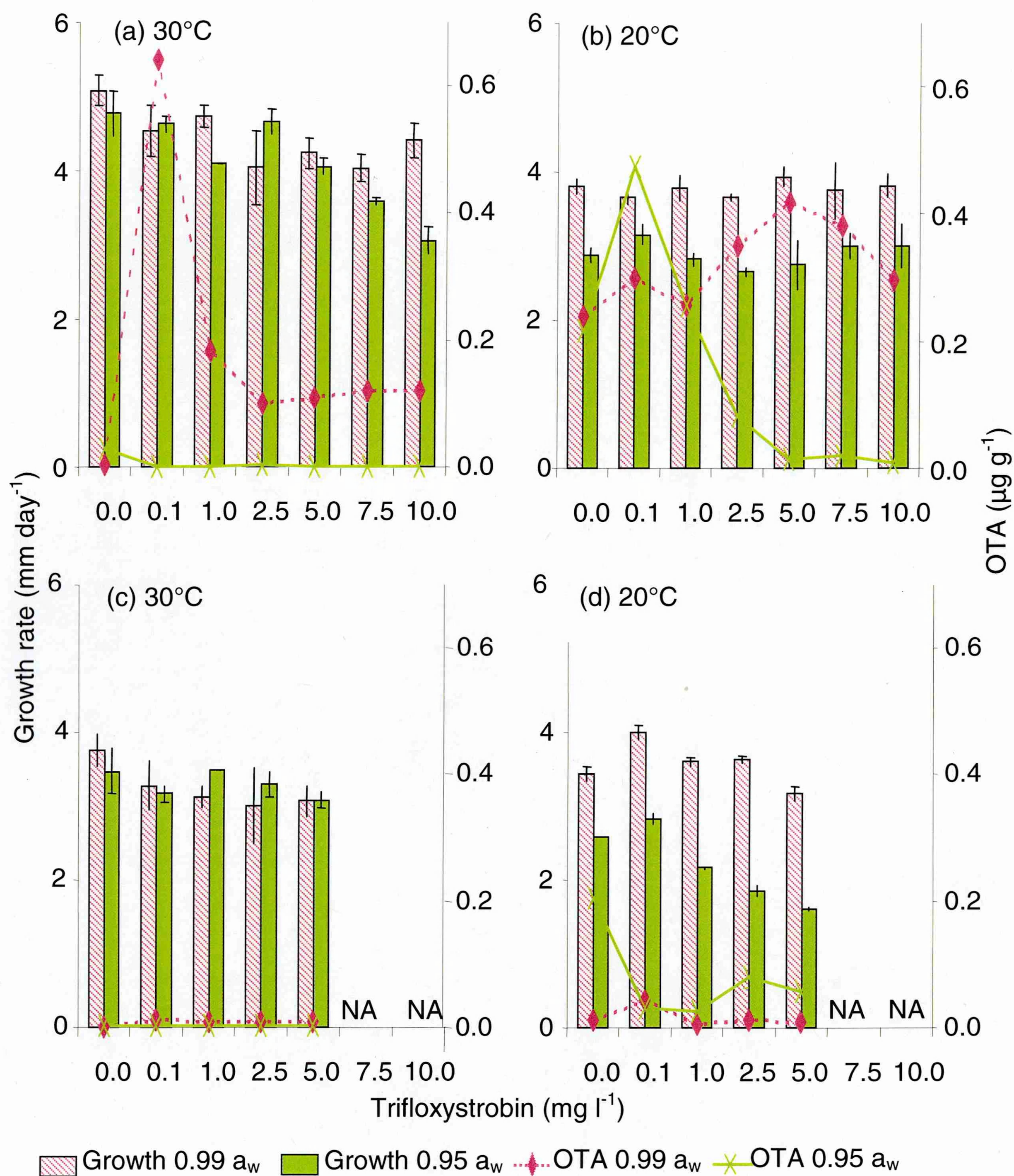


Figure 3.31 Growth and ochratoxin A production by *A. carbonarius* strain Italy MPVP A 1102 (a,b) Portugal 01VAS 219 (c,d) grown on two different glycerol modified media to 0.99 and 0.95 a_w at 30°C and 20°C in relation to a range of Trifloxystrobin concentrations (0-50 mg l^{-1}). Bars indicate standard errors of the mean concentrations. NA, no growth at this concentration.

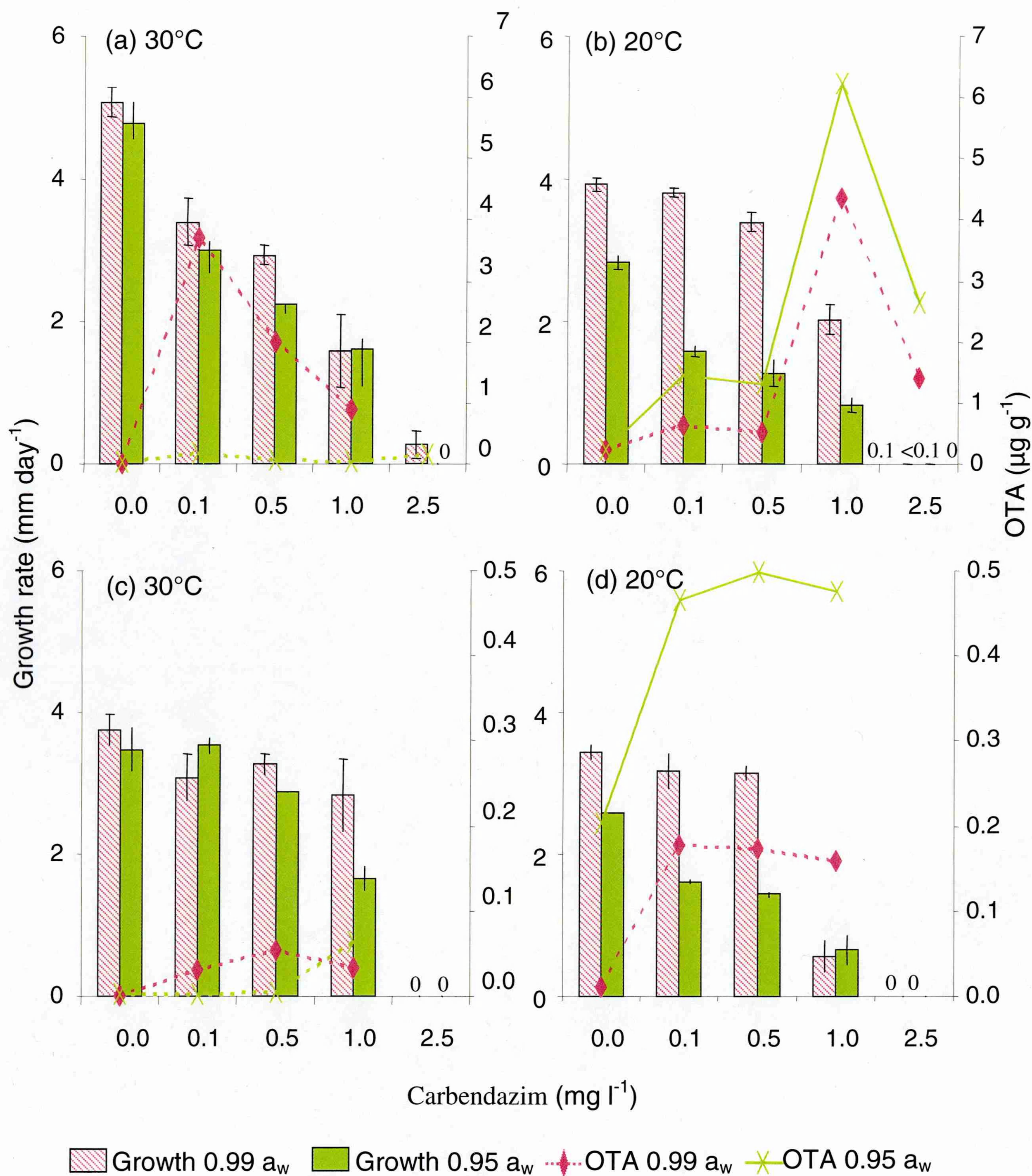


Figure 3.32 Growth and ochratoxin A production by *A. carbonarius* strain Italy MPVP A 1102 (a,b) Portugal 01VAS 219 (c,d) grown on two different glycerol modified media to 0.99 and 0.95 a_w at 30°C and 20°C in relation to a range of Carbendazim concentrations (0–2.5 mg l^{-1}). Bars indicate standard errors of the mean concentrations.

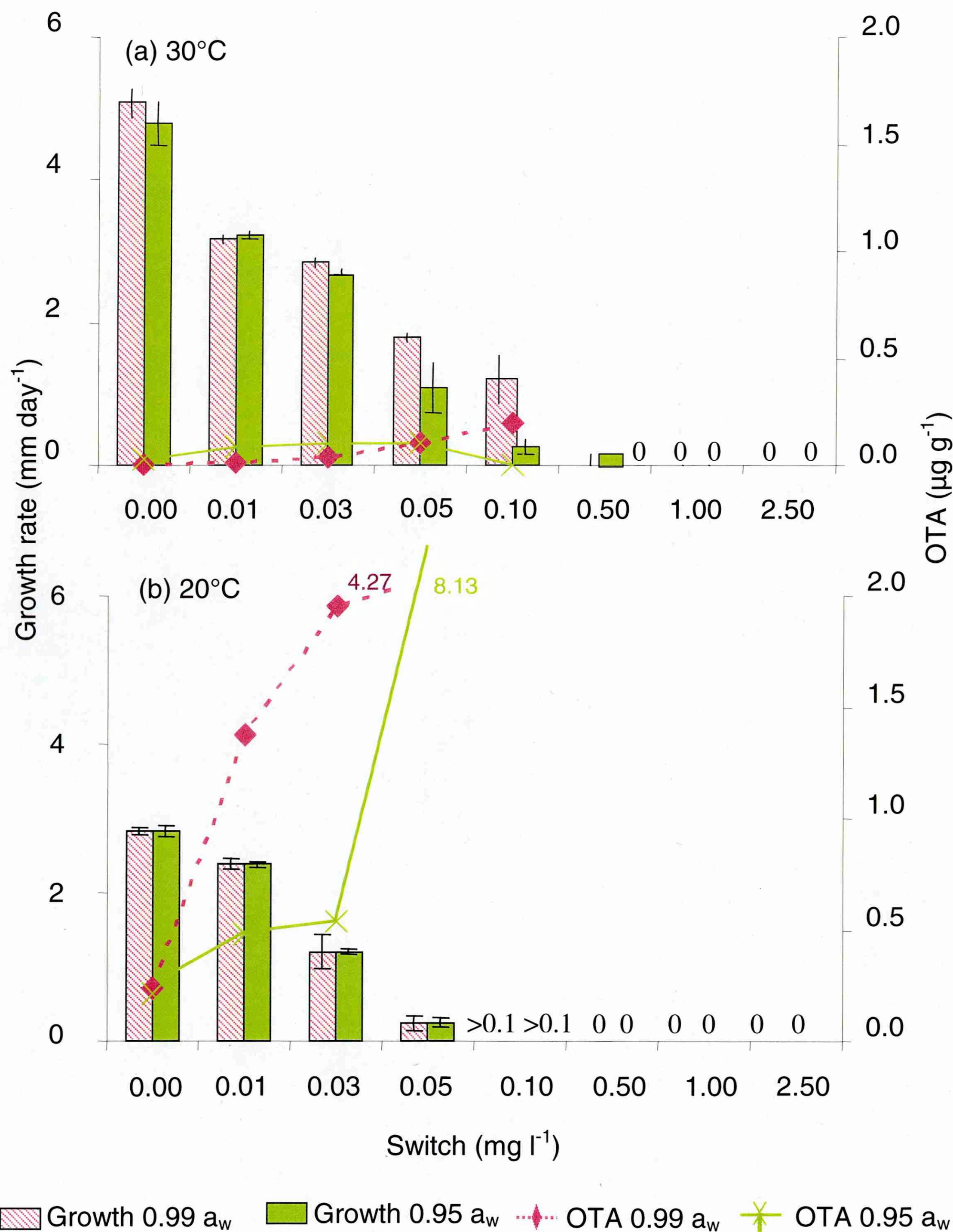


Figure 3.33 Growth and ochratoxin A production by *A. carbonarius* strain Italy MPVP A 1102 (a,b) Portugal 01VAS 219 grown on two different glycerol modified media to 0.99 and 0.95 a_w at 30°C and 20°C in relation to a range of Switch concentrations (0–0.25 mg l^{-1}). Bars indicate standard errors of the mean concentrations.

3.8.2 Effects of other control agents on growth and ochratoxin A production

Several natural occurring control agents have been suggested as possible fungicides. Two such suggested agents are Catechin and Resveratrol. The effects of them on growth rates and OTA production are show in Figure 3.34-3.35. Neither of the two agents showed any difference in growth rates across the range of concentrations examined which is born out by the statistics (Table 3.10).

The SGM is based on berries from early varaison with 50 mg l^{-1} of Catechin. This study modified SGM to include no Catechin. OTA production on Catechin modified SGM (Figure 3.34) at 30°C showed very little variation in control. At 20°C OTA increased with increasing levels of Catechin with OTA production at 150-200 mg l^{-1} of Catechin being double the control levels. At 15°C, OTA production was greatly increased compared to control levels with the highest concentrations of Catechin being >10 times the levels at 0-50 mg l^{-1}. Resveratrol treatment had no real effect on growth or in OTA production (Figure 3.35).

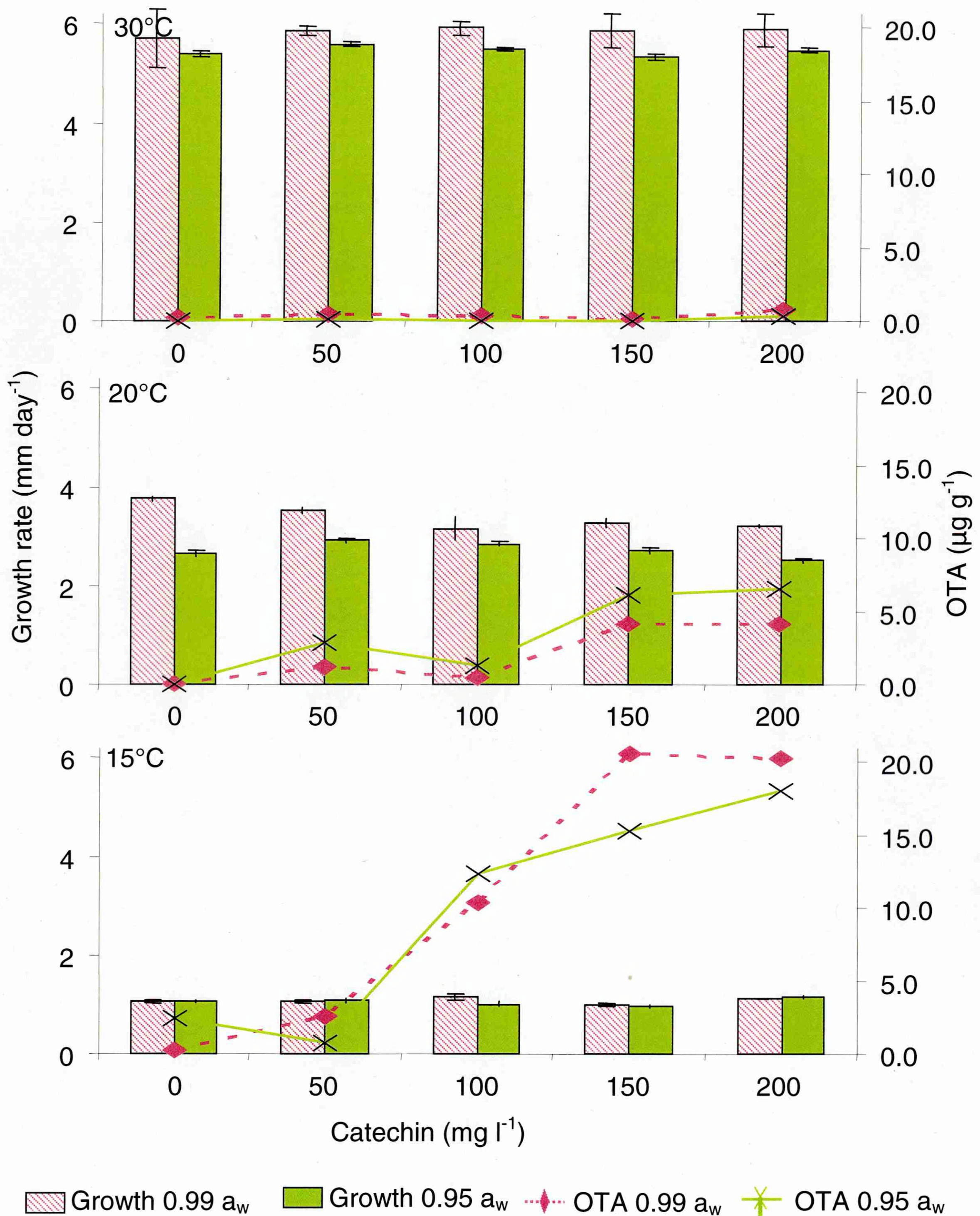


Figure 3.34 Growth rates and ochratoxin A production by *A. carbonarius* (MPVPA 1102) at two different water activities (0.95-0.99) at 15- 30°C and a range of Catechin concentrations (0 – 200 mg l^{-1}). Bars indicate standard errors of the mean.

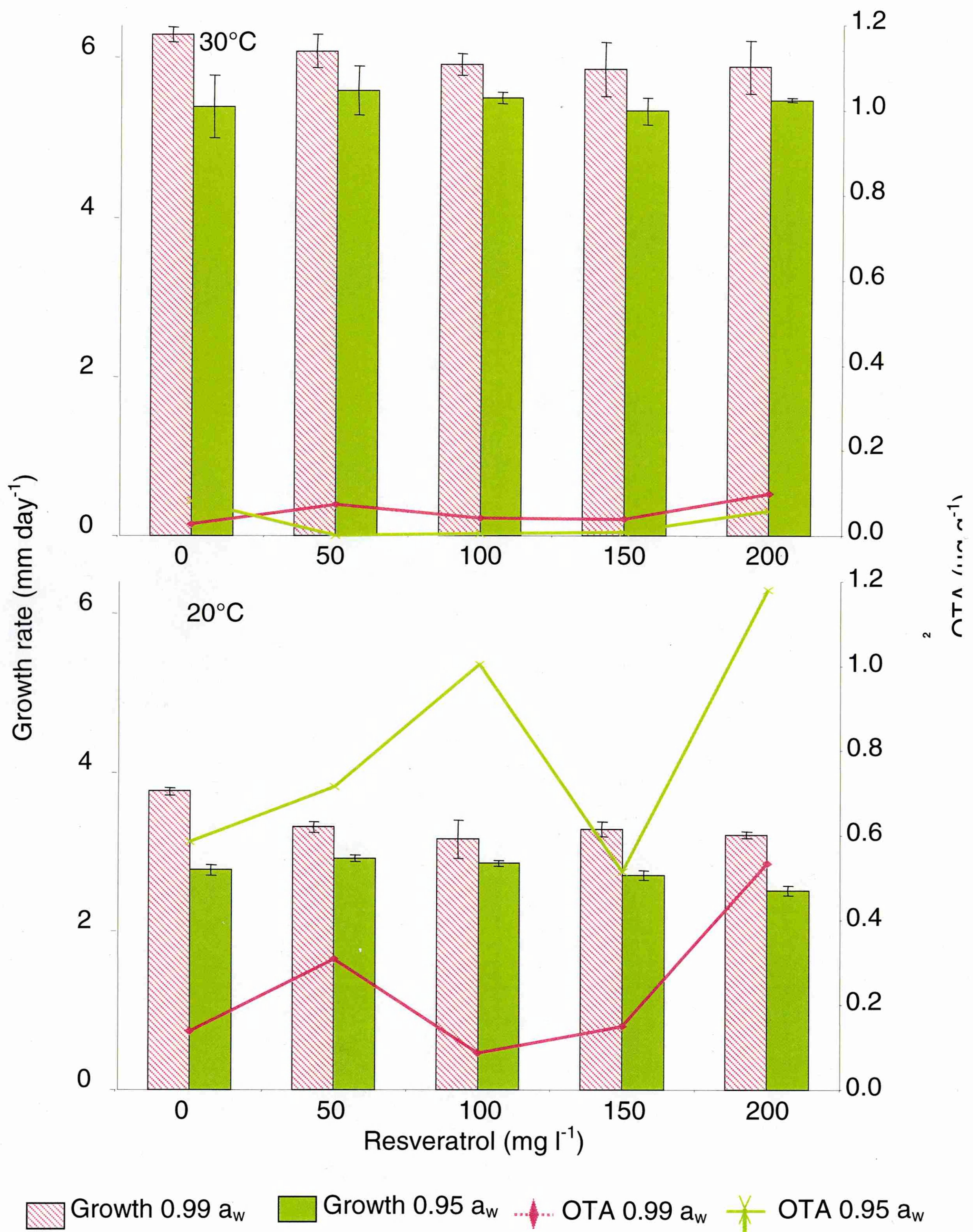


Figure 3.35 Growth rates and ochratoxin A production by *A carbonarius* (MPVPA 1102) at two different water activities (0.95-0.99) and 15- 30°C with a range of resveratrol concentrations (0 – 200 mg l^{-1}). Bars indicate standard errors of the mean.

Table 3.10 Summary One way ANOVA of growth of two strains of *A. carbonarius* (MPVP A 1102 (1102); 01UA s219 (219) at two temperature 20, 30°C and 0.95, 0.99 water activities, over a range of fungicide concentrations. * Indicates factors which was significantly different (p<0.05) (for details see Appendices H-M).

Source	DF	SS	MS	F	P	SS	MS	F	P
	1102					219			
Copper Sulphate	2.0	0.3	0.2	1.0	0.367	0.1	0.1	1.0	0.390
Trifloxystrobin	5	3.1	0.6	4.3	<0.001*	3	0.6	0.2	0.334
Carbendazim	3	68.7	22.9	143.0	<0.001*	69.6	23.2	388.0	<0.001*
Switch	2	19.1	9.6	176.2	<0.001*	N/A	N/A	N/A	N/A
Catechin	0.3	0.1	0.8	0.555	0.3	N/A	N/A	N/A	N/A
Resveratrol	4	0.7	0.2	1.4	0.248	N/A	N/A	N/A	N/A

N/A No data

3.9 *IN VITRO* STUDIES ON SODIUM METABISULPHITE ($Na_2S_2O_3$), AND CONTROLLED ATMOSPHERES

Figure 3.36 shows the effect of $Na_2S_2O_2$ treatments on the growth rate and OTA production of *A. carbonarius* (MPVA 1102). The use of $Na_2S_2O_2$ notably affected the growth rates at all three a_w with no growth possible at > 1000 mg kg^{-1}. Growth rates decreased with increasing $Na_2S_2O_2$concentrations, with the exception of 0.99 and 0.93 a_w at 100 mg kg^{-1} $Na_2S_2O_2$ resulted in both increased growth compared to the control and 200 mg kg^{-1}. Table 3.11 shows the comparisons of LD_{50} and LD_{90} on growth rates. OTA production after 10 days growth on RGJM was not detected at levels >700 mg kg^{-1} $Na_2S_2O_2$. (Figure 3.36b) In general OTA levels decreased with increasing $Na_2S_2O_2$, with the exception of 100 mg kg^{-1} at 0.99, 0.93 a_w which both had a marked decrease in OTA production compared the control and 200 mg kg^{-1}. Tables 3.13 and 3.14 gives the LD_{50} and LD_{90} levels required for growth and OTA production.

Figure 3.37 shows the effects of increased levels of CO_2 on germination after 24 and 48 hours in air and 25% CO_2 no marked difference was observed. Germination after 24 hours in50 % CO_2 was < 50% at all a_w levels. After 48 hours there was no notable difference between germination rates with all levels examined except at 50 % CO_2 and 0.93 a_w where germination was <5%. Effects of CO_2 on growth rates are shown in (Figure 3.38). Growth rates at 25% CO_2 were less then half of than in air with no measurable growth at 50% CO_2. OTA production was examined after 10 days incubation on RGJM. OTA levels showed a slight decrease, but in general the levels were fairly consistent across all CO_2 levels examined. The only noticeable difference was at 25%, 0.99 a_w were there was stimulation in OTA production.

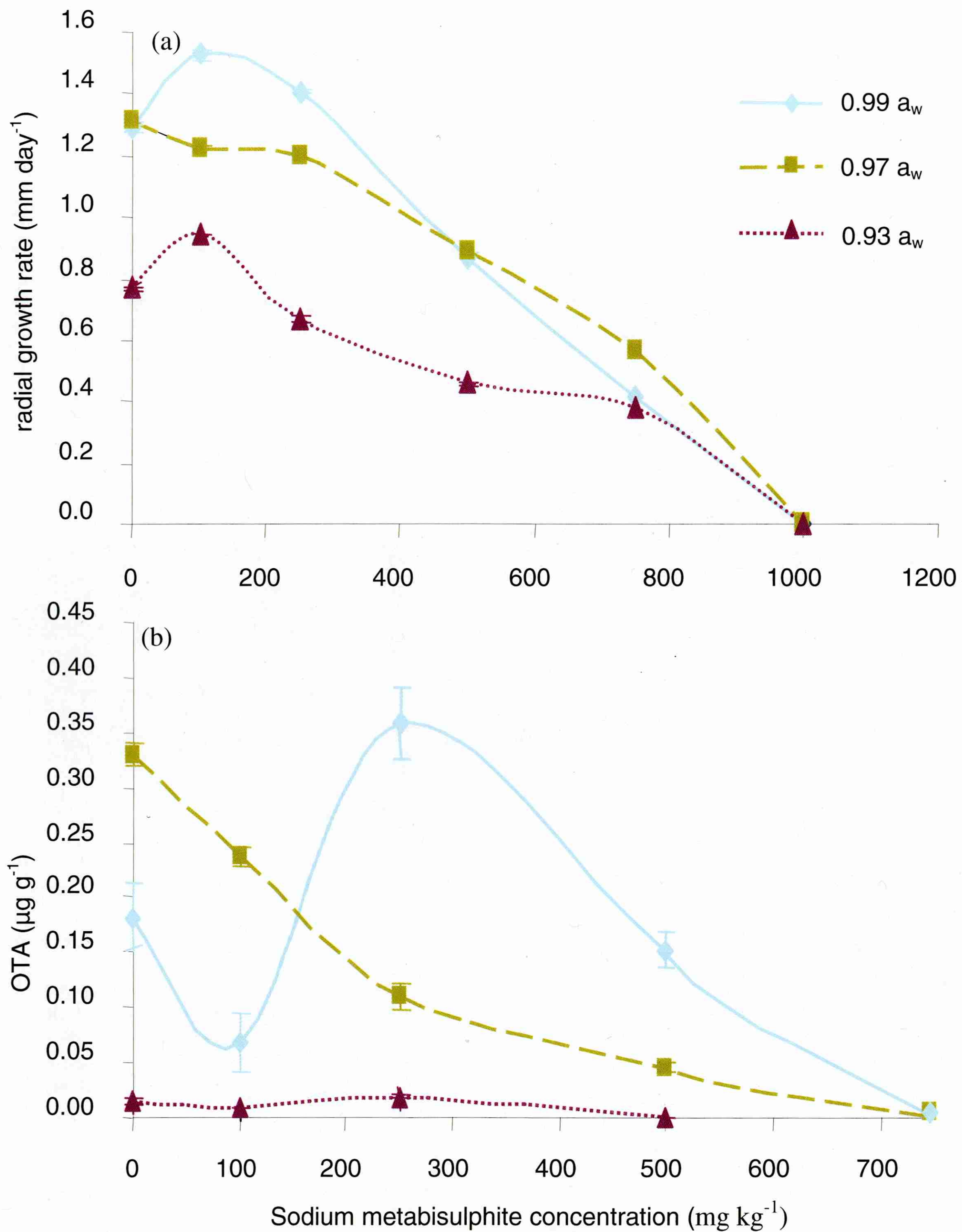


Figure 3.36 Effect of Sodium metabisulphite concentration on (a) growth and (b) ochratoxin A by *A. carbonarius* (MPVP A 1102) on a red grape juice medium modified to three water activity levels at 25°C. Bars indicate standard error of the means.

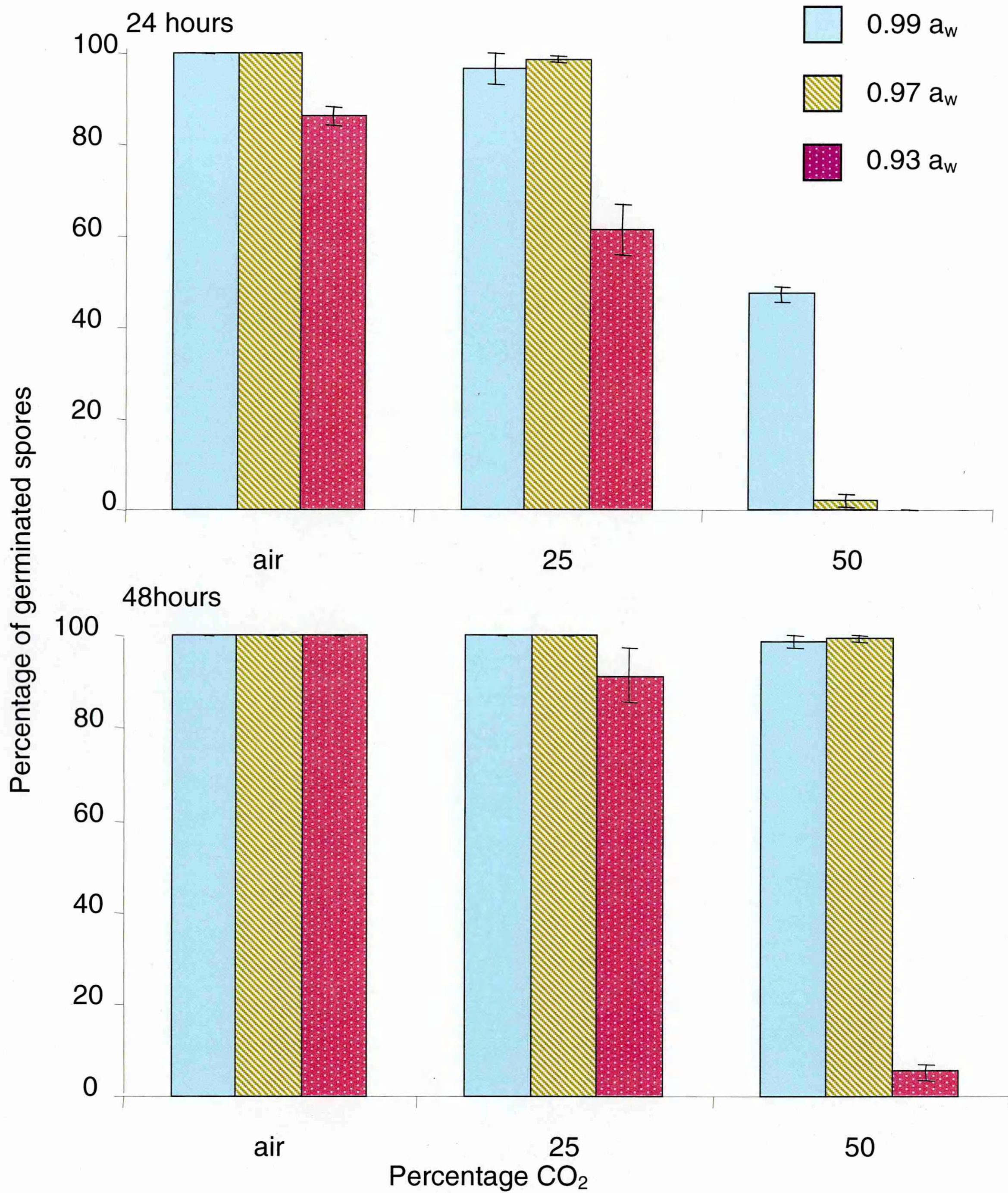


Figure 3.37 Effect of CO_2 concentration on germination of conidia of *A. carbonarius* (MPVP A 1102) on a red grape juice medium modified to three water activity levels at 25°C. Bars indicate standard error of the means.

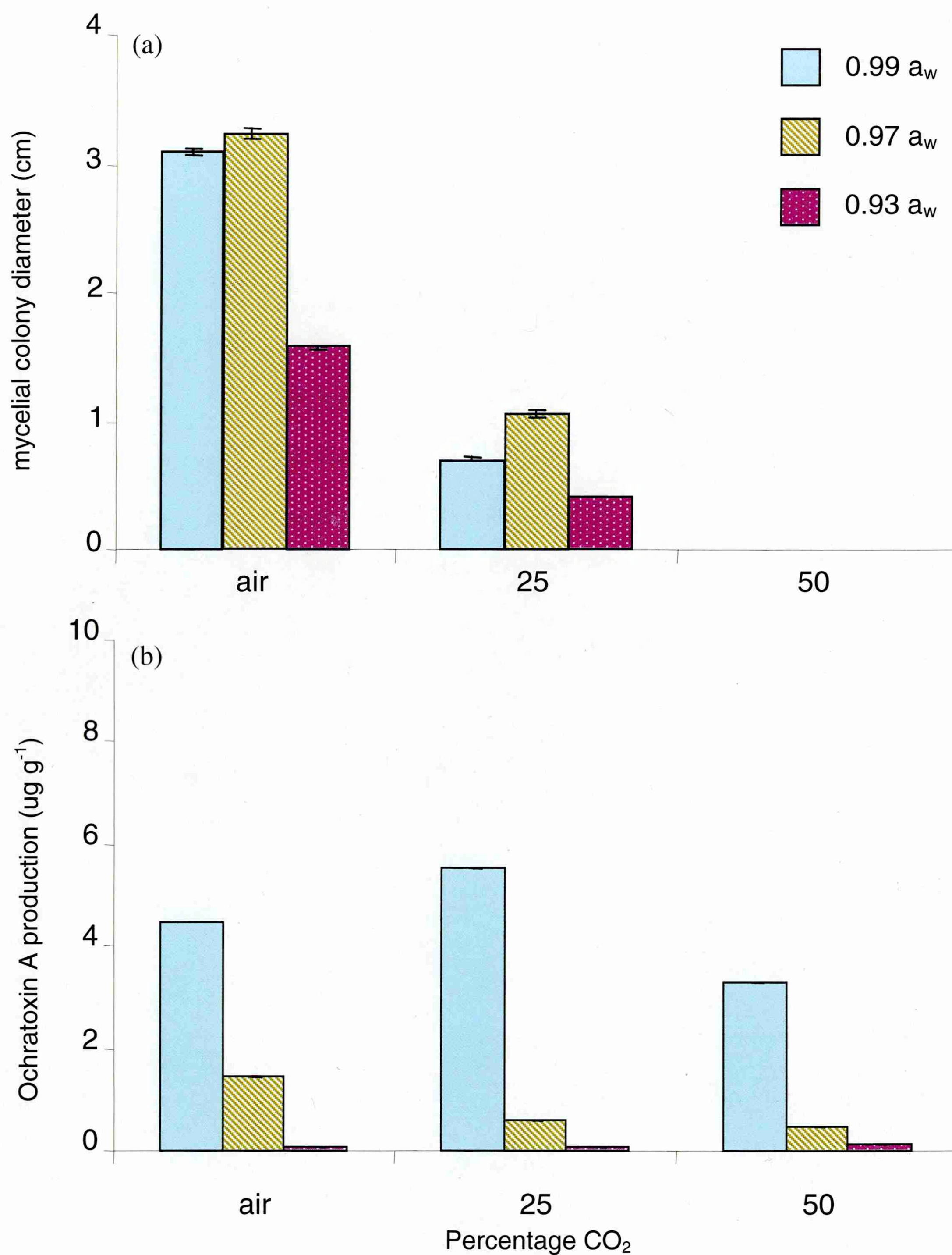


Figure 3.38 Effect of CO_2 on (a) growth and (b) ochratoxin A by *A. carbonarius* (MPVP A 1102) on red grape juice medium modified to three water activity levels at 25°C. Bars indicate standard error of the means.

Table 3.11 The LD_{50} and LD_{90} concentrations of NaMBS required for inhibiting growth of f *A. carbonarius* (MPVA P A 1102).

	$Na_2S_2O_2$ (mg kg^{-1})	
Water activity	LD_{50}	LD_{90}
0.99	600	950
0.97	680	975
0.93	805	1000

Table 3.12 The LD_{50} and LD_{90} concentrations of NaMBS required for inhibiting ochratoxin A production by *A. carbonarius* (MPVA P A 1102).

	$Na_2S_2O_2$ (mg kg^{-1})	
Water activity	LD_{50}	LD_{90}
0.99	600	690
0.97	170	620
0.93	420	480

3.10 COMPETITION BETWEEN *ASPERGILLUS CARBONARIUS* AND GRAPE FUNGI

In the vineyard *A. carbonarius* seldom occurs on it own but as a mixed community of other yeasts, filamentous fungi and bacteria. The interactions between different fungi have been shown to have an impact on growth, and toxin production, partially on cereal grain and maize (Magan & Lacey, 1984b; Lee & Magan, 2000). The aims of this study were to:-

1. Determine the competitiveness of *A. carbonarius* against a range of other grape fungi on SGM agar under various temperatures and water activity levels.

2. Investigate the effects of interspecific interactions under different environmental stresses on OTA production by *A. carbonarius.*

3.10.1 Interactions of *A. carbonarius* against common grape fungi

Plate 3.3 shows examples of dual culture plates used to determine macroscopic interactions between *A. carbonarius* and other grape fungal species. The interactions changed as temperature and a_w were changed (Table 3.13).

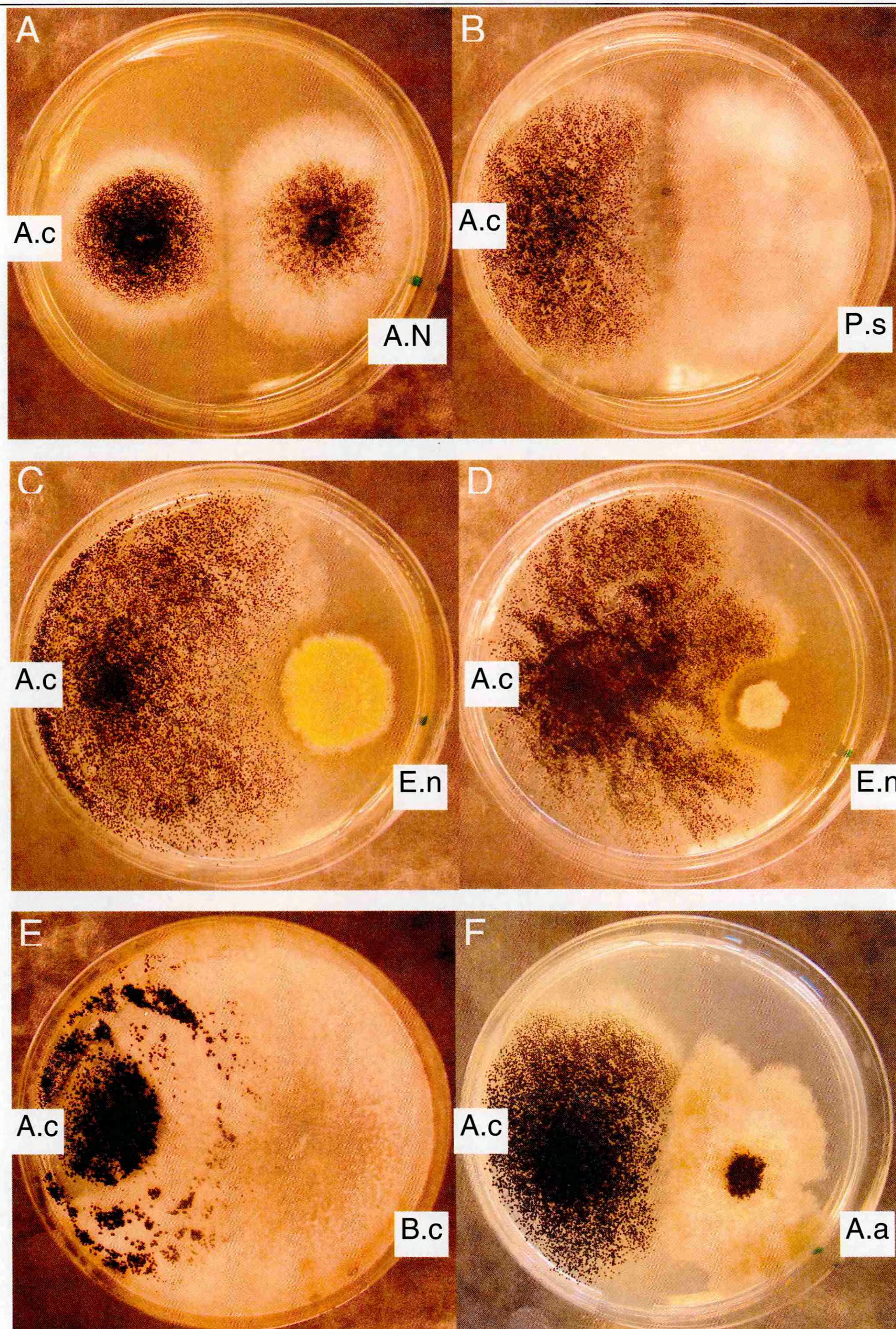
Three different interactions were found in the study; at 30°C *A. carbonarius* generally dominated all other fungi scoring 4-0. The exceptions were the other *A.* section *Niger* fungi and *E. nigrum*. At 20°C there was a change with *A. carbonarius* only able to dominate the pink yeast. Against all other fungi *A. carbonarius* was mutually antagonistic either on contact scoring 2-2, whilst with *E. nigrum* this was mutually antagonistic at a distance, scoring 3-3.

Growth rates of *A. carbonarius* varied when grown as a duel culture with other grape fungi. When grown against most of the species at 20°C there was a slight reduction in growth rates and a slight increase at 30°C when compared to the control (Table 3.16).

3.10.2 Ochratoxin A production of *A. carbonarius* when grown against other common grape fungi

Figure 3.39-3.41 compares OTA production in presence and absence of competitive species under different environmental factors. OTA production was greatest at 20°C

and 0.99 a_w with stimulation occurring when grown against most other species. OTA production decreased with lowering a_w and increasing temperature, with the lowest OTA production at 30°C and 0.93 a_w. In general, there was a stimulation of OTA production at 0.99 a_w. However, there was a reduction in OTA production at 0.93 and 0.95 a_w when compared to *A. carbonarius* alone. Interaction with *B. cinerea* was one of the few interactions which consistently resulted in more OTA than *A. carbonarius* alone. Competition with pink yeast and *A.* section *Niger* uniseriate produced a reduction in OTA by *A. carbonarius* than when grown alone.



Type of interaction and interaction score

A-B Mutual antagonism on contact both score 2 . Interaction score 2-2

C-D Mutual antagonism at a distance both score 3 Interaction score 3-3

E-F Dominance of *A. carbonarius* dominance species score 4 species. Interaction score 4-0

Plate 3.3 Examples of interaction between *A. carbonarius* (MPVP A 1102) and other common grape fungi. **A**. 0.99 a_w 20°C, **B**. 0.99 a_w 25°C, **C**. 0.95a_w 25°C, **D**. 0.99 a_w 25°C, **E**. 0.99 a_w 20°C, **F**. 0.99 a_w 20°C. Species key: A.c, *A. carbonarius*; A.N, bi *Aspergillus* section *Niger* biseriate; .p.s *Phoma* spp; B.c, *B. cinerea* A.a, *A. alternaria.*

Table 3.13 Interaction and Index of Dominance (I_D) scores for *A. carbonarius* vs various vineyard fungi. Synthetic grape juice medium at three a_w levels (0.93-0.99) incubated at 20-30°C for 15 days.

	30°C			25°C			20°C			
a_w/spp.	0.99	0.95	0.93	0.99	0.95	0.93	0.99	0.95	0.93	I_D
Cladosporium spp.	4/0	4/0	4/0	4/0	4/0	2/2	4/0	2/2	2/2	30/6
Epicoccum nigrum	2/2	2/2	2/2	3/3	3/3	3/3	3/3	3/3	3/3	24/24
Aureobasidium pullulans	4/0	4/0	4/0	4/0	4/0	2/2	2/2	2/2	1/1	27/7
Aspergillus sectiom Niger biseriate	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	18/18
Botrytis cinerea	4/0	4/0	4/0	4/0	4/0	4/0	2/2	2/2	2/2	32/6
Alternaria alternaria	4/0	4/0	4/0	4/0	4/0	2/2	2/2	2/2	2/2	28/8
Aspergillus sectiom Niger uniseriate	2/2	2/2	2/2	2/2	3/3	2/2	3/3	3/3	2/2	21/21
Phoma spp.	4/0	4/0	4/0	4/0	4/0	2/2	2/2	2/2	2/2	28/8
Pink yeast	4/0	4/0	4/0	4/0	4/0	4/0	4/0	4/0	4/0	36/0
I_D	30/6	30/6	30/6	31/7	32/8	19/15	24/16	22/18	20/16	48/24

Table 3.14 Interaction and Index of Dominance growth rates for *A.carbonarius* when grown in competition with various vineyard fungi. Synthetic grape juice medium at three a_w levels (0.93-0.99) incubated at 20-30°C for 15 days.

	30°C			25°C			20°C		
a_w/spp.	0.99	0.95	0.93	0.99	0.95	0.93	0.99	0.95	0.93
Aspergillus carbonarius	5.1	4.8	4.0	5.0	4.5	3.5	3.9	2.8	2.2
Cladosporium spp.	5.4	5.8	6.6	5.2	5.0	3.2	3.5	1.9	1.2
Epicoccum nigrum	N/A	N/A	N/A	5.7	4.4	2.3	5.9	3.4	2.5
Aureobasidium pullulans	5.9	6.3	4.7	3.8	5.0	3.7	2.6	1.6	2.4
Aspergillus sectiom Niger biseriate	2.2	2.2	2.4	3.6	2.2	1.5	3.6	2.5	0.9
Botrytis cinerea	7.1	7.6	4.2	4.8	4.4	3.6	2.9	2.6	1.2
Alternaria alternaria	6.5	5.8	4.1	5.3	4.6	4.1	3.6	3.8	1.9
Aspergillus sectiom Niger uniseriate	4.5	3.4	4.0	4.9	3.3	2.7	3.6	2.2	0.9
Phoma spp.	5.2	4.9	6.4	4.7	3.5	2.6	3.6	2.2	1.0
Pink yeast	6.7	5.8	4.2	4.9	4.6	1.8	3.7	2.6	1.4

3.11 FIELD WORK

The colonisation of berries by fungi started soon in the field and increased from setting (75-85 %) to harvest (100 %) in all regions and over both years (Figure 3.44). The most common mycoflora isolated from grapes, in decreasing order, were: *Alternaria*, yeasts, *Aspergillus*, *Botrytis*, *Epicoccum*, *Cladosporium*, *Rhizopus*, *Penicillium*, *Fusarium*, *Mucor*, *Phoma*, *Trichoderma* and *Ulocladium*. No statistically significant differences were found between both years and among the regions, therefore and as an example, the fungi infecting berries at each sampling date in 2003 in La Rioja region is shown in Figure 3.42. *Alternaria* was the highest component of the natural flora on the surface of fresh grapes, followed by yeasts. The number of *Aspergillus*, *Botrytis*, *Epicoccum*, *Rhizopus* and yeasts was statistically higher when approaching harvest, with the exception of *Alternaria*, which decreased from 95 % to 70 % in the later growth stages. The remaining species were rarely isolated without following any trend.

A total of 464 and 648 *Aspergillus* belonging to the section *Nigri* were isolated in 2002 and 2003, respectively, distributed in the four regions sampled. The number of these moulds found in the sampling near harvest was significantly higher than the number of these moulds found in the first or second sampling for the four regions (Figure 3.43). According to analysis of variance (data not shown), significant differences were found between both years due to the number of strains isolated, as more *A.* section *Nigri* were found in 2003. Grapes from Costers del Segre were significantly the most contaminated every year, with approximately 300 strains isolated in 2003 and around 180 in 2002 at the third sampling period, followed by Utiel-Requena ones with around 100 isolates in both years at the third sampling period, meanwhile no statistical differences were found between Penedés/Conca de Barberà and La Rioja region.

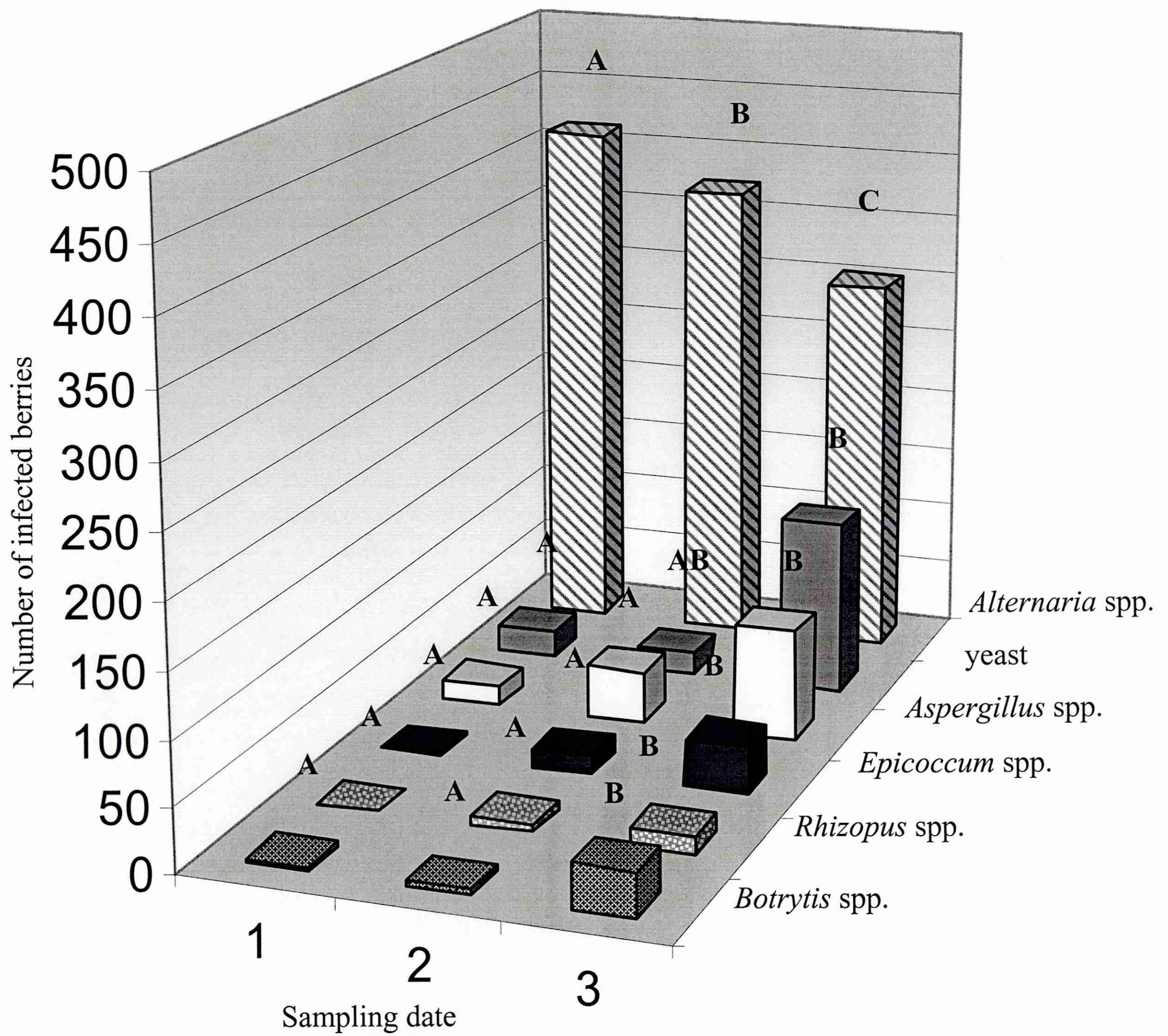


Figure 3.42 Number of berries infected by different fungi plated on DRBC, at each sampling date: (1) one month after setting, (2) veraison and (3) harvest, in 2003 in La Rioja region. Different letters over bars mean significant differences in the number of these fungi along the sampling periods.

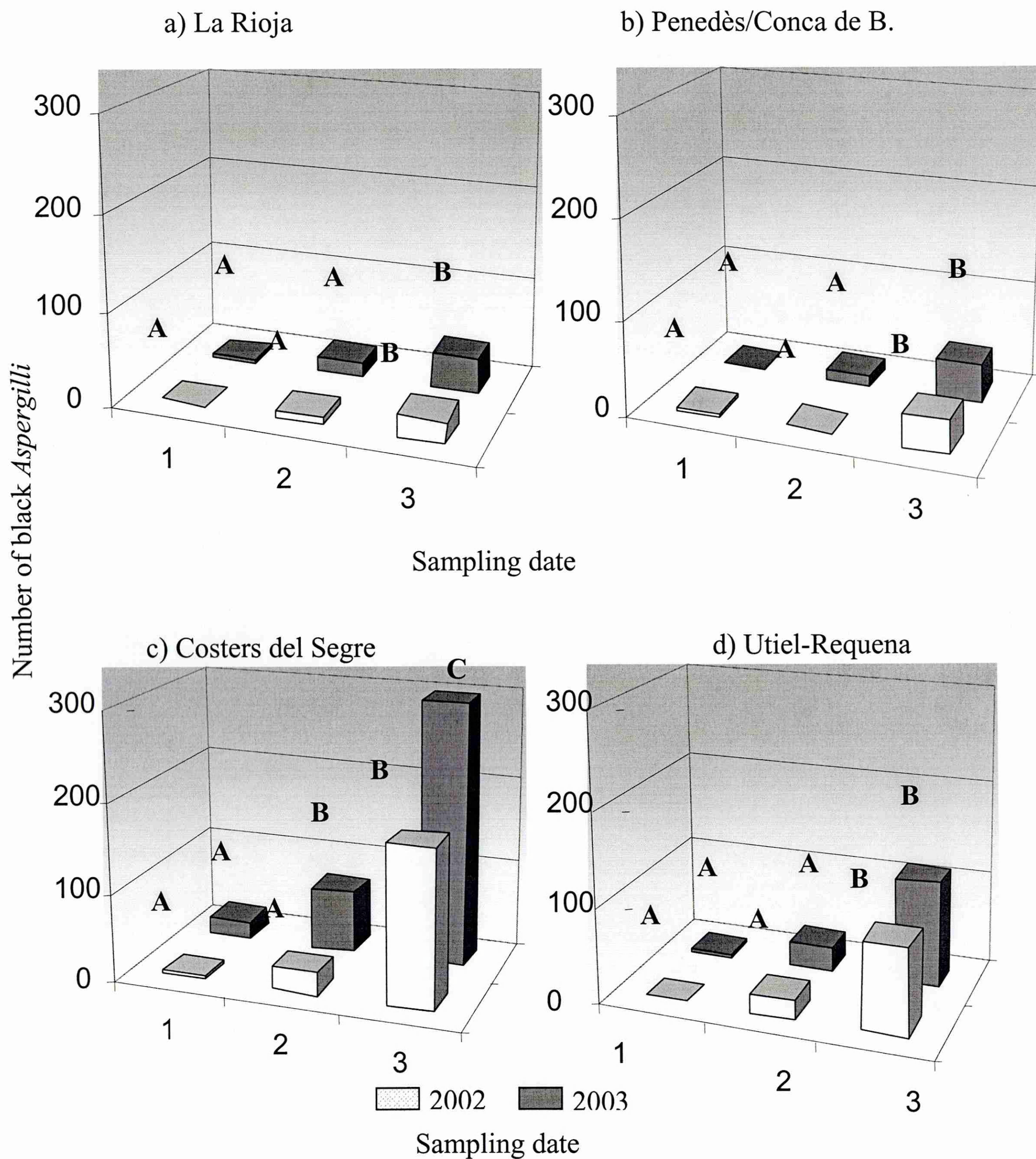


Figure 3.43 Number of black *Aspergilli* found on grapes plated on DRBC in 2002 and in 2003 in four wine-making regions of Spain, at three sampling periods: (1) one month after setting, (2) veraison and (3) harvest. Different letters over bars mean significant differences in the number of these fungi along the sampling periods.

Figure 3.44 shows the distribution of the black *Aspergilli* isolates, resulting *A. niger* aggregate the most common species (75 % in 2002 and 53 % in 2003) followed by *A. carbonarius* (7 % in 2002 and 29 % in 2003), meanwhile the percentage of uniseriates was similar in both years (18%).

No *P. verrucosum* was detected, while only a few *A. ochraceus* were isolated (19 in 2002 and 31 in 2003), most of them at harvest time, approximately 15 % of these strains were OTA-producers.

In 2002, 7 % of the total number of *A.* section *Nigri* strains isolated were OTA positives, meanwhile 25 % positives were found in 2003. Among the black *Aspergilli* groups, *A. carbonarius* was the one which presented the highest percentage of OTA-positive strains (82 % in 2002 and 76 % in 2003). A low percentage of *A. niger* aggregate isolates produced OTA (2 % in 2002 and 5 % in 2003) and no toxin was detected in any of the uniseriate strains in both years (Table 3.15). Furthermore, more than 95 % of the total number of positive isolates produced low amounts of OTA (<2.5 $\mu g\ g^{-1}$), although some strains were found to produce OTA at high levels (2.5 - 25 $\mu g\ g^{-1}$). The most toxigenic strains proved to be *A. carbonarius* in both years. OTA was not detected in any of 2002 and 2003 must samples analysed.

Table 3.16 shows 2001-2003 meteorological data in the sampling months for each region, used to be correlated with fungi isolated from grapes. A significant positive correlation between the number of black *Aspergilli* isolated, and the temperature in the field in the months preceding harvest was found (Table 3.16). Although not significantly, relative humidity (R.H.) contributed to the infection of these fungi, mainly in uniseriates.

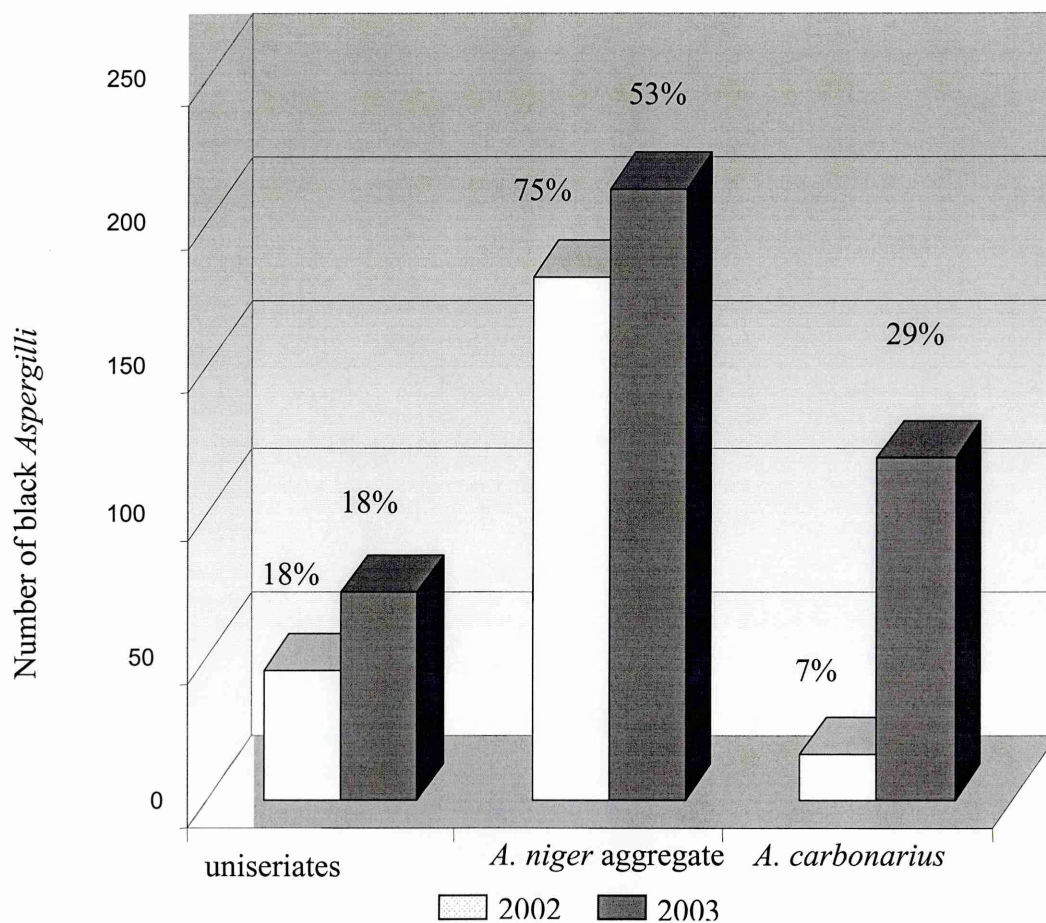


Figure 3.44 Number and percentages of black *Aspergilli* isolated from grapes in 2002 and in 2003, classified into three groups.

Table 3.15 Number of black *Aspergilli* isolates of each group found in each region at each sampling date in 2002 and 2003 and their ochratoxigenic ability on Czapek yeast agar.

		uniseriate		*A. niger* aggregate		*A. carbonarius*	
Region	S	2002	2003	2002	2003	2002	2003
P/CB	1	0 (0)	0 (0)	1 (0)	2 (0)	0 (0)	0 (0)
UR	1	0 (0)	0 (0)	6 (0)	11 (0)	0 (0)	0 (0)
R	1	4 (0)	4 (0)	12 (0)	28 (0)	0 (0)	0 (0)
CS	1	0 (0)	12 (0)	2 (0)	6 (0)	0 (0)	0 (0)
P/CB	2	15 (0)	34 (0)	10 (0)	15 (1)	0 (0)	1 (1)
UR	2	24 (0)	14 (0)	71 (0)	33 (2)	1 (1)	0 (0)
R	2	0 (0)	0 (0)	0 (0)	4 (2)	0 (0)	0 (0)
CS	2	0 (0)	0 (0)	15 (0)	16 (1)	0 (0)	6 (0)
P/CB	3	1 (0)	0 (0)	53 (0)	58 (5)	1 (0)	0 (0)
UR	3	0 (0)	0 (0)	3 (1)	2 (-)	7 (6)	7 (7)
R	3	0 (0)	2 (0)	2 (1)	8 (-)	0 (0)	0 (0)
CS	3	1 (0)	6 (0)	6 (1)	28 (-)	8 (7)	104 (82)
TOTAL		45 (0)	72 (0)	181 (3)	211 (11)	17 (14)	118 (90)

S, sampling (1, one month after setting; 2, veraison; 3, harvest);
P/CB, Penedès/Conca-Barberà; UR, Utiel-Requena; R, La Rioja; CS, Costers del Segre;
(-) not tested for OTA.
Numbers in brackets are the number of fungi producing OTA above the detection limit (0.01 $\mu g\ g^{-1}$ CYA).

Table 3.16 Mean of 2001, 2002 and 2003 meteorological data of each region at each sampling period (1, June; 2, July; 3, August).

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

Table 3.17 Correlation between meteorological parameters with ochratoxigenic fungi isolated from grapes, by using the coefficients of Pearson.

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

* significant $p<0.05$

**significant $p<0.001$

3.12 EFFECT OF SPORE CONCENTRATION ON GRAPE INFECTION

Grapes are naturally contaminated with fungi but, little is known about how long the spore will remain viable until the conditions are right for germination.

Figure 3.45 showed that at 100% ERH two concentrations of spores 1×10^3 and 1×10^8 had no notable difference in the amount of colony forming units (CFU's) that were obtained after 5-20 days. At 93% ERH the lower spore concentration yielded lower CFU than 1×10^8 but both concentrations produced higher colony forming units (CUF) than the original spore suspension. This was due to the grapes becoming infected but also natural contamination with another *A.* section *Nigri* as found.

It was also noted that after 5 days grapes started to show grape rot around the stalk in some grapes whilst other remained healthy. Figure 3.46 shows uninfected grapes in contact with infected grapes with no sign of infection up to >7 days. It was also noted that most of the infected grapes started showing a browning colour around the stalk spreading through the grape before the skin ruptured and fungal growth was visible.

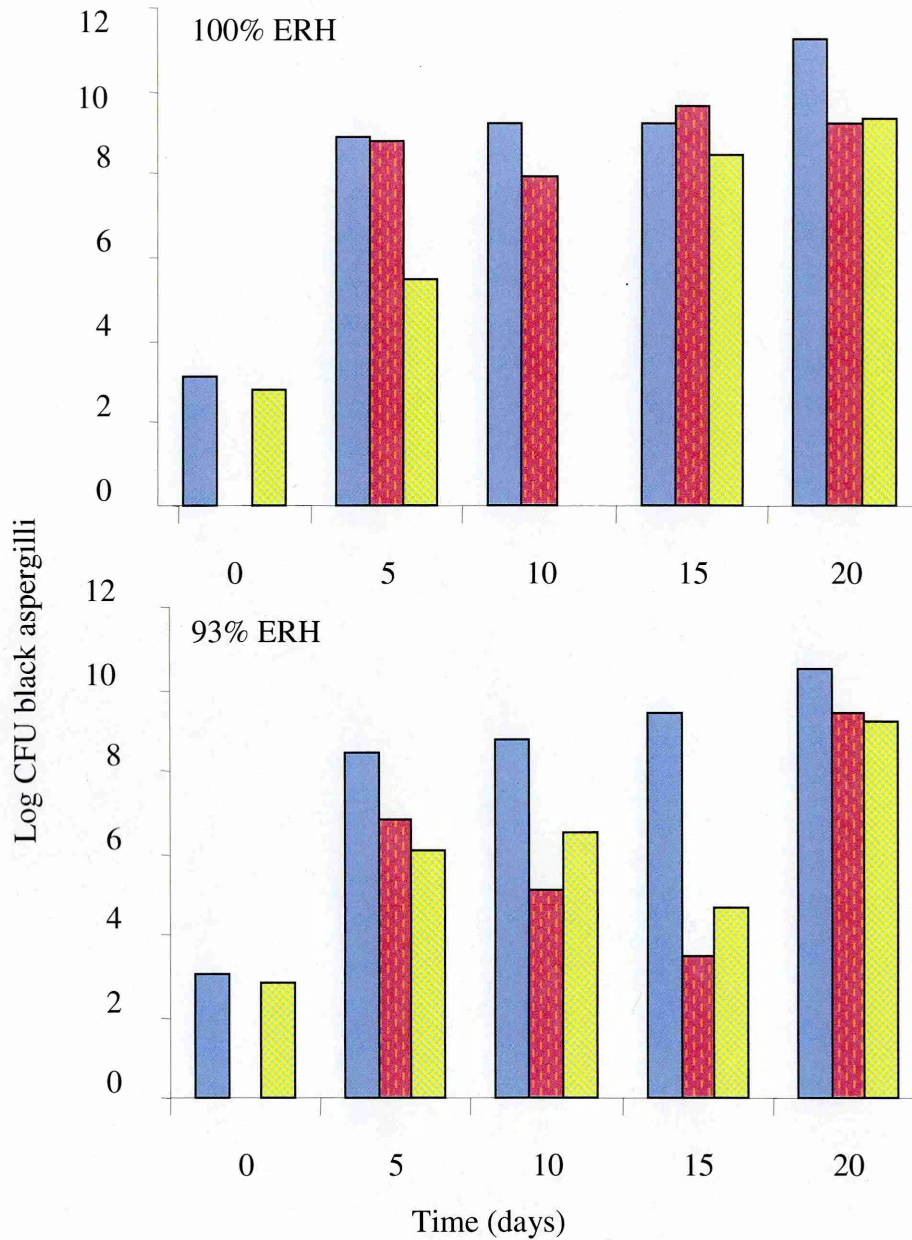


Figure 3.45 Colony forming units of black *aspergilli* from organic grapes dipped in *Aspergillus carbonarius* (1102) Spores 1×10^3 Spores 1×10^8 and no spores, after 0-20 day at 30°C at 93% or 100% ERH.

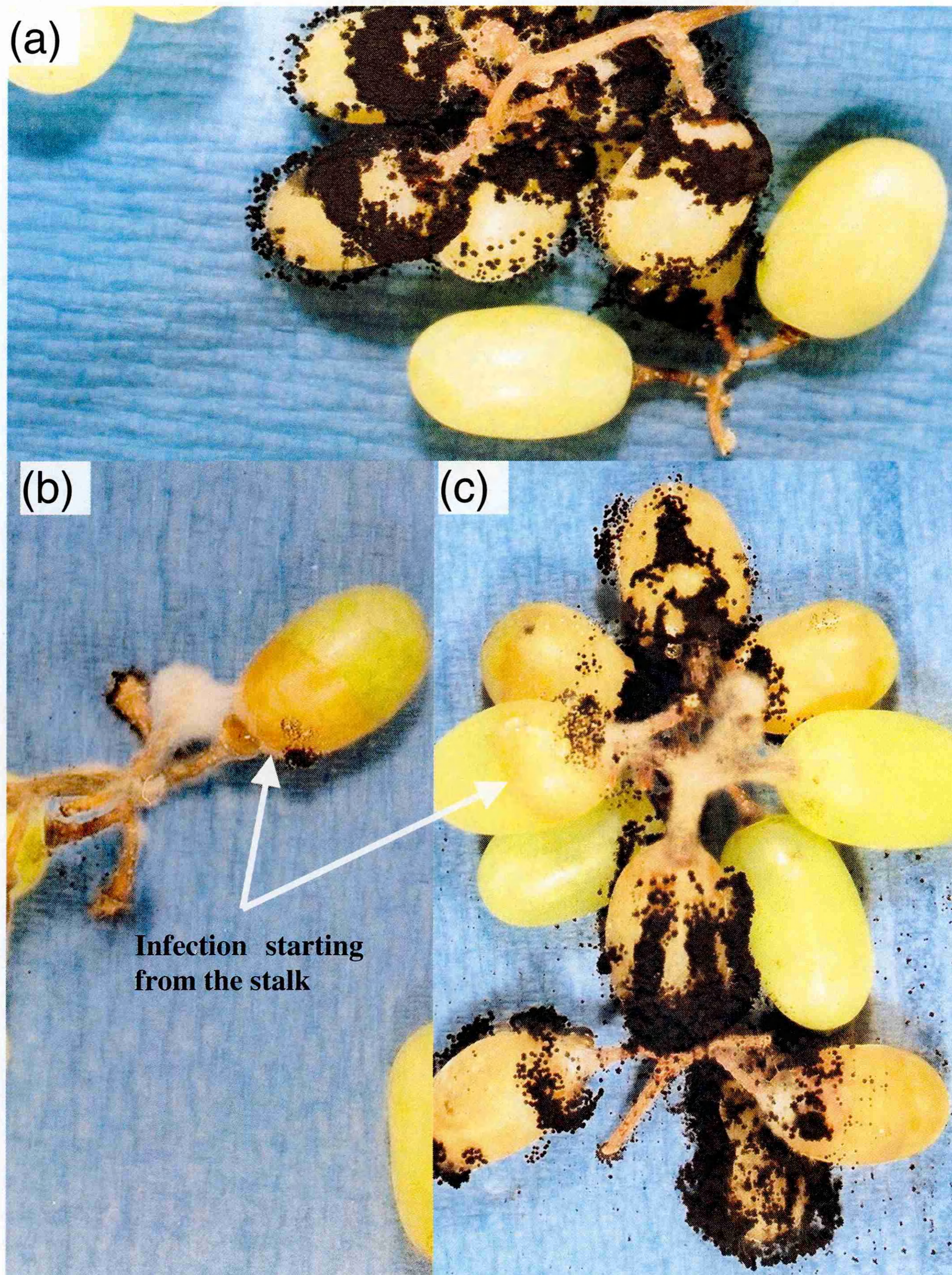


Figure 3.46 Black *aspergilli* infecting grapes (a,c) healthy grapes in contact with infected grapes, (b,c) infection starting from the pedestal then breaking through the grape skin. Incubated in controlled chambers at 30° for 15 days at 100% ERH.

CHAPTER 4
DISCUSSION

4.1 EFFECTS OF ENVIRONMENT CONDITIONS ON GROWTH OF *ASPERGILLUS CARBONARIUS* AND *ASPERGILLUS* SECTION *NIGRI* GROUP

Studies on the impact of environmental factors on the different growth phases of *A. carbonarius* are important as they provide a good indication of the speed at which colonisation and establishment could occur on relevant nutrient substrates. Grape products are a very rich medium and can be colonised by wide variety of microorganisms. It is thus an advantage for a toxigenic species to grow rapidly to exclude competitors from the grape niche (Magan & Lacey, 1984b; Marin *et al.*, 1998e). This study has provided detailed profiles on germination and growth in relation to various temperatures, water activities, pH levels and different gas compositions of strains of *A. carbonarius* and *A.* section *Nigri* group for the first time. Many of the individual and interacting factors were also found to be statistically significant.

4.1.1 Effects on germination and germ tube extension

This study has examined, for the first time, the detailed effect of temperature × 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°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.* (1998a) showed that isolates from grain germinated at a minimum of 0.82-0.83 a_w at optimum temperatures with mycelial growth occurring over the range 0.83-0.995 a_w with optimum conditions at 0.995 a_w/35°C. Temperature ranges were 10-40°C at optimum a_w conditions.

In this study the ability of conidia to germinate on grape flesh and to a lesser extent on SGM at 40°C would suggest the capacity for effective establishment, even at very high temperatures over a wide water availability range ($a_w > 0.90$). However, between 20-35°C at $a_w > 0.90$ on SGM and grape flesh measurements of germ tube extensions after 24 hours were impossible. The germ tube extension was extensive and had started to form branching hyphae. Under optimum conditions on grape flesh, conidiophores and young conidia were being produced rapidly.

In contrast, germination on grape skin occurred more slowly than on grape flesh or *in vitro* on agar media. After 12-18 hours germination appeared to remain constant or was reduced. The most probable reason for this is that the organic grapes had a natural wax coating on the skin to prevent water loss and provides protection from fungal colonisation. It is thus probable that under optimum conditions germination on grape skin initially occurred but with no nutrients available, after exhausting endogenous nutrients the germ tubes probably died. If the cells were dead, the stain would not easily be adsorbed into the cell making it much harder to observe and measure. The wax coating would also account for observations in the population

studies under different modified atmosphere storage conditions. Healthy grapes would be in contact with infected grapes for >7 days with out any visible sign of infection. Infection was most common around the stork of the grape and then spreading through the inside of the grape before erupting through the skin. Bellí (2006) also found grapes that were damaged had a greater infection rate than non-damaged grapes.

4.1.2 Effects of a_w and temperature on growth rates

This study compared the impact of $a_w \times$ temperature regimes on growth of strains of *A. carbonarius* and *A.* section *Nigri* from different countries in Europe and from Israel for the first time. Both factors affected the lag phase prior to growth and mycelial extension of the strains. While the pattern of effects of environmental factors was similar, the growth rates of strains varied significantly. Generally, growth was reduced by up to 50% at 0.93 a_w. Growth was also to a large extent inhibited in *A. carbonarius* at 0.88 a_w for most strains even after 56 days incubation. There was some variation in the growth rates between countries, and also within countries. 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*.

These results are very different from those obtained previously for strains of the *A. niger* group prior to the taxonomic differentiation of the species in the section *Nigri* and the production of OTA by species. Studies of *A. niger* by Ayerst (1969) and Marin *et al.* (1998a) isolated from spoiled grain showed wider a_w ranges of 0.83–0.995 with optimum growth at 0.995 a_w and 35°C. Temperature ranges were found to

be 10–40°C at optimum water availability. OTA producing strains of *A. ochraceus* were found to grow optimally at 0.995 and 30°C with similar a_w limits of about 0.83–0.85 a_w and a temperature range of 10–35°C. The other important OTA producer is *P. verrucosum*, which is common in temperate climatic conditions and grows over the range 5-35°C (optimally at 25°C), and the a_w range of 0.995 – 0.83 (optimum of 0.95 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 different 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 4.0 and 7.0 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. There has been very little work on the growth for *A. carbonarius* in relation to pH (Magan & Lacey, 1984a; Muhlencoert *et al.*, 2004; Esteban *et al.*, 2005). The slow growth rates found at pH 2.7 on SGM are interesting when compared to germination on grape flesh where conidia were produced in *ca* 30 hrs. SGM is based on grapes at early veraison whereas the grape flesh was from ripe table grapes. The difference in pH was 0.5 but the difference in available nutrients would probably be significant, and be much higher in the ripe table grapes. It is also possible that *A. carbonarius* was able to change the hydrogen ions modifying the ecological niche (grape tissue) to a pH more suitable for growth (Deacon, 2005).

4.2 EFFECTS OF ENVIRONMENT CONDITIONS ON OTA PRODUCTION BY *ASPERGILLUS CARBONARIUS* AND *ASPERGILLUS* SECTION *NIGRI* GROUP

4.2.1 Effects of a_w and temperature on growth rates

In the present study, the production of OTA over a range of interacting environmental conditions provides important information as it could assist in predicting the possible risk of OTA production and in choosing appropriate harvesting dates to minimize entrance into wine and vine fruit production.

This study confirms that optimum environmental conditions for OTA production by *A. carbonarius* strains are very different from the optimum for germination and growth. Thus optimum OTA production was at 15-20°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 on SGM is produced after 5 days (Bellí *et al.*, 2004d), after 7-14 days (Mitchell *et al.*, 2003) and after 14 days but this was on unmodified yeast extract sucrose agar and Czapek yeast (Cabañes *et al.*, 2002) although some was detected after 7 days. There is also no mention of whether the inoculation was by spread plating, triple or single point inoculation which would have lead to different dynamics on the plate especially in the first few days. The amount of OTA produced varied markedly between strains from different countries. For example, isolates from Spain produced 4-5 $\mu g\ g^{-1}$, while others produced $< 1\ \mu g\ g^{-1}$ medium (Italy, Portugal) at optimum a_w conditions.

Studies of *A. carbonarius* on irradiated coffee berries by Joosten *et al.* (2001) found that most OTA production was at 25, 27 and 30°C and 0.99 a_w, with production strongly reduced at 0.94 a_w after 14 days incubation. However, only a few environmental conditions were tested, and the nutritional status of coffee berries is very different from grape-based media or grapes.

The surface response curves produced in the present study on growth and OTA production may provide very useful guidelines for facilitating effective management of predicting risk from growth and OTA production during ripening and harvesting of grapes and during transport for wine production. Over long incubation periods it has been observed that some decrease in OTA production can occur under some treatment conditions (Mitchell *et al.*, 2003; Bellí *et al.*, 2004d; Mitchell *et al.*, 2004). Previous studies have suggested that *A. carbonarius* itself, or other black Aspergilli could degrade OTA in media resulting in the formation of breakdown products such as ochratoxin alpha (Abrunhosa *et al.*, 2002; Varga *et al.*, 2002). Other inputs into the system such as fungicides and pesticides may further stress these fungi influencing both growth and more importantly, OTA production.

OTA production by *P. verrucosum* occurs over a narrower range than that for growth with a minimum a_w of 0.82-0.83 and optimum at 0.91 and about 16-17°C (Sanchis & 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 these limits and ranges for growth are very different from those for *A. carbonarius*.

Although it was found that higher concentrations of OTA were produced at pH 7.0 than 4.2, the neutral pH is not a condition which normally exists in grape juice, wine or grape products. The pH of vine fruits is about 3.5. The higher production of OTA at pH 2.8 and 7.0 may reflect marginal environmental conditions for growth and this stress could explain the higher production at these pH levels than at 4.2.

The most important findings are that the optimum conditions for OTA production are generally very different from those for growth. This study has demonstrated the contrasting impact of both a_w and temperature on growth and OTA production using a wide range of strains from different climatic regions. Marked differences have been found for eight strains of *A. carbonarius* examined for growth and OTA production even though strains were from the same country. This shows the importance of building a database to determining the geographical regions with the environmental conditions which represent a risk from OTA contamination.

4.3 PARTITIONING OF OTA IN SPORES, BIOMASS AND MEDIUM

This study has shown for the first time that partitioning of OTA into spores is significantly higher than that secreted into the medium by isolates of *A. carbonarius*. This is different from other OTA producing species such as *A. ochraceus*. Thus OTA is probably present in the outer layer of the spores as demonstrated by Stormer *et al.* (1998) for citrinin.

Other studies have demonstrated the presence of mycotoxins in fungal conidia (Stormer *et al.*, 1998; Fischer *et al.*, 2000; Skaug *et al.*, 2001) Conidia collected from cultures of *P. verrucosum* and *A. ochraceus* contained 0.4–0.7 and 0.02–0.06 pg OTA per conidium, respectively (Skaug *et al.*, 2001) Moreover, a recent study by Fischer *et al.* (2000) has proven that approximately 60% of the mycotoxins found in culture extracts of 205 *Penicillium* and *Aspergillus* species were also found in the conidial extracts, indicating that a series of metabolites and mycotoxins are not only excreted by the fungi into the substrate, but also can be expected to be attached to or present in the conidia.

As already shown conidia can be formed in *ca* 30 hrs, so the presence of spores as contaminants on the grape surface and in must could be an important source of OTA contamination of products as well as increase the possibility of inhalation of the conidia and any associated health problems that this may cause.

4.4 EFFECTS OF FUNGICIDES AND OTHER CONTROL AGENTS

Aspergillus carbonarius has not been considered a major problem on grapes until the last 5 years although it was known to cause grape rot in India (Gupta, 1956). In the literature *A. carbonarius* has seldom been identified as a major cause of crop loss. Only in the last few years has its importance grown with the connection between contamination of wine with OTA (Zimmerli & Dick, 1996; Heenan *et al.*, 1998; Cabanes *et al.*, 2002). Fungicides for the control of other food-spoilage mycotoxigenic fungi have been studied, e.g. *Fusarium* spp. (Moss & Frank, 1985;

Mathies & Buchenauer, 1996; Magan *et al.*, 2003), *A. flavus* and *A. ochraceus* (Munimbazi *et al., 1997)*, and *B. cinerea* (Slawecki *et al., 2002).* The only other data on fungicides and *A. carbonarius* in relation to grapes is that by Bellí (2006) and like the present study, the fungicides were not designed specifically for *A. carbonarius.* However Belli (2006) did not examine the efficacy of fungicides in relation to a_w, only temperature.

Studies on the effect on copper sulphate showed some variations from the control levels with some stimulation in OTA but it had no significant effect on controlling growth. Bellí (2006) studied copper and sulfur individually, and showed an increase in growth and OTA in the presence of sulfur and a reduction in OTA with copper respectively. This variation emphasizes the need to carry out studies on a range of *A. carbonarius* strains from several regions to ensure that useful information can be obtained which includes that on strain variations.

The present study found that Trifloxystrobin was effective in inhibiting OTA production at levels less than the recommended rate. This fungicide is claimed to act by preventing spore germination (*Sherald et al.*, 1993) but was not previously examined in relation to *A. carbonarius*, where no inhibition of germination was observed. Bellí (2006) also found no inhibition of growth by this fungicide but did find suppression of OTA at the manufacturers recommended levels, but these studies were only carried out on unmodified media (0.99 a_w).

Carbendazim was effective at inhibiting growth at concentrations >2.5 mg l^{-1}, which is less than the recommended rate by the manufacturers. Bellí (2006) found total inhibition at manufactures recommended levels. The concern with using this

fungicide for control of *A. carbonarius* is that at lower concentrations OTA production was stimulated. Wine grapes are tightly bunched and the fungicide may not penetrate the center therefore if the application is not applied correctly there is thus a real possibility of greater OTA contamination.

Switch (cyprodinil and fludioxonil) was the most effective fungicide at inhibition growth of strains of *A. carbonarius* over a range of a_w levels at the recommended application rates in the present study. At 25% of the recommended rate some growth was observed. Similarly, Bellí (2006) found fungicides with the active ingredient cyprodinil were the best inhibitors of growth. However, Switch was also one of the fungicides that resulted in the largest stimulation in OTA (up to 8.13 $\mu g\ g^{-1}$), especially when applied at suboptimal inhibitory concentrations. Ochratoxin A stimulation occurred at the marginal environmental conditions for growth. This suggests again that application of the fungicide needs to be made by ensuring an even dose across the entire canopy.

4.4.1 Other control agents

Resveratrol is known as a grape vine secondary metabolite and thought to be associated with a response to injury or due to fungal attack (Fremont, 2000). There is interest in using resveratrol as a natural control agent, an alternative too many fungicides, as well as having health benefits. It is known to have antioxidant and anti-microbial properties (Pinto *et al.*, 1999; Fremont, 2000). Resveratrol has been used to prolonged shelf-life of grapes and soft fruits due to its fungicidal properties (Jimenez *et al.*, 2005). The effects on *A. carbonarius* were limited, with no real difference noted between treatments and control. This is contrary to what was found by Cairns-Fuller (2004) that resveratrol (200-500 $\mu g\ g^{-1}$) was the most effective antifungal for

controlling OTA production by *A. ochraceus* and *P. verrucosum* in stored grain. The lack of efficacy against *A. carbonarius* could be due to its natural prevalence in grape skin.

Catechin is also a naturally occurring product found in grapes but better associated with green tea. It is also is linked with antioxidants (Meyer *et al.*, 1998). Below 100 mg kg^{-1} catechin had no effect on growth or OTA production. At 150 mg kg^{-1} and all three temperatures and a_w levels, a stimulation in OTA was found. There is no other literature on efficacy of this compound against mycotoxigenic fungi in the literature. At 30°C the trend would appear to support a stimulation in production, but at other temperatures the trend was not so clear. More work would be needed before any conclusions could be reached.

4.5 COMPETITIVENESS AND THE EFFECTS OF COMPETING MYCOFLORA ON GROWTH AND OCHRATOXIN A PRODUCTION BY *ASPERGILLUS CARBONARIUS*

Filamentous fungi, yeasts and bacteria are all likely to be present on the surface of grapes, each species competing for nutritional resources. Thus interactions between fungal species are inevitable, and this has been shown in previous studies (Marin *et al.*, 1998d; Lee & Magan, 1999b, 2000; Edwards *et al.*, 2001; Marin *et al.*, 2001) Interspecific interactions have also been shown to be affected by environmental factors such as temperature, a_w and nutrient status. Thus it is important to understand

how *A. carbonarius* interacts with other fungal species and whether any stimulation or suppression of growth and or OTA production occurs.

The production of aflatoxins and cyclopiazonic acid by *A. flavus* is thought to provide a competitive advantage and increase the chances of survival and colonisation of substrates (Gqaleni *et al.*, 1997). It is also believed that *Fusarium* spp. may use the same principle in the production of fumonisins in maize (Marin *et al.*, 1998b). This theory may also be extended to the production of OTA by *A. carbonarius.* Cooke & Whipps (1993) suggested that fungi use primary and secondary strategies for colonizing ecological niches. Fungi can thus be C-selected, S-selected, or ruderal (R-selected) or use merged secondary strategies (C-R, S-R, C-S, C-S-R). These aspects must be considered in fungal competitiveness, in terms of both primary resource capture and secondary combat. High spore production, fast germination and growth rates as well as enzyme production can lead to the capturing of resources. Combat is the ability of the species to maintain hold on a resource, preventing other fungi from dominating it or dominating other fungi attempting to capture an already colonised resource.

Cuero, *et al.* (1987) showed that aflatoxin production by *A. flavus* was increased by *Bacillus amyloliquefaciens* and *Hyphopichia burtonii* when grown on irradiated maize and rice under certain environmental conditions. *Fusarium* spp. have also been shown to stimulate in fumonisin production by interaction with *A. ochraceus* particularly at 0.98 a_w (Marin *et al.*, 1998c). Stimulation could have been due to *A. ochraceus* attempting to maintain occupation of nutrient areas and prevention of competitors from occupying the niche (Lee & Magan, 1999b).

This study is the first to look at interactions of *A. carbonarius* and other grape fungi on SGM. The effects of a_w and temperature on the possible dominance of one species over another and the effect on OTA production, has been examined. In this study *A. carbonarius* at the highest temperature tested (30°C) dominated all non-Aspergilli species except *E. nigrum*. *Aspergillus carbonarius* has a higher optimum temperature than all of the species it was able to dominate. There was also an increase in speed of growth and in many cases *A. carbonarius* grew over competitors at 0.99 and 0.95 a_w, especially the yeast and *A. pullulans*. *Aspergillus carbonarius* clearly had a competitive advantage and prevented its competitors from becoming established. This was supported by the Index of Dominance scores.

At the lower temperatures tested (20-25°C) *A. carbonarius* growth conditions were not optimum and sometimes competitiveness was lost, except against the yeasts. Most of the species were thus mutually antagonistic on contact. Both *E. nigrum* and *A.* section *Nigri* uniseriate showed signs of being antagonistic at a distance. These two species have a wide optimum growth range and in the case of *E. nigrum* a higher optimal a_w as well (Magan & Lacey, 1984a).

4.5.1 Effects of interactions on OTA production

With nine different competitive fungi and nine different environmental conditions the results were mixed, with both stimulation and suppression of OTA production being observed. At 30°C, 0.99 a_w, OTA production was decreased when *A. carbonarius* was grown in the presence of all competitive fungi except *A. pullulan, B. cinerea, A. alternaria* and the pink yeast. It was these four competitive fungi that stimulated growth of *A. carbonarius* when grown against each other.

At 20°C 0.99 a_w there was stimulation of OTA production by *A. carbonarius* when grown in the presence all species except the pink yeast, and *A.* section *Nigri* uniseriate. The opposite occurred at 20°C, 0.95 a_w where OTA production by *A. carbonarius* was suppressed by interaction with all species except the *Cladosporium* spp. There was no effect when grown with *A.* section *Nigri uniseriate.* At 30°C, *A. carbonarius* has most competitive. The question arises as to whether OTA is produced as a defence mechanism. When *A. carbonarius* was grown at 20°C and 0.99 a_w, neither of these condition were optimum for growth, and therefore when it encounters a competitor it may produce OTA as a defensive measure to maintain occupation of the niche. While 0.95 a_w is optimum for growth of *A. carbonarius,* it was more stressful for the competitive fungi (Magan & Lacey, 1984a; Pitt & Hocking, 1997) and a reduction in OTA production occurred. A stimulation in OTA occurred at 0.95 a_w when *A. carbonarius* was in competition with *cladosporium* and *A.* section *Nigri* uniseriate,

4.5.2 Efficacy of $Na_2S_2O_2$ for control of germination, growth and OTA production

This study examined the efficacy of $Na_2S_2O_2$ on control of germination, growth and OTA production by strains of *A. carbonarius*. Generally, spore germination occurred over a wide range of $Na_2S_2O_2$ concentrations, although germ tube extension was significantly controlled. For complete inhibition of mycelial growth at least 750-1000 mg kg^{-1} was required, regardless of the a_w level used. The OTA production appeared to be inhibited by up to 750 mg kg^{-1}. At 0.95 a_w the concentration required for inhibition was lower (500 mg kg^{-1}). However, at lower $Na_2S_2O_2$ concentrations (100,

250 mg kg^{-1}) *A. carbonarius* growth was stimulated This is similar results for strains of *A. carbonarius* from vine fruits (Dekanea, 2005).

Previously, studies (Magan, 1993b; Magan, 1993a) examined the effect of different concentration of SO_2 in solution on the growth of some *Penicillium* and *Aspergillus* spp. The growth of *Aspergillus* species (*A. flavus*, *A. ochraceus*, *A. terreus*) were inhibited by 50 mg kg^{-1} dissolved SO_2 on a malt extract-based medium. Some *Penicillium* species and *Aspergillus niger* were tolerant of up to 250 mg kg^{-1}. In contrast, growth of *Penicillium* spp. was stimulated by 100 mg kg^{-1}. Moreover, phyllosphere fungi such as *Cladosporium herbarum* and *E. nigrum* were tolerant of up to 200 mg kg^{-1} while *A pullulans* and *B. cinerea* were inhibited by this level.

Generally, the information collected from previous studies suggest that the threshold SO_2 concentration may vary considerably between fungi. Those differences could be due to the fact that several factors influence the efficacy of SO_2. Particularly, the tolerance of *Penicillium* species to high concentrations of SO_2 has been suggested to be due to their ability to actively transport the SO_2 into the mycelia (Tweedie & Segel, 1970). Furthermore, King *et al.* (1981) showed that SO_2 binding substances enabled yeasts to be tolerant to higher concentration of SO_2. In the present study it is possible that a percentage of SO_2 was absorbed and bound to the glucose substance reducing its antifungal activity.

Furthermore, the relationship between the concentration of available SO_2 and effects on fungi is influenced by formulation of the product and critically by pH. The medium pH in all studies was set at 4.2 by using a buffer. The solubility products of SO_2 in

water vary with the different pH level (Babich & Stotzky, 1974). The significant point is that the toxicity of these products also differs, with greatest efficacy of the undissociated sulphurous acid (H_2SO_3) > bisulphite (HSO_3^-) > sulphite (SO_3^{-2}). In the present study it was mainly in the bisulphite form, which has moderate toxicity of the solubility products, which would have affected *A. carbonarius*.

It is important to point out that the total amount of $Na_2S_2O_2$ added is different from the final amount of SO_2 generated into the substrate. Thus, the comparison between studies where different forms of SO_2 are used, at different pH levels and with various substrates and contact times is difficult and requires careful comparison and interpretation.

4.5.3 Efficacy of elevated CO_2 exposure and water activity on control of growth phases and OTA production

Brown (1922) suggested that the concentration of CO_2 and O_2 required to inhibit spore germination is influenced by nutrient status and spore concentration. The germination of *B. cinerea* spores was inhibited in 1% O_2 without nutrient but 20% of spores germinated in presence of some nutrient. Moreover, depending on the nutrient and spore concentration, 10-20% CO_2 was necessary for inhibition of spore germination.

The present study has demonstrated that the mycelial extension phase was influenced by exposure to elevated CO_2 concentrations used, at least under the exposure periods used. Specifically, 25% CO_2 reduced mycelial extension by about 1/3 while 50% CO_2,

regardless of the a_w level, completely inhibited growth after 5 days incubation. However, after 10 days some growth had occurred.

Information is limited on the effects of CO_2 on growth and OTA production by *A. carbonarius* (Dekanea, 2005). However, information does exist for some other species. Recently, Cairns-Fuller *et al.* (2005) demonstrate that the growth and OTA production by *P. verrucosum* were higher in air, followed by 25 and 50% CO_2, regardless of the a_w level tested. Additionally, they demonstrated that significant inhibition of growth and OTA production occurred with 50% CO_2 and 0.90-0.995 a_w.

In the present study and in terms of OTA production the results were quite different when compared to those for growth. Water activity played a major role in decreasing OTA production. A a_w level of 0.93 significantly reduced OTA production regardless of the CO_2 concentration. It is worthwhile noting that 50% CO_2 in combination with 0.97 a_w also reduced OTA these are similar to Dekanea (2005).

There are a number of studies in the literature that point to the crucial role of a_w in influencing mycotoxin production (Sanchis & Magan, 2004). Recent studies have suggested that *P. verrucosum* growth can certainly occur under some conditions at 0.80 a_w but OTA production was probably limited to about 0.83 a_w on wheat grain (Lindblad *et al.*, 2004). They also suggested that 1000 CFUs g^{-1} grain could be correlated with a threshold at which OTA would be at the legislative level. Anything higher than this would represent a high risk. This type of correlation has not been done for grapes for wine or during the production of vine fruits and needs

investigation to identify the level of risk and develop appropriate prevention strategies.

Studies have been carried out on the effect of CO_2 on other mycotoxigenic fungi. For example, studies by Paster *et al.* (1983) reported that OTA production by *A. ochraceus* was completely inhibited by >30% CO_2 on agar-based media after 14 days. Work with *F. sporotrichioides* showed that T-2 toxin production could be reduced by 80% with 50% CO_2/20% O_2, but growth was not affected by <60% CO_2 *in vitro* (Paster *et al.*, 1986; Paster & Menasherov, 1988). Contaminated maize stored at 26°C for 14 days at 22% moisture content in 60% CO_2/20% O_2 reduced T-2 production completely, with 40% CO_2/5% O_2 treatment resulting in only trace amounts. Production of zearalenone by *F. equiseti* was almost completely inhibited by >20%CO_2 with either 20 or 5% O_2 in grain (Paster *et al.*, 1991).

In the present study the O_2 level reaches about 3-5% at the same time as CO_2 was increased. In 21% O_2 *Eurotium* spp. grew in 50 and 79% CO_2, although not in 85% CO_3 and 3% O_2. Moreover, *A. alternata*, *Rhizopus stolonifer*, and *C. herbarum* grew in up to 45% CO_2, with O_2 being present. In contrast, the growth of some fungi can be stimulated by 4-20% CO_2 even in low O_2 atmospheres (Wells & Uota, 1970; Magan & Lacey, 1984c). Furthermore, Epstein *et al.* (1970) showed that both in air and in controlled atmospheres of 10% CO_2 and 1.8% O_2, aflatoxin was produced.

Diener and Davis (1977) made a systematic study of CO_2 and O_2 and how these gases affected aflatoxin production. When O_2 concentration was decreased from 21 to 15 % there was no effect on aflatoxin production and a marked inhibition occurred only

when the O_2 concentration was decreased < 5%. Moreover, aflatoxin production was decreased by 25% when the CO_2 elevated to 20% although it had no visible effect on growth and sporulation. The opposite happened in the present study where although 25% CO_2 reduced growth it caused only a limited reduction in OTA accumulation.

Previously studies have observed stimulation in terms of growth. For example, Stotzky and Goos (1965) showed that the *F. roseum* growth was stimulated by 10% CO_2 but was inhibited by 45-50% CO_2. Growth of *A. alternata* was stimulated at 5% CO_2 (in air) at 0.95 a_w and at 10% CO_2 at 0.90 a_w but not at all at 0.98 a_w. All these studies suggest that mycotoxigenic fungi have different responses to exposure to CO_2 in relation to growth and toxin production and factors such as water activity, temperature, nutrients and times of exposure all need to be considered in establishing effect control regimes.

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

4.6 FIELD WORK

Mould growth is strongly inhibited in ethanol and anaerobic activities during fermentation (Otteneder & Majerus, 2000). If growth is inhibited in the wine making process then the origin of the OTA and contamination should be in the field. Therefore emphasis on the prevention of OTA entering the human food chain was concentrated on the field and the identification and timing of when OTA is produced.

4.6.1 Grape microflora

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

4.6.2 Effects of sampling time and environmental conditions

Da Rosa *et al.* (2002) suggested that the diversity of grape mycoflora depends on grape variety, degree of berry maturity, physical damage, viticulture practices and climatic conditions. This study found a positive correlation between the number of black *aspergilli* isolated from grapes and temperature but not from variety.

It is known that optimum temperatures for growth of *A. niger* aggregate *in vitro* are between 30–37°C; meanwhile the optimum for *A. carbonarius* and uniseriate strains are 30°C (Mitchell *et al.*, 2003; Bellí *et al.*, 2004c). In addition, a_w has been demonstrated to have an effect on *in vitro* growth of strains of black *aspergilli*, with the highest levels (0.98–0.995 a_w), similar to that of grapes, being the optimum in most cases (Bellí *et al.*, 2004b). The highest number of black *aspergilli* were detected at harvest in all four regions and in both years. The same trend as was found in a similar trial carried out in 2001 (Bellí *et al.*, 2004b). Black aspergilli species are ubiquitous saprophytes found in soils around the world (Klich & Pitt, 1988).

Another factor that was significant in the sampling was time, notably more berries were infected just before harvest. This is when the berries are more susceptible to fungal infection as the sugar content increases and the berry texture softens (MAPA, 1998). All of this, together with the increasing temperatures in the month preceding harvest, sometimes > 30°C, could influence black *aspergilli* development. It has also been found that physiological stress due to drought, too much water and cold weather make vines more susceptible to fungal infection (Stander & Steyn, 2002; Battilani *et al.*, 2003a).

The general pattern of colonisation by fungal species of grapes was not significantly different in 2001, 2002 and 2003; thus results can be considered representative of the situation in the sampled areas. However, more black *aspergilli* were isolated in 2003 than in the 2 previous years, probably because 2003 was an extremely hot year in Spain. High temperatures could also explain the higher number of black *aspergilli* found in Costers del Segre in 2002 and 2003.

The percentages of uniseriate isolates, *A. niger* aggregate and *A. carbonarius* (21, 60 and 19%, respectively) found in a survey of Italian grapes in 1999–2000 (Battilani *et al.*, 2003b), were very similar to those found in the present study. High incidence of *A. niger* aggregate was also found by Da Rosa *et al.* (2002) in a mycofloral survey of wine grapes from Argentina and Brazil. Less *A. carbonarius* were found, but 25% of these were OTA producers (18–234 μg g^{-1}) on CYA.

4.6.3 OTA detection in must

OTA was not detected in any of the must samples analysed, and therefore no correlation between the incidence of OTA-producing strains in grapes and OTA in musts could be established from this study. A similar study in 2001 in the same area found, 15% of the musts contained low amounts of OTA: with five samples contained between 0.091– 0.293 ng ml^{-1} and one sample containing 0.813 ng ml^{-1} (Bellí *et al.*, 2004c). Results from other countries have found greater amounts for contaminated musts but also with variations between years. An Italian study into OTA content in grapes, found a correlation between temperature, rain and relative humidity as the main factors that influenced OTA production in grapes (Battilani & Pietri, 2002). Sage *et al.* (2002) also found a strong correlation between these factors, as eight of eleven must samples were contaminated with OTA (10–461 ng ml^{-1}) and a significant number of *A. carbonarius* strains were previously isolated from grapes.

4.6.4 Other possible sources of OTA contamination

With the variation in musts contaminated with OTA in comparison to OTA found in red wine or juice, there would appear to be another factor for OTA contamination in the final product. Otteneder & Majerus (2000) noted that fermentation would prevent

fungal growth but they also noted that red wines grapes are mashed than put aside for several days before fermenting or pressing for juice. There conditions are ideal for growth of *A. carbonarius.* These conditions would link in with other findings in this study that conidia can be formed in *ca* 30 hours under optimum conditions and the majority of OTA from *A. carbonarius* was found in the spores.

If the grapes are contaminated with *A. carbonarius* there is a possibility that the OTA found in wine is not from the field but as a result germination and growth of *A. carbonarius* of the time taken from harvesting the grape to the fermentation process prevents metabolic activity. No information is available to show at what point in the fermenting process *A. carbonarius* would be totally killed, but there is no reason to believe that growth would stop instantly when fermentation begins.

CHAPTER 5
CONCLUSIONS AND FURTHER WORK

5.1 CONCLUSIONS

The following conclusions can be drawn from the experimental work of this study:

1. This study has demonstrated that conidial germination and germ tube extension are more rapid on grape tissue than *in vitro* on agar or on grape skin. This suggests that damage or wounds could be a method of rapid establishment in grapes.
2. The profiles for growth and OTA production in relation to interactions between a_w and temperature have been determined for the first time for a range of strains for four different European countries.
3. Optimum condition for growth of *A. carbonarius* was identified 30°C and at an intermediate water activities (0.98 a_w) with no growth below 10°C and at < 0.88 a_w.
4. Two dimensional profiles constructed of *A. carbonarius* strains showed for the first time that optimal OTA production was markedly different from that for growth and were 0.95 a_w and 15-20°C.
5. Commonly used fungicides for grapes used had a variable efficacy against strains of *A. carbonarius*. At sub-optimal concentrations they could result in stimulation of growth and OTA production. This suggests that recommended rates pf application must be adhered to prevent a stimulation of OTA contamination of grapes.
6. Interspecific interactions showed that no common grape fungi could dominate *A. carbonarius* under a range of environmental factors. The Index of Dominance of the mycotoxigenic species was predominantly higher than other

competing fungi. Interactions with other species resulted in both inhibition and stimulation of OTA depending on the environmental conditions.

7. The effect of Sodium metabisulphite suggested that effective control could be achieved of OTA production with about 750 mg kg^{-1}. However, about 1000 mg kg^{-1} was required for effective control of growth.
8. Modified atmospheres of 50% CO_2 only partially inhibited germination and growth of *A. carbonarius*. OTA production was more significantly inhibited by changing of a_w, than by CO_2 concentration suggesting that > 50% CO_2 is needed for effective control.
9. *A. carbonarius* was the predominant OTA producing species isolated from vineyards with no *P. verrucosum* and only a few strains of *A. ochraceus*. No OTA was detected in must samples. Climate had a significant effect on black aspergilli populations.
10. The data accumulated in this research project will be utilised for the development of models which will use climatic data and detailed ecological data on *A. carbonarius* to help predict high and low risk regions and years for OTA contamination in grapes for wine making.

5.2 FURTHER WORK

Possible further work based on the findings of this study.

1 The effect on germination of common competitive grape fungi in the presence of OTA.

2 Quantity and density of spore production at different environmental conditions. With the majority of the OTA found in spores is the key factor to OTA levels,

the relationship between the quantity of spore in a colony rather than the amount of OTA in a spore.

3 Investigate OTA levels in grapes/must from the harvest to fermentation when metabolism stops in *A. carbonarius*.

4 Improved knowledge of the effects of fungicides and interaction on growth and OTA over a wider variation of *A. carbonarius* strains, to build up a database that can be used for risk prevention.

5 Molecular approaches for tracking *A. carbonarius* in the field and during production of vine fruits.

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APPENDIX

Appendix A

Buffer (Fisher UK)
2N KOH

Appendix B

SYNTHETIC GRAPE MEDIUM (all chemicals Fisher UK except †Sigma-Aldrich)
70 g D (+) glucose
30 g D (-) fructose
7 g L (-) tartaric acid
10 g L (-) malic acid
0.67 g $(NH_4)_2HPO_4$
0.67 g KH_2PO_4
1.5 g $MgSO_4$*7 H_2O
0.15 g NaCl
0.15 g $CaCl_2$
0.0015 g $CuCl_2$
0.021 g $FeSO_4$*7 H_2O
0.0075 g $ZnSO_4$*7 H_2O
0.05 g (+) catechin hydrate†
Water until 1000 ml.

Appendix C

DRBC (Dichloran Rose Bengal Chloramphenicol) (all chemicals Sigma-Aldrich Spain)
10 g glucose
5 g peptone
1 g KH_2PO_4
0.5 g $MgSO_4$*7H_2O
0.5 g rose bengal
1 ml dicholoran
1 ml chloramphenicol
15 g agar
1 lt of Reverse osmosis water

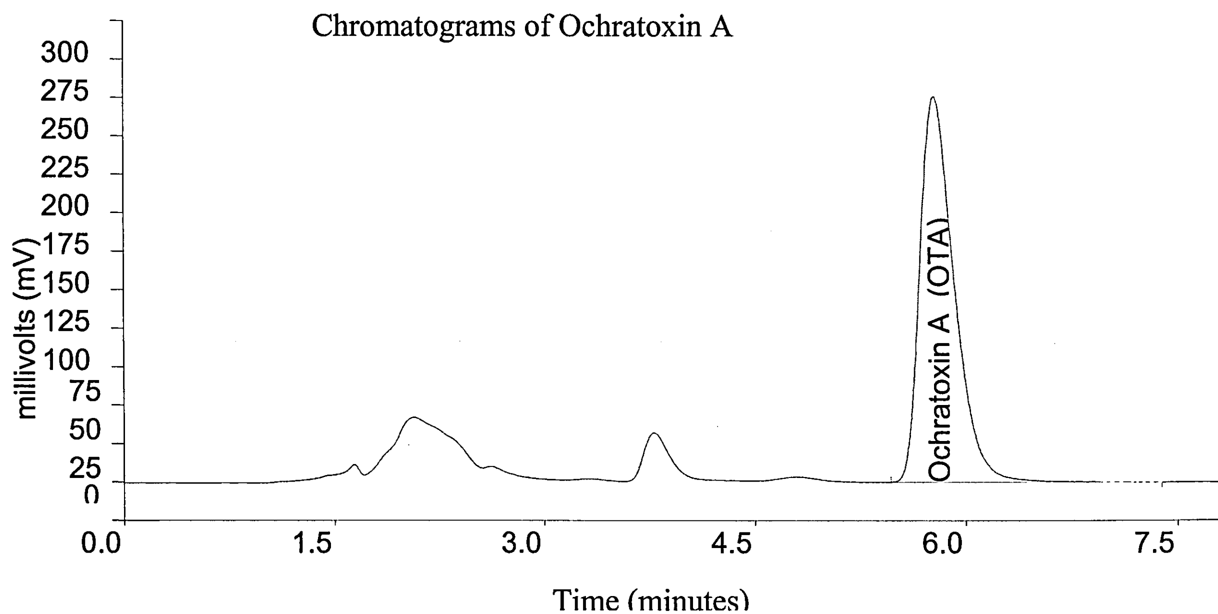
Appendix D

(Dallyn & Fox, 1980)

All amounts are for those to be added to 100 mls water

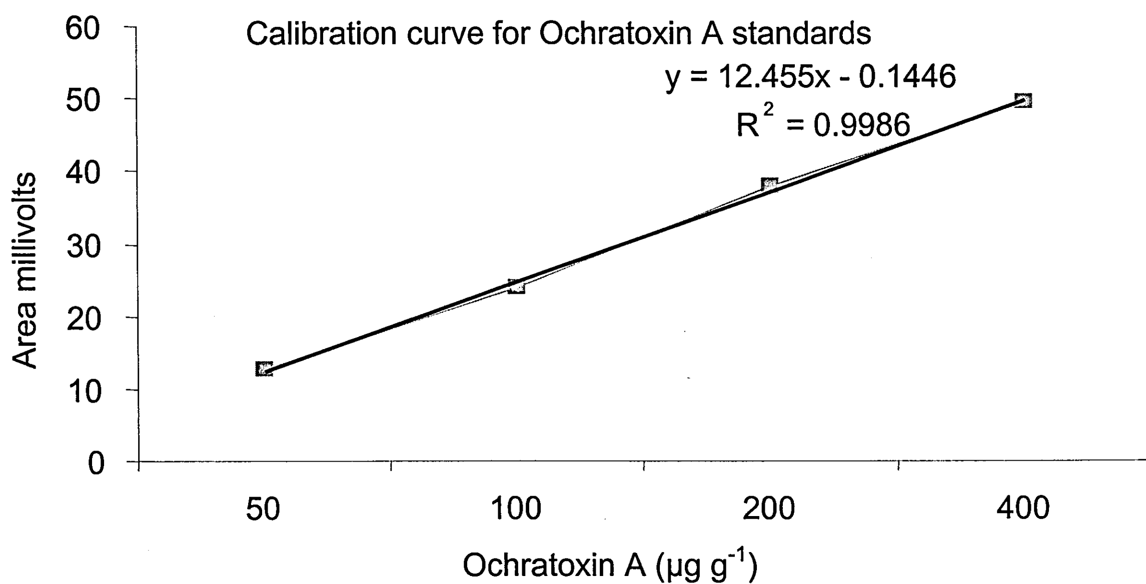
Water activity	g^{-1} glycerol
0.995	2.76
0.991	4.61
0.982	9.20
0.971	14.72
0.964	18.40
0.955	23.00
0.937	32.20
0.928	36.80
0.919	41.40
0.910	46.00
0.901	50.60
0.892	55.20
0.884	59.80
0.867	69.00
0.850	78.20
0.841	82.80
0.809	101.20
0.793	110.40
0.747	138.00
0.705	165.60

Appendix E Chromatograms with Ochratoxin A detection and integration repot showing ochratoxin A detection at 5.78 minutes and a standard calibration cuve of ochratoxin A standards



Intergration Report

Sample No.	Retention Time minutes	Name	Area mV*min	Amount %
1	5.78	OTA	70.8218	100.00



Appendix F

CZ (Czapek) (all chemicals Sigma-Aldrich Spain)
50 ml solution A
50 ml solution B
1 ml solution a
1 ml solution b
15 g agar
30 g sucrose
900 ml Reverse osmosis water
The above-mentioned solutions have to be considered as follow:
Solution A
40 g $NaNO_3$
10 g KCl

10 g $MgSO_4 \times 7\ H_2O$
0.2 g $FeSO_4 \times 7\ H_2O$
1 lt of Reverse osmosis water
Solution B
20 g K_2HPO_4
1 lt of Reverse osmosis water
Solution a
1 g $ZnSO_4 \times 7\ H_2O$
100 ml Reverse osmosis water
Solution b
0.5 g $CuSO_4 \times 7\ H_2O$
100 ml of Reverse osmosis water

Appendix G

CYA (CZAPEK YEAST AGAR) (all chemicals Sigma-Aldrich Spain)
Same as CZ with the addition of:
5 g yeast extract

Appendix H One way ANOVA of growth of two strains of *Aspergillus carbonarius* Italy MPVP A 1102 (1102) and Portugal 01UA s219 (219) at two temperature 20, 30°C and 0.95, 0.99 water activities, over a range of copper sulphate concentrations (0-50 g l^{-1}). * Indicates factors which was significantly different ($p<0.05$)

		1102				219			
Source	DF	SS	MS	F	P	SS	MS	F	P
Intercept	1.0	588.5	588.5	3694.8	<0.001*	397.1	397.1	5536.4	<0.001*
a_w	1.0	5.2	5.2	32.7	<0.001*	2.3	2.3	31.5	<0.001*
Temperature	1.0	16.6	16.6	104.3	<0.001*	2.8	2.8	39.6	<0.001*
Fungicide	2.0	0.3	0.2	1.0	0.367	0.1	0.1	1.0	0.390
a_w × Temperature	1.0	1.9	1.9	11.7	0.002	3.4	3.4	47.9	<0.001*
a_w × fungicide	2.0	0.1	0.1	0.4	0.649	0.1	0.0	0.5	0.643
Temperature × fungicide	2.0	0.0	0.0	0.1	0.866	0.1	0.1	0.8	0.449
a_w × Temperature × fungicide	2.0	0.0	0.0	0.1	0.865	0.3	0.2	2.2	0.129
Total	35.0	28.1				10.9			

Appendix I One way ANOVA of growth of two strains of *Aspergillus carbonarius* Italy MPVP A 1102 (1102) and Portugal 01UA s219 (219) at two temperature 20, 30°C and 0.95, 0.99 water activities, over a range of Trifloxystrobin concentrations (0 – 10 g l^{-1}). * Indicates factors which was significantly different (p<0.05).

			1102				219**		
Source	DF	SS	MS	F	P	SS	MS	F	P
Intercept	1	992.2	992.2	6817.8	<0.001*	1	450.8	450.8	<0.001*
a_w	1	17.1	17.1	117.8	<0.001*	1	0.7	0.7	0.051
Temperature	1	3.7	3.7	25.3	<0.001*	1	2.3	2.3	0.001*
Fungicide	5	3.1	0.6	4.3	<0.001*	3	0.6	0.2	0.334
a_w × Temperature	1	0.8	0.8	5.6	0.022*	1	4.1	4.1	<0.001*
a_w × fungicide	5	4.9	1.0	6.7	<0.001*	3	0.2	0.1	0.762
Temperature × fungicide	5	1.6	0.3	2.2	0.071	3	0.0	0.0	0.978
a_w × Temperature × fungicide	5	2.7	0.5	3.7	0.006*	3	0.2	0.1	0.704
Total	71	40.9				47	13.9		

** Portugal strain not grown at 0.0075-0.0010

Appendix J One way ANOVA of growth of two strains of *Aspergillus carbonarius* Italy MPVP A 1102 (1102) and Portugal 01UA s219 (219) at two temperature 20, 30°C and 0.95, 0.99 water activities, over a range of Carbendazim concentrations (0 – 2.5g l^{-1}). * Indicates factors which was significantly different ($p<0.05$).

		1102				219			
Source	DF	SS	MS	F	P	SS	MS	F	P
Intercept	1	163.6	163.6	1021.9	<0.001*	162.8	162.8	2723.8	<0.001*
a_w	1	0.0	0.0	0.0	0.921	5.0	5.0	83.3	<0.001*
Temperature	1	4.4	4.4	27.6	<0.001*	1.3	1.3	21.8	<0.001*
Fungicide	3	68.7	22.9	143.0	<0.001*	69.6	23.2	388.0	<0.001*
a_w × Temperature	1	1.3	1.3	8.4	0.007*	0.0	0.0	0.8	0.371
a_w × fungicide	3	0.1	0.0	0.2	0.890	3.1	1.0	17.3	<0.001*
Temperature × fungicide	3	2.1	0.7	4.3	0.011*	1.2	0.4	6.8	0.001*
a_w × Temperature × fungicide	3	0.5	0.2	1.1	0.346	4.4	1.5	24.6	<0.001*
Total	47	82.3				86.6			

Appendix K One way ANOVA of growth of *Aspergillus carbonarius* Italy MPVP A 1102 (1102) at two temperature 20, 30°C and 0.95, 0.99 water activities, over a range of Switch concentrations (0 – 2.5 g l^{-1}). * Indicates factors which was significantly different ($p<0.05$).

Source	DF	SS	MS	F	P
Intercept	1	168.5	168.5	3106.6	<0.001*
a_w	1	2.1	2.1	38.1	<0.001*
Temperature	1	9.9	9.9	182.3	<0.001*
Fungicide	2	19.1	9.6	176.2	<0.001*
a_w × Temperature	1	0.5	0.5	9.6	0.005*
a_w × fungicide	2	1.2	0.6	11.3	<0.001*
Temperature × fungicide	2	0.8	0.4	7.2	0.004*
a_w × Temperature × fungicide	2	2.2	1.1	20.3	<0.001*
Total	35	37.1			

Appendix L One way ANOVA of growth of *Aspergillus carbonarius* Italy MPVP A 1102 at two temperature 20, 30°C and 0.95, 0.99 water activities, over a range of Catacin concentrations (0 – 200 g l^{-1}). * Indicates factors which was significantly different ($p<0.05$).

Source	DF	SS	MS	F	P
Intercept	966.0	966.0	8502.1	0.000	966.0
a_w	311.7	155.9	1371.8	0.000	311.7
Temperature	3.4	3.4	30.3	0.000	3.4
Fungicide	0.3	0.1	0.8	0.555	0.3
a_w × Temperature	1.6	0.8	6.8	0.002	1.6
a_w × fungicide	0.6	0.1	0.6	0.748	0.6
Temperature × fungicide	0.2	0.0	0.4	0.841	0.2
a_w × Temperature × fungicide	0.1	0.0	0.2	0.996	0.1
Total	324.7				324.7

Appendix M One way ANOVA of growth of *Aspergillus carbonarius* Italy MPVP A 1102 at two temperature 20, 30°C and 0.95, 0.99 water activities, over a range of Resveratrol concentrations (0 – 200 g l^{-1}). * Indicates factors which was significantly different ($p<0.05$).

Source	DF	SS	MS	F	P
Intercept	1	1162.6	1162.6	10047.9	<0.001*
a_w	1	103.8	103.8	897.3	<0.001*
Temperature	1	5.8	5.8	50.0	<0.001*
Fungicide	4	0.7	0.2	1.4	0.248
a_w × Temperature	1	0.0	0.0	0.4	0.537
a_w × fungicide	4	0.1	0.0	0.3	0.888
Temperature × fungicide	4	0.6	0.1	1.2	0.312
a_w × Temperature × fungicide	4	0.1	0.0	0.2	0.942
Total	59	115.7			

Appendix N One way ANOVA of growth of *Aspergillus carbonarius* Italy MPVP A 1102 at three pH 2.6.-7.0 at 20 and 0.88-0.99 water activities. * Indicates factors which was significantly different ($p<0.05$).

Source	DF	SS	MS	F	P
Intercept	1	186.3	186.3	3584.6	<0.001*
a_w	4	21.8	5.5	105.1	<0.001*
pH	2	79.2	39.6	761.9	<0.001*
$a_w \times$ pH	8	14.9	1.9	35.9	<0.001*
Total	44	117.5259			

PUBLICATIONS

Refereed papers

Mitchell D., Aldred D., and Magan N. (2003) Impact of ecological factors on the growth and ochratoxin A production by *Aspergillus carbonarius* from different regions of Europe. *Aspects of Applied Biology,* **68,** 109–116.

Mitchell D., Parra R., Aldred D. (2004) Water and temperature relations of growth and ochratoxin A production by *Aspergillus carbonarius* strains from grapes in Europe and Israel *Journal of Applied Microbiology,* **97,** 439-445

Belli, N., Mitchell, D., Marin, S., Alegre, I., Ramos, A. J., Magan, N. & Sanchis, V. (2005) Ochratoxin A-producing fungi in Spanish wine grapes and their relationship with meteorological conditions. *European Journal of Plant Pathology,* **113,** 233-239.

Pateraki, M., Dekanea, A., Mitchell, D., Lydakis, D. & Magan, N. (2005). Ecology and control of ochratoxin in grapes and dried vine fruits. *BCPC Crop Science and Technology, 5B, 403-410.*

Pateraki, M, Dekanea, A., Mitchell, D., Lydakis, D. & Magan, N. (2006). Efficacy of sulphur dioxide, controlled atmospheres and water availability on in vitro germination, growth and ochratoxin A production by strains of *Aspergillus carbonarius* from grapes and vine fruits. *Postharvest Biology and Technology.* Submitted.

Atoui A., Mitchell D., Mathieu F., Magan M., & Lebrihi A. (2006) Partitioning of ochratoxin A in mycelium and conidia of *Aspergillus carbonarius* and the impact on toxin contamination of grapes and wine. *Applied and Environmental Microbiology. Submitted.*

Conference poster/abstract

Mitchell, D., Aldred, D. & Magan, N. (2004). Ecophysiology of germination, growth and ochratoxin production by strains of *Aspergillus carbonarius* from grapes. IUPAC Symposium on Mycotoxins and Phycotoxins. July, 2004. Washington DC, USA.

Pateraki M., Dekanea A., Mitchell D., Lydakis D., Magan N (2005) Potential for control of *Aspergillus. carbonarius* strains from vine fruits using sulphur dioxide or controlled atmosphere storage *International workshop ochratoxin A in grapes and wine: prevention and control program marsala Sicily, Italy, 20–21 October, 2005*

Pateraki M., Dekanea A., Mitchell D., Lydakis D., Magan N (2005) Abiotic factors determine germination, growth and ochratoxin A production by *Aspergillus carbonarius* strains from the Mediterranean basin *International workshop ochratoxin A in grapes and wine: prevention and control program marsala Sicily, Italy, 20–21 October, 2005*

Mitchell D., Bellí N., Marin S., Aldred D., Magan N., Sanchis V. (2004) Impact of ecological factors on growth and ochratoxin production by *Aspergillus carbonarius* from grapes *British Mycological Society, Annual Scientific Meeting: Fungi in the Environment, Nottingham, 13-15 September, 2004*

Aspects of Applied Biology 68, 2003
Mycotoxins in Food Production Systems

Impact of ecological factors on the growth and ochratoxin A production by *Aspergillus carbonarius* from different regions of Europe

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Summary

This study investigated the effects of water activity (a_w; 0.88-0.987) and temperature (10-35°C) on growth of strains of *Aspergillus carbonarius* isolated from wine grapes from four different European countries. The effect of these conditions on ochratoxin A (OTA) over a period of 56 days was also determined. Optimum growth was found to be 35°C for all strains tested. No growth occurred below 15°C. The optimum a_w varied from 0.93-0.987 depending on the strain, with the widest a_w tolerance at 25°C. There was no direct relationship between growth, environmental factors and country of origin of individual strains. OTA production was greater at 25°C than 20°C and was detectable after 7-14 days growth.

Key Words: Water activity, temperature, ochratoxin A, *Aspergillus carbonarius*, grapes, growth

Introduction

Ochrotoxin A (OTA) is a mycotoxin which has received increasing attention because of its potent nephrotoxic effects. OTA has been classified into group 2b, by IARC (1993), a possible human carcinogenic, and is known to be carcinogenic to rodents. It has also been found to have teratogenic, immunotoxic and possible genotoxic properties, and been implicated in the human disease Balkan Endemic Nephropathy and the development of urinary tract tumours in humans (Pohland *et al.,* 1992; Kuiper-Goodman, 1995; Creppy, 1999).

The occurrence of OTA in cereals has been well documented and for some time it was thought to be the major source of OTA in humans and animals. Over the last few years OTA has been found in other food commodities such as, coffee beans, cocoa, beans and pulses (Jõrgensen, 1998; Pohland *et al.,* 1992; Kuiper-Goodman & Scott, 1989). OTA has also been detected in wine, grape juice, beer and dried fruits (Majerus & Otteneder, 1996; Zimmerli & Dick, 1996). OTA was originally described by Van der Merwe *et al.,* (1965) as a metabolite of *Aspergillus ochraceus* (Wilhelm) but has since been found to be produced by other species such as *Penicillium verrucosum* (Dierckx) and *A. carbonarius* (Bainier) Thom.

A. carbonarius has been found to be responsible for OTA production in grapes and wine (Battilani & Pietri, 2002; Cabanes *et al.,* 2002). However, very little information is available on the ecology of this species. This is mainly due to the practice of grouping all black aspergilli into the *A. niger* group. Fungal growth and OTA production can be affected by different environmental factors, the two most important being water availability (water activity, a_w) and temperature (Scott, 1957). Temperature and a_w are the principle factors influencing germination, growth and sporulation of

spoilage fungi (Magan & Lacey, 1984). Changes in a_w and temperature also affect the growth and ability of species to compete with one another (Magan & Lacey, 1985a; 1985b), and can have an impact on OTA production (Ramakrishna *et al.,* 1996; Lee & Magan, 2000).

There has been much work carried out on the effects of a_w and temperature on fungal growth and OTA production in stored products. However, there has been very little work on a_w and temperature effects on members of the *A. niger* aggregate, and practically none on *A. carbonarius* either pre- or post-harvest. The objectives of this study were to determine (a) effect of a_w and temperature on growth of isolates of *A. carbonarius* from different countries, and (b) temporal and environmental effects on OTA production.

Materials and Methods

Fungal species

Nine *A. carbonarius* strains isolated from grapes in four different European countries were examined in this study (Table 1). Strains were kindly supplied by: Departamento Engenharia Biologica, Universidade do Minho, Portugal; Agricultural University of Athens, Faculty of Crop Sciences and Production, Greece; Department of Postharvest Science, ARO The Volcani Center, Israel; Catholic University Piacenza, Faculty of Agriculture, Italy

Table 1 *Strains of* Aspergillus carbonarius *used in the study with the country of origin and their ability to produce Ochratoxin A*

Origin	Code	OTA Producer
Italy	MPVP A 1102	Yes
	MPVP A 933	No
Portugal	01UAs294	No
	01UAs219	Yes
	01UAs263	Yes
Israel	1-4-1-9-10.8	Yes
	1-4-1-9-7.7	No
Greece	G 458	Yes
	G 444	No

Media and water activity modification

A synthetic grape juice medium (SGM) was prepared consisting 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 0.021, $ZnSO_4$*7 H_2O 0.0075g, (+) Catechin hydrate 0.05, agar 25g for 1litre of medium. This reflected a range of grape varieties and variations of sweetness within the growing season. This medium was adjusted with 2M KOH to pH 4.0 – 4.2.

Experiments were carried out at 0.987-0.88 by addition of glucose or glycerol. The a_w of the unmodified medium was 0.987 a_w and used as the control. The a_w was checked using an Aqualab®

WP4 (Decagon Devices, Inc Washington USA) connected to a PC using Hyperterminal software (Higraeve Inc Michigan USA).

Ecological studies on isolates of Aspergillus carbonarius

Growth rate studies were carried out under various ecological conditions using glucose/glycerol modified SGM between 0.88-0.987 a_w and at 10, 15, 25, and 35°C, for a period of up to 56 days. All treatments and replicates were inoculated centrally with a small loop of spore suspension (10^5 spore ml^{-1}). All plates were stored in a controlled environment to ensure no fluctuation in temperature and a_w occurred.

Temporal studies were carried out to determine optimal time of production of OTA on glucose/glycerol modified SGM between a_w 0.88-0.987 at 20 and 25°C. Sub-samples were removed at 7 day intervals up to 56 days, and frozen until analysis.

Statistical analyses of the data

Diametric growth rate was measured in two directions at right angles to each other. The radial extension rate was plotted against time and the growth rate was calculated using linear regression (mm day^{-1}) at each different a_w and temperature, for each replicate and treatment. ANOVA followed by either a LSD or Tukey B analysis was carried out using SPSS for windows (version 11.0.0 Chicago, Illinois, USA) to compare differences between glucose/glycerol substrate and growth rates between different strains.

Ochratoxin A extraction and analysis

The method used was adapted from Bragulat *et al.,* (2001). Six agar plugs were removed from each plate using a cork borer (4.5mm diameter) across the radius of each colony. The plugs were placed into a 2 ml microtube (Eppendorf, UK) and weighed. 1ml of HPLC grade methanol (Fisher, 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.

The HPLC system used consisted of a Millipore Waters 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 (57% acetonitrile , 41% water and 2% acetic acid) was 1 ml min^{-1} Analysis of the results was carried out on a computer with Kroma system 2000 software (Bio-tek Instruments, Milan, Italy).

Results

Optimum growing conditions

The effect of varying temperature and a_w considerably changed both the growth rate and also the lag phase prior to growth. Generally, lag phases increased with decreasing temperatures. At 35°C growth reached the exponential phase within 1-3 days at all a_w levels, whereas at 15°C this was not observed until 5 days after inoculation at the higher a_w levels. Under the same conditions at lower a_w levels this did not occur until at least 12+ days after inoculation. Fig. 1 shows the effect of

temperature, a_w and solute type on growth at different steady state a_w levels for a strain of *A. carbonarius* from Portugal (01UAs294). Maximum growth occurred at 35°C and 0.98 a_w with a notable decrease at 0.987 a_w. Between 0.95-0.98, the growth rate of most strains at 25°C was 25% less that at 35°C. Solute type had a small effect on growth. There were some differences but not a large variation, with the optimum growth range for both glucose/glycerol modified media being at 35°C and 0.98 a_w. Similar results were found for all strains on glucose/glycerol modified media. Statistically LSD there was no significant difference between growth rates on glucose/glycerol modified media at 15°C or 35°C but for a few strains there was a significant difference (P=0.05) at 25°C between 0.90-0.987 a_w. There were a large proportion of the strains that did not show growth at 0.88 a_w and were, therefore excluded from the statistical analysis. In an analysis of growth at 15°C there was a significant difference at all a_w treatments. At 25°C between 0.98-0.987 a_w there was no statistically significant difference, but there was at all other a_w levels. At 35°C and < 0.95 a_w there was a significant difference in growth.

The critical point at which growth will not occur, and the optimum growth rate varie depending on temperature and a_w for individual strains. No strain grew at 10°C whereas at 15°C growth was only possible at the higher a_w levels. Only 2 strains grew at <0.93 a_w. Between 0.93 and 0.95 a_w growth was reduced by about 20% of that at 0.98 a_w and 35°C. This was not the case at 15 and 25°C where growth was significantly reduced at <0.95 a_w. Some strains grew at a_w levels as low as 0.85. The a_w x temperature ranges for growth of all *A. carbonarius* strains from different European countries are summarised in Table 2. The strains from the four countries had a wide variety of growth patterns and optimum requirements (Fig 2). There were six significantly related groups of growth between the strains. Only the strains from Israel were statistically significantly similar, with all other strains from the same country having significantly different growth rates (Table 3).

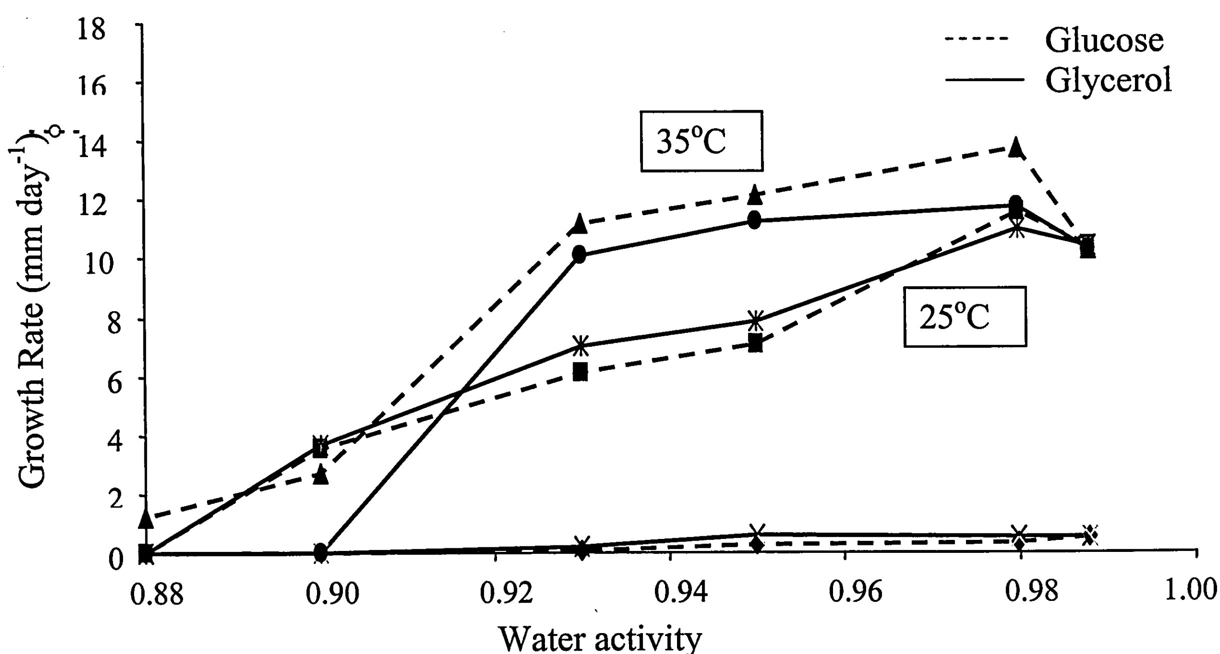

Fig. 1. compares growth of Aspregillus. carbonarius *(01UAs219) over a range of water activitys (0.88-0.987) and at temperatures (15, 25, 35°C)*

Effect of time and water activity on Ochratoxin A

A temporal study was carried out to find the maximum production time of OTA in relation to environmental factors. Examples of these results for *A. carbonarius* (01UAs219) are shown in Fig. 3. The maximum amount of OTA, when grown at 25°C, occurred between 7 and 21 days depending on a_w levels. At <0.90 a_w no OTA production was detected. For both species there were fluctuations in the levels of OTA production at different a_w levels and at different times. Generally more toxin was produced at higher a_w levels compared to lower a_w levels and at 25°C compared to the same conditions at 20°C

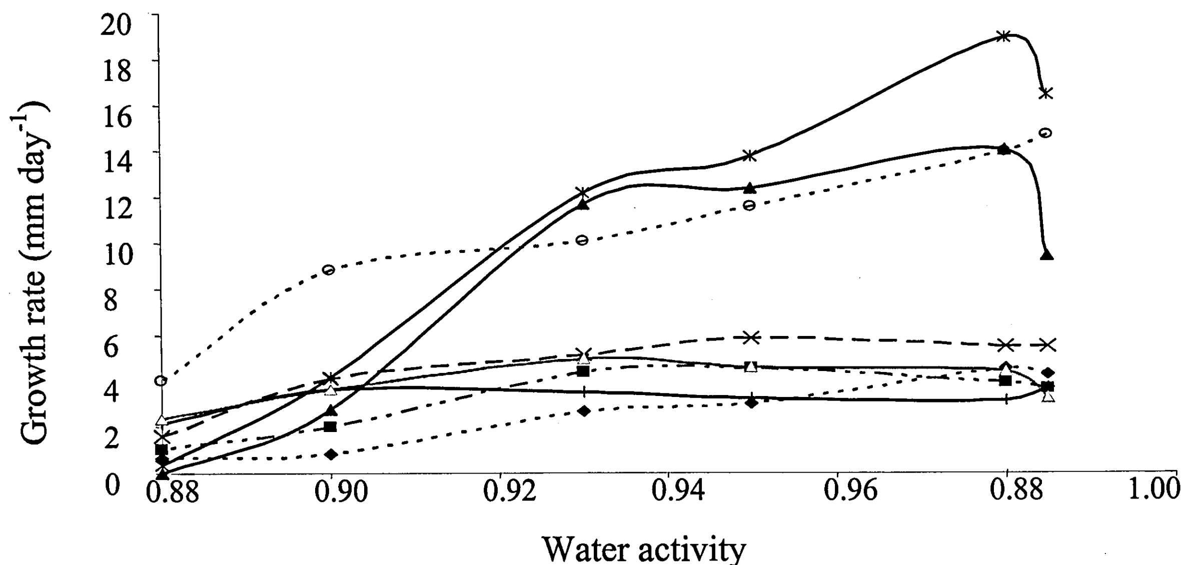

Fig. 2. Comparison of growth rates of strains of Aspergillus carbonarius *grown on glycerol modified media at 35°C at 0.88-0.987 a_w from different European countries at 35°C. Key to strains: MPVP A1102, MPVP A 933, 01UAs294, 01UAs219, 01UAs263, 1-4-1-9-7.7, G 458, G 444,*

Table 2 Temperature and water requirements of Aspergillus carbonarius *from 4 Europe countries*

		Temperature (°C)		Water activity for growth (a_w)		Maximum radial growth rate (mm day^{-1})
		Range	Optimum	Minimum	Optimum	
Portugal	01UAs294	15-35	25-35	0.90	0.93-0.98	11.8
	01UAs263	15-35	35	0.85	0.95-0.987	17.3
	01UAs219	15-35	35	0.88	0.98	11.5
Italy	MPVP A 933	15-35	25	0.88	0.93-0.987	5.2
	MPVP A1102	15-35	35	0.88	0.95-0.98	4.4
Israel	1-4-1-9-10.8	15-35	35	0.90	0.95	4.8
	1-4-1-9-7.7	15-35	35	0.90	0.90-0.98	4.0
Greece	G 444	15-35	35	0.88	0.90	5.9
	G 458	15-35	35	0.85	0.987	13.9

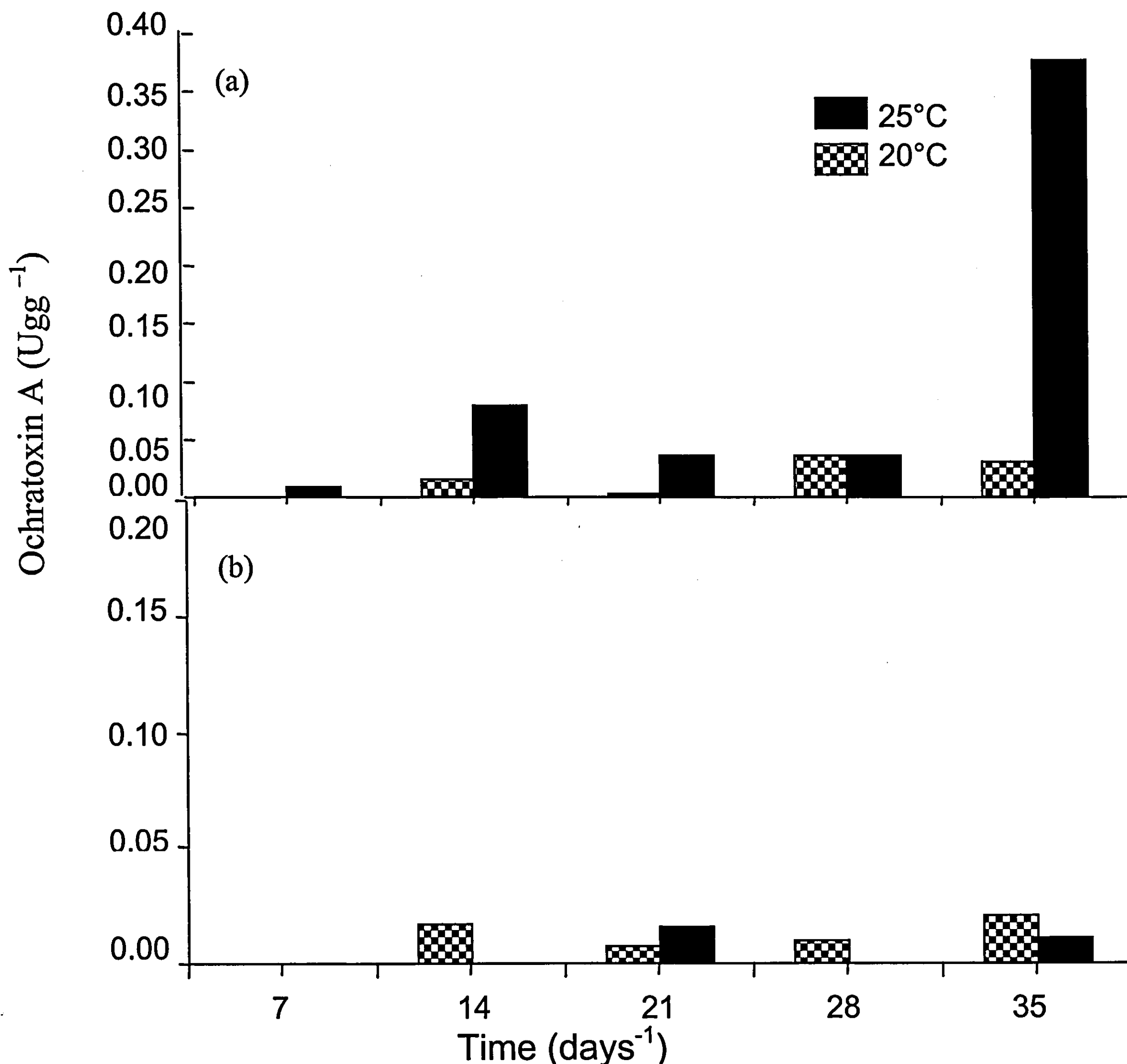

Fig. 3 Ochratoxin A production by Aspergillus carbonarius *(01UAs219) grown on synthetic grape juice on glycerol modified media at (a) 0.98 a_w and (b) 0.93 a_w at 20°C and 25°C over a period of 35 days*

Table 3 Homogeneous subsets of a Tukey B comparisons between growth rate (mm day^{-1}) means of different strains from all countries at all temperatures and 0.93-0.987 a_w (p=0 .05)

	Strains	N	Subset 1	2	3	4	5	6
Israel	1-4-1-9-7.7	24	2.5482					
	1-4-1-9-10.8	24	2.6205					
Italy	MPVP A 933	36		3.1896				
Greece	G 444	24		3.3393				
Italy	MPVP A 1102	36			4.3669			
Portugal	01UAs219	24				6.3686		
Greece	G 458	24				6.7610	6.7610	
Portugal	01UAs294	36					6.9794	
Portugal	01UAs263	36						9.6386

Discussion

Water activity plays a significant role in the growth of *A. carbonarius*. Growth rates at <0.93 a_w are up to 50% slower than at higher a_w levels. Growth at the lowest a_w was either severely limited or completely inhibited. *A. carbonarius* appears to have a narrower growth range in comparison with *A. niger* aggregates and closely related species, although they do fall in the range of other spoilage fungi (Magan & Lacey, 1984). Growth at a_w levels between 0.93-0.98 a_w were optimal for most strains at all temperatures, but outside of this range growth was significantly reduced similar to work described by Joosten *et al.,* (2001). Below 0.90 a_w growth for most strains was sporadic with only a few showing good growth at 0.88 a_w and work is under way to find the lower limits for these strains.

Temperature had a marked effect on the ability to grow. No growth occurred at 10°C, while only limited growth was observed at 15°C at > 0.95 a_w. Most strains of *A. carbonarious* grew best at 25/35°C down to 0.88 a_w. There was a large variation in the growth rates not only between the countries of origin, but also within countries. This has shown the importance of using a wide range of strains from different regions reflecting the climates and sub-climates within such a large area.

Ochratoxin A production by *A. carbonarius* (01UAs219) appears to be greater at 25°C compared to 20°C. This is at variance with other work (Battilani, personal communication) where 15°C was found to be optimum for Italian strains. Ochratoxin A production can be detected after seven days at 25°C and 14 days at 20°C at most a_w levels. Although the experiment was run for 56 days the amounts of OTA produced varied showing in particular a cyclic pattern; the first peak between 7 and 14 days and a second peak detected around 28 days. This cyclical pattern continued up until the end of the experiment at 56 days. The OTA may be degraded chemically or biologically by fungal degradation (Abrunhosa *et al.,* 2001) over the incubation period. Production of OTA from *A. carbonarius* requires less than 21 days for detection in the medium; this is significantly less time than is needed for the production of OTA by *P. verrucosum* on wheat grain agar, where up to 28 days is needed (Cairns, personal communication). Lower a_w conditions appear to inhibit OTA production. Studies are in progress to develop two dimensional profiles for germination, growth and OTA production in relation to temperature and water availability in vitro and in situ on grapes.

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Water and temperature relations of growth and ochratoxin A production by *Aspergillus carbonarius* strains from grapes in Europe and Israel

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ABSTRACT

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Aims: This study investigated the *in vitro* effects of water activity (a_w; 0·85–0·987) and temperature (10–40°C) on growth and ochratoxin A (OTA) production by two strains of *Aspergillus carbonarius* isolated from wine grapes from three different European countries and Israel on a synthetic grape juice medium representative of mid-veraison (total of eight strains).
Methods and Results: The synthetic grape juice medium was modified with glycerol or glucose and experiments carried out for up to 56 days for growth and 25 days for OTA production. The lag phase prior to growth, growth rates and ochratoxin production were quantified. Statistical comparisons were made of all factors and multiple regression analysis used to obtain surface response curves of $a_w \times$ temperature for the eight strains and optimum growth and OTA production by *A. carbonarius*. The lag phase increased from <1 day at 25–35°C and 0·98 a_w to >20 days at marginal temperatures and water availabilities. Generally, most *A. carbonarius* strains grew optimally at 30–35°C, regardless of solute used to modify a_w, with no growth at <15°C. The optimum a_w for growth varied from 0·93 to 0·987 depending on the strain, with the widest a_w tolerance at 25–30°C. There was no direct relationship among growth, environmental factors and country of origin of individual strains. Optimum conditions for OTA production varied with strain. Some strains produced optimal OTA at 15–20°C and 0·95–98 a_w. The maximum OTA produced after 10 days was about 0·6–0·7 μg g^{-1}, with a mean production over all eight strains of 0·2 μg g^{-1} at optimum environmental conditions.
Conclusions: This work demonstrates that optimum conditions for OTA production are very different from those for growth. While growth rates differed significantly between strains, integration of the OTA production data suggests possible benefits for use of the information on a regional basis.
Significance and Impact of the Study: Very little detailed information has previously been available on the ecology of *A. carbonarius.* This knowledge is critical in the development and prediction of the risk models of contamination of grapes and grape products by this species under fluctuating and interacting environmental parameters.

Keywords: *Aspergillus carbonarius*, grapes, growth, ochratoxin A, surface response curves, temperature, water availability.

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INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin which has received increasing attention because of its potent nephrotoxic, immunosuppressive effects. It has been classified as a possible group 2b human carcinogen (IARC 1993). The occurrence of OTA in cereals has been well documented and for some time it was thought to be the major source of OTA in humans and animals. Over the last few years, OTA has been found in other food commodities including coffee beans, cocoa, beans and pulses (Kuiper-Goodman and Scott 1989; Pohland *et al.* 1992; Jörgensen and Rasmundsen 1996; Jörgensen 1998; Joosten *et al.* 2001). OTA has also been detected in wine, grape juice, beer and dried fruits (Zimmerli and Dick 1996).

Traditionally it was thought that OTA was produced by isolates of *Aspergillus ochraceus* and *Penicillium verrucosum* (Pitt and Hocking 1997). However, in the mid-1990s *Aspergillus* section *Nigri* were identified as being able to produce ochratoxin in grapes and wine for the first time (Belli *et al.* 2004). It was subsequently shown that within this group *A. carbonarious* was predominantly responsible for the production of OTA in grapes and wine (Bragulat *et al.* 2001; Battilani and Pietri 2002).

Fungal growth and OTA production are markedly affected by different environmental factors, the two most important being water availability (water activity, a_w) and temperature influencing germination, growth and sporulation (Magan and Lacey 1984). Changes in a_w and temperature have been shown to affect the growth of *A. ochraceus* and *A. niger* isolates (Ayerst 1969; Marin *et al.* 1998) and ability to compete with other spoilage fungi and influence OTA production (Ramakrishna *et al.* 1996; Magan *et al.* 2003).

Aspergillus carbonarius has been isolated in a range of southern European regions. At the present time no studies have compared the effect of water availability and temperature on the growth of such isolates and identified the conditions conducive to growth and OTA production. Such information is important in developing realistic forecasting systems for predicting risk of colonization of grapes by *A. carbonarius* and OTA production. One recent study has compared OTA production by *A. carbonarius* and other section *Nigri* strains from Italy and Spain in relation to water activity and time, but only at 25°C (Belli *et al.* 2004). They suggested that 5–10 days was optimum for OTA production. The objectives of the present work were to compare two strains of *A. carbonarius* from four different trans-European regions (Portugal, Italy, Greece, Israel) with regard to the effect of $a_w \times$ temperature on lag phases prior to growth, growth rates and on temporal production of OTA. Surface response curves for optimum and marginal conditions for growth and OTA production were identified.

MATERIALS AND METHODS

Fungal isolates

In this study *A. carbonarius* strains isolated from grapes in Italy (MPVA A 1102, Ital; MPVP A 933, Ita2); Portugal (01UAs219, Portl; 01UAs263, Port2); Greece (G 458,Grel; G 444, Gre2) and Israel (1-4-1-9-10·8, Isrl; 1-4-1-9-7·7, Isr2). Identification of isolates was confirmed by Dr Z. Lawrence (CABI BioSciences, Egham, Surrey, UK) and were shown to produce OTA on Czapek yeast autolysate agar using the method developed by Bragulat *et al.* (2001).

Medium, water activity modification and inoculation

Studies were conducted *in vitro* on a synthetic grape juice medium (SGM) representative of grape composition at early veraison. This consisted of D(+) glucose 70 g, D(−) fructose 30 g, L(−) tartaric acid 7 g, L(−) malic acid 10 g, $(NH_4)_2HPO_4$ 0·67 g, KH_2PO_4 0·67 g, $MgSO_4 \cdot 7H_2O$ 1·5 g, NaCl 0·15 g, $CaCl_2$ 0·15 g, $CuCl_2$ 0·0015 g, $FeSO_4 \cdot 7H_2O$ 0·021 g, $ZnSO_4 \cdot 7H_2O$ 0·0075 g, (+) Catechin hydrate 0·05 g, agar 25 g in a litre of medium. This was adjusted with 2 M KOH to pH 4·0–4·2.

All experiments were carried out over the range of 0·987–0·85 a_w by addition of either glucose or glycerol. The a_w of 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 and 40°C. The a_w of all cooled treatment media were checked using a Aqualab® WP4 (Decagon Devices, Inc., Pullman, WA, USA) connected to a PC using Hyperterminal software (Higraeve Inc., Monroe, MI, USA) and found to be within a_w of treatment values.

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 media 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.

Two types of measurement were made: (a) the lag phase (in days) prior to mycelial growth (>0·5 mm diameter) and (b) measurement of mycelial extension. Temporal mycelial extension rates were measured daily in two directions at right angles to each other until the medium was fully colonized. 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, for each replicate and treatment.

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 (4·5 mm diameter) across the radius of each colony every 5 days for 20 days. 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, Histon, UK) and weighed. One millilitre of HPLC grade methanol (Fisher, Loughborough, UK) was added and the samples shaken and incubated at room temperature for 90 min. The extracts were filtered (Millex® HV 13 mm; Millipore Corporation, Bellerica, MA, USA) directly into amber HPLC vials (Jaytee Biosciences Ltd, Whitstable, 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) (excitation 330 nm, emission 460 nm). The samples were separated using a C18 Luna Spherisorb ODS2 column (150 × 4·6 mm, 5 μm) (Phenomenex, Macclesfield, UK), with a guard column of the same material. Run time for samples was 12 min with OTA detection after 5·75 min. 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 × temperature was examined by ANOVA using Statistica (Statsoft, Tulsa, OK, USA). 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 the surface response curves were obtained using the above programme.

RESULTS

Effect of a_w × temperature on lag phase and growth of isolates of *A. carbonarius*

Figure 1 shows an example of the effect of different temperatures on the lag times prior to growth initiation at different steady-state a_w levels on a strain from Italy (MPVA 1102). Very short lag phases occurred at 25–35°C (<1 day) and 0·985 to 0·95 a_w. At lower temperatures the lag times increased to >20 days, especially at 0·93–0·88 a_w. Similar trends were found for all strains studied (data not shown).

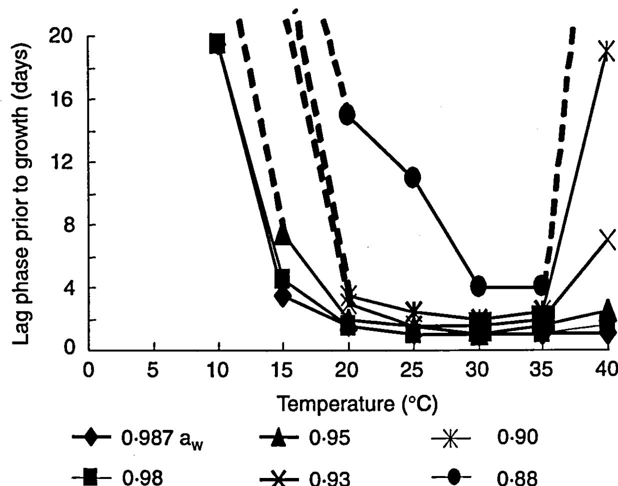

Fig. 1 Relationship between temperature and water activity (a_w) level on the lag phase prior to growth of a strain of *A. carbonarius* on a synthetic grape juice medium. Dotted lines indicate extrapolation

Figure 2 compares growth rates in relation to water availability at three different temperatures using media modified with either glucose or glycerol for a strain of *A. carbonarius* from Portugal (01AUs263). For this isolate maximum growth occurred at 35°C and 0·98 a_w with a decrease under wetter conditions (0·987 a_w). Between 0·95 and 0·98, the growth rate of most strains at 25°C was 25% less than at 35°C. Solute type had a small effect on growth.

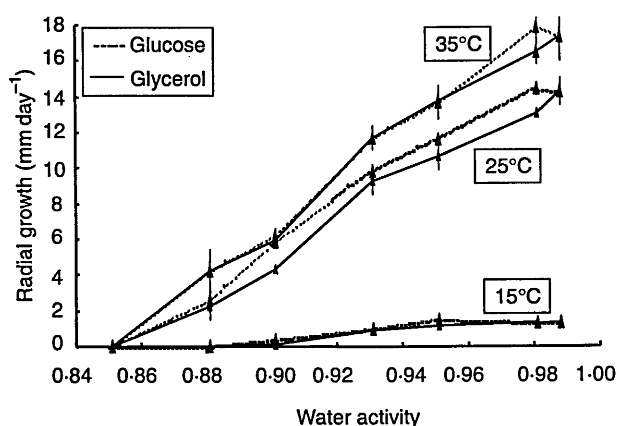

Fig. 2 Effect of water activity (a_w) and solute used (glucose, glycerol) on growth rates of a strain of *A. carbonarius* (Port2) at three different steady-state temperature conditions on a synthetic grape juice medium

Similar patterns in relation to a_w were observed for all the strains examined although growth rates varied. Statistically there was no significant difference between growth rates on glucose/glycerol modified media at 15 or 35°C but for a few strains there was a significant difference ($P = 0{\cdot}05$) at 25°C between 0·90 and 0·987 a_w (data not shown). A large proportion of the strains did not show any growth at 0·88 a_w. In an analysis of growth at 15°C there was a significant difference at all a_w treatments. No strains grew at 10°C.

Comparisons were made of growth of strains from each of the four countries examined at different a_w levels at 25°C (Fig. 3). There was no consistent pattern of growth for strains from different countries or between strains from the same country. Statistical comparison showed that for only a few strains within a country were there were statistically significant differences in growth rates (Table 1). Figure 4 shows three examples of the surface response profiles for growth of strains of *A. carbonarius* from Italy, Israel and Greece respectively. This confirms that optimum growth was at higher temperatures (25–35°C) and intermediate a_w levels (0·97–0·985).

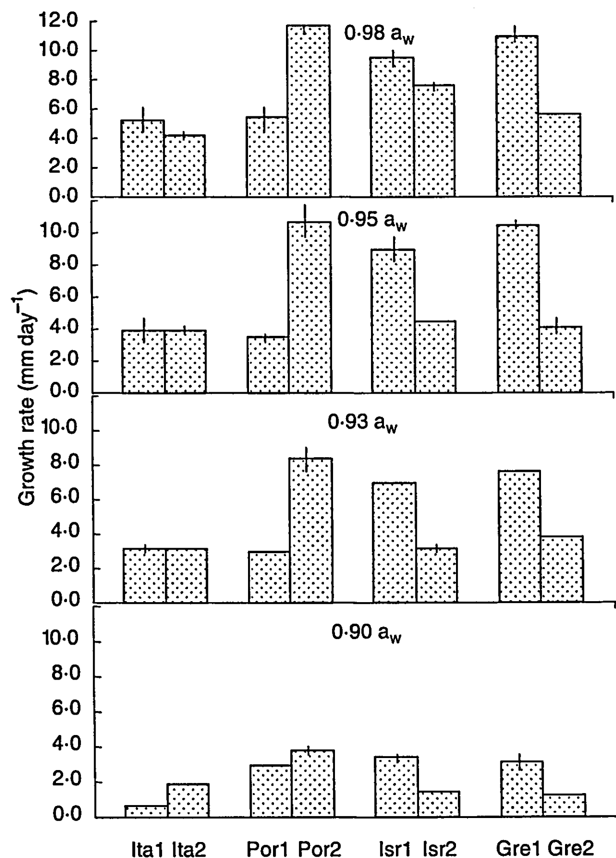

Fig. 3 Comparison of growth rates of two strains each of *A. carbonarius* from Italy, Portugal, Israel and Greece in relation to four steady-state water activity (a_w) conditions at 25°C on a synthetic grape juice medium. Bars indicate standard errors. For key to strains see Materials and Methods

Effects of a_w and temperature on ochratoxin production

Comparison of OTA production by all strains in relation to three a_w levels at a steady-state temperature (20°C) after 10 days incubation is shown in Fig. 5. Significant differences between amounts of OTA produced by an individual strain, and between strains from the same country or different countries were observed. Statistical analysis showed that the overall, the mean OTA production by seven of the eight strains over all treatment conditions were relatively similar. Table 2 shows the statistical comparison for all eight strains and the statistically significant effect of

Table 1 Statistical comparisons of mean growth rates of strains of *Aspergillus carbonarius* in relation to temperature × water availability for all strains using growth rate as the variable for analysis

Strains	{Ita1} (M = 2·3000)	{Ita2} (M = 3·3597)	{Por1} (M = 1·9100)	{Por2} (M = 3·8155)	{Isr1} (M = 1·8671)	{Isr2} (M = 3·8172)	{Gre1} (M = 3·8172)	{Gre2} (M = 3·3552)
{Ita1}		0·286	0·669	0·098	0·648	0·120	0·110	0·288
{Ita2}	0·286		0·132	0·635	0·134	0·645	0·645	0·996
{Por1}	0·669	0·132		**0·031**	0·963	**0·037**	**0·037**	0·133
{Por2}	0·097	0·635	**0·031**		**0·034**	0·998	0·998	0·631
{Isr1}	0·647	0·134	0·962	**0·034**		**0·040**	**0·040**	0·134
{Isr2}	0·110	0·645	**0·037**	0·998	**0·040**		1	0·642
{Gre1}	0·110	0·645	**0·037**	0·998	**0·040**	1		0·642
{Gre2}	0·288	0·996	0·133	0·631	0·135	0·641	0·641	

$P = 0{\cdot}05$.
Figures in bold show statistically significant differences between strains.
M, overall mean of a single strain over all conditions. For key to strains see Materials and methods.

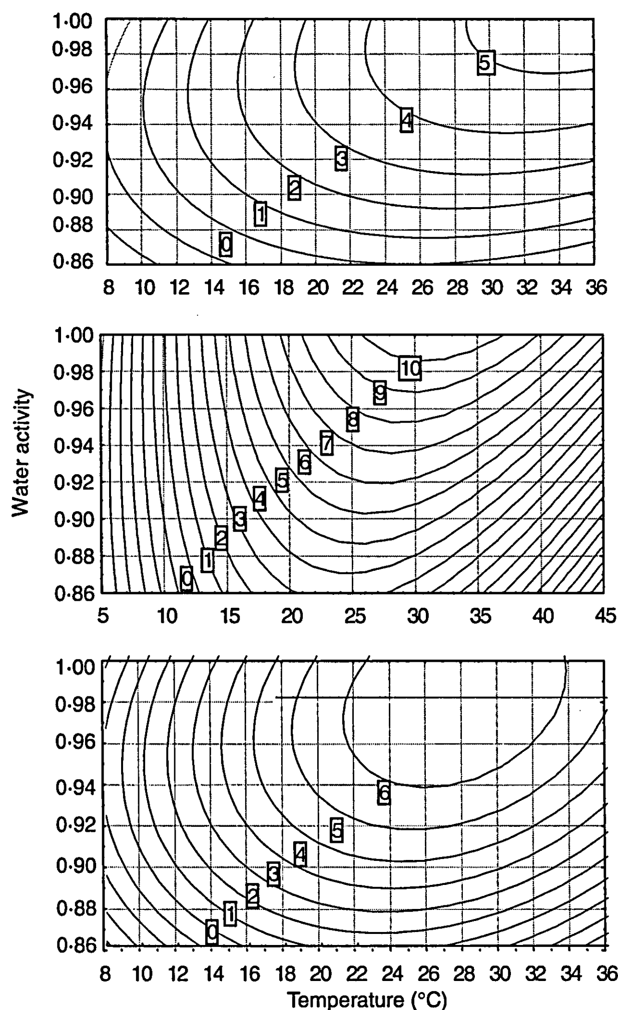

Fig. 4 Surface response curves for three strains of *A. carbonarius* from Italy (Ita1), Portugal (Port) and Israel (Isr1) in relation to water activity and temperature. The numbers on the contour lines refer to growth rates (mm day^{-1}). For key to strains see Materials and Methods

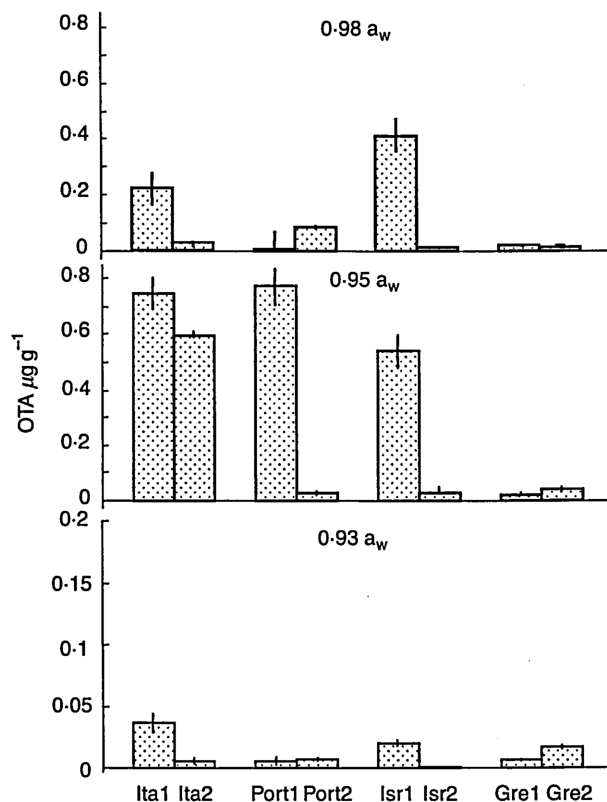

Fig. 5 Comparison of ochratoxin production ($\mu g\ g^{-1}$) of two strains each of *A. carbonarius* from Italy, Portugal, Israel and Greece in relation to three steady-state water activity (a_w) conditions at 20°C on a synthetic grape juice medium. For key to strains see Materials and Methods

single factors of a_w, temperature, and two-way interactions of $a_w \times$ temperature.

The data for all eight strains were used to develop a surface response curve to identify the optimum conditions of a_w and temperature and the range of conditions for production of different quantitates of OTA (Fig. 6). In contrast to results for growth this clearly identified 15–20°C and 0·95–0·98 a_w as optimum conditions for OTA production.

Table 2 Statistical comparison of strains, water activity, temperature and water activity × temperature effects on ochratoxin A production by eight strains of *Aspergillus carbonarius*

Treatments	Standard error	Significance
Ita1	0·087	0·053
Ita2	0·087	0·310
Port1	0·096	0·127
Port2	0·096	0·532
Isr1	0·096	0·203
Isr2	0·096	0·071
Gre1	0·096	0·641
Gre2	0·0960	0·000
Water activity	0·034	0·000
Temperature	0·034	0·049
A_w × temperature	0·017	0·000

DISCUSSION

This study compares the impact of $a_w \times$ temperature regimes on growth of strains of *A. carbonarius* from different countries in Europe and from Israel for the first time. Both factors affected the lag phase prior to growth and mycelial extension of the strains. While the pattern of effects of environmental factors was similar, the growth rates of strains varied significantly. Generally, growth was

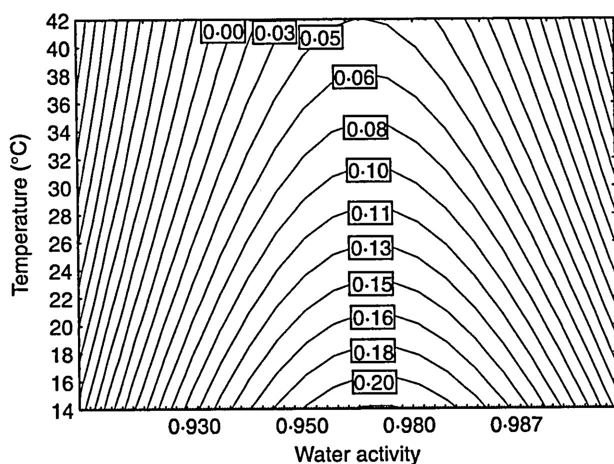

Fig. 6 Surface response curve for OTA production by *Aspergillus carbonarius* based on data for all eight strains from the four countries in relation to water activity and temperature. The numbers on the contour lines refer to mean mycotoxin production ($\mu g\ g^{-1}$)

reduced by up to 50% at ≤0·93 a_w. Growth was also to a large extent inhibited at 0·88 a_w for most strains even after 56 days incubation. There was some variation in the growth rates between countries, and also within countries. This shows the importance of using a wide range of strains from different climatic regions for building a database to determining environmental conditions where risk from OTA contamination may occur. These results are also very different from those obtained previously for isolates of the *A. niger* group prior to the detailed taxonomic knowledge of species in the section *Nigri* and the production of OTA by species in this section. Studies of *A. niger* by Ayerst (1969) and Marin *et al.* (1998) isolated from spoiled grain showed wider a_w ranges of 0·83–0·995 with optimum growth at 0·995 a_w and 35°C. Temperature ranges were found to be 10–40°C at optimum water availability. In comparison, OTA producing strains of *A. ochraceus* were found to grow optimally at 0·995 and 30°C with similar a_w limits of about 0·83–0·85 a_w and a temperature range of 10–35°C. These limits and ranges for growth are very different from those for *A. carbonarius*.

Temporal studies have shown that OTA was initially produced after 5–10 days incubation on the SGM medium. Previous studies by Cabanes *et al.* (2002) found that *A. carbonarius* isolates produced maximum OTA on unmodified yeast extract sucrose agar and Czapek yeast agar after about 14 days although some was detected after 7 days. In contrast, Belli *et al.* (2004) suggested that 5 days may be optimum for OTA production by *A. carbonarius* strains from Spain and Italy. They found OTA production of 4–5 $\mu g\ g^{-1}$ of medium for a strain from Spain after 5 days with a subsequent decrease over time. However, the temperature (25°C) used now appears to be suboptimal for OTA production by many *A. carbonarius* strains. In the present study, the production of OTA over a range of environmental conditions provides important information as it could assist in predicting the possible risk of OTA production and in choosing appropriate harvesting dates to minimize entrance into wine and vine fruit production. The present study has also demonstrated the contrasting impact of both a_w and temperature on growth and OTA production by the eight strains examined. The most important findings are that the optimum conditions for OTA production are generally very different from those for growth. Studies of *A. cabonarius* on irradiated coffee berries by Joosten *et al.* (2001) found that most OTA production at 25, 27 and 30°C and 0·99 a_w, with production strongly reduced at 0·94 a_w after 14 days incubation. However, only a few environmental conditions were tested, and the nutritional status of coffee berries is very different from grape-based media or grapes. The surface response curves presented in the present study on growth and OTA production may provide very useful guidelines for facilitating effective management of predicting risk from growth and OTA production during ripening and harvesting of grapes and during transport for wine production.

Over long incubation periods it has been observed that some decrease in OTA production can occur under some treatment conditions (Belli *et al.* 2004). Previous studies have suggested that *A. carbonarius* itself, or other black *Aspergilli* could degrade OTA in media resulting in the formation of breakdown products such as ochratoxin α (Abrunhosa *et al.* 2002; Varga *et al.* 2002). Other inputs into the system such as fungicides and pesticides may further stress these fungi influencing both growth and more importantly, OTA production.

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

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Abstract

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

Introduction

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

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

Little information exists on mycoflora and potential OTA-producing fungi in Spanish wine grapes. This study focused on the identification of the common mycoflora in wine grapes from four important grape growing regions of Spain to study their progression during grape ripening in 2002 and 2003, with particular interest in ochratoxigenic species and their ability to produce OTA. An initial study was carried out by our team in 2001, and the results have been recently published (Bellí et al., 2004a). In the present study the objective was to correlate the fungal populations isolated over the 3 years with the meteorological conditions in the vineyards.

Materials and methods

Field sampling

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

Mycoflora determination

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

Screening of fungi for OTA production

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

OTA in musts

At the last sampling time of both years, the same ten bunches collected from each vineyard for the mycoflora study were crushed and the resulting musts ($n = 40$) were analysed for OTA using the method of the Office International de la Vigne et du Vin (Bezzo et al., 2002). Briefly, 100 ml of each sample (pH 7.4 with NaOH 4 M) were centrifuged (3830 g, 15 min) and filtered (Whatman No. 1). Afterwards, they were passed through an immunoaffinity column (Ochraprep, Rhône Diagnostics Technologies, Glasgow, UK) at 2–3 ml min^{-1}. The column was then washed with 20 ml of distilled water (5 ml min^{-1}) and finally dried in an air stream (2 min). Desorption was carried out with 1.5 ml of methanol/acetic acid

(98/2) solution. The eluate was evaporated to dryness at 40 °C under a stream of nitrogen and redissolved in 2 ml of mobile phase (acetonitrile 48%-sodium acetate 4 mM/acetic acid (19/1) 52%). About 25 µl of each final sample were injected into a HPLC system equipped with a fluorescence detector (Waters 474) (λ_{exc} 230 nm; λ_{em} 458 nm) and a C_{18} column (Waters Spherisorb 5 µm, ODS2, 4.6 × 250 mm). The analysis was performed under isocratic conditions at a flow rate of 1 ml min^{-1}. Detection limit and retention time were 0.05 µg l^{-1} and 11.5 min, respectively.

Statistical analysis

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

Results

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

According to analysis of variance, all single factors: year, sampling date and region and their interactions, presented significant differences in the number of black aspergilli isolated ($P < 0.0001$). A total of 464 (7.7%) and 648 (10.8%) black aspergilli were isolated in 2002 and 2003, respectively, distributed in the four regions sampled. The number of these moulds found at harvest was significantly higher than were found in the first or second sampling for the four regions (Figure 2). Grapes from Costers del Segre were significantly the most contaminated every year, with approximately 300 strains isolated in 2003 and around 180 in 2002 at harvest, followed by those from Utiel-Requena with around 100 isolates in both years at harvest. However, no statistical differences were

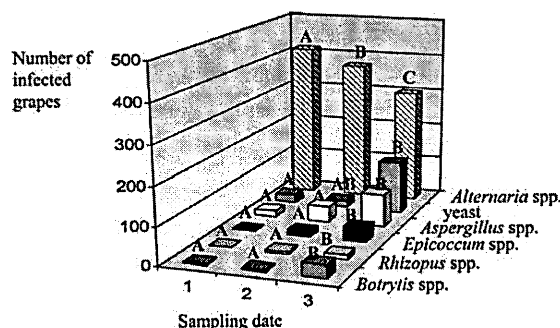

Figure 1. Number of grapes infected by different fungi from a total of 500 grapes plated on DRBC, at each sampling date: (1) 1 month after setting, (2) veraison and (3) harvest, in 2003 in La Rioja region. Different letters over bars indicate significant differences in the number of these fungi between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

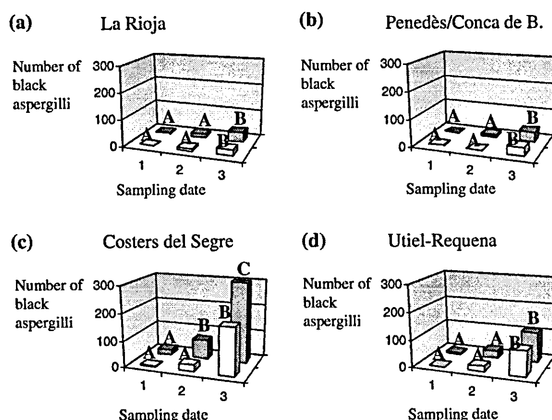

Figure 2. Number of black aspergilli found on grapes plated on DRBC in □ 2002 and in ■ 2003 in four wine-making regions of Spain, at three sampling periods (2000 grapes per sampling period): (1) 1 month after setting, (2) veraison and (3) harvest. Different letters over bars indicate significant differences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

found between Penedés/Conca de Barberà and La Rioja regions.

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

In 2002, 7% of the total number of black aspergilli strains isolated produced detectable levels of OTA in culture, whereas 25% were detected in 2003. Among the black aspergilli groups, *A. carbonarius* presented the highest percentage of OTA-positive strains (82% in 2002 and 76% in 2003). A low percentage of *A. niger* aggregate isolates produced OTA (2% in 2002 and 5% in 2003) and no toxin was detected in any of the uniseriate strains in either year (Table 1). Furthermore, more than 95% of the total number of positive isolates produced low amounts of OTA ($< 2.5\ \mu g\ g^{-1}$), although some isolates were found to produce OTA at higher levels ($2.5–25\ \mu g\ g^{-1}$). The most toxigenic ones were *A. carbonarius* isolates in both years. However, subsequent analysis of musts detected no OTA. Fungi isolated from grapes were correlated to the meteorological data in each sampling month for each region in 2002 and 2003 detailed in Table 2. A positive correlation between the number of black aspergilli isolated and the temperature in the field in the months preceding harvest was found (Table 3). High relative humidity (R.H.) also contributed to the infection of these fungi, mainly for the uniseriate group.

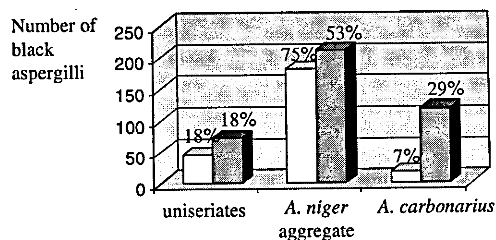

Figure 3. Number of black aspergilli isolated from grapes in □ 2002 and in ■ 2003, classified into three groups and percentage of each group among all black aspergilli isolated.

Discussion

The main moulds causing secondary rots of grapes are black aspergilli, *Alternaria*, *Rhizopus*, *Cladosporium* and *Penicillium*. These are generally associated with vine trash on soil, leaves, leaf buds and other residues in the field (MAPA, 1998). These fungi were the dominant genera isolated from grapes in the present survey as well as in another study in Argentina and Brazil by Da Rocha Rosa et al. (2002). They found that yeasts were a major

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

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

Numbers in brackets are the total number of black aspergilli isolated / the number of isolates producing OTA above the detection limit (0.01 $\mu g\ g^{-1}$ CYA).
[a]S, sampling (1, 1 month after setting; 2, veraison; 3, harvest).
[b]P/CB, Penedès/Conca-Barberà; UR, Utiel-Requena; R, La Rioja; CS, Costers del Segre.
[c](–) not tested for OTA.

component of the fungal population, and *Alternaria, Aspergillus* and *Botrytis* were frequently isolated. *Alternaria* and *Aspergillus* were also the most frequent moulds of the mycoflora of Argentinean grapes isolated by Magnoli et al. (2003). *Penicillium, Cladosporium* and *Botrytis* prevailed in Portuguese grapes (Abrunhosa et al., 2001). Da Rocha Rosa et al. (2002) suggested that the diversity of grape mycoflora depends on grape variety, degree of berry maturity, physical damage, viticulture practices and climatic conditions.

A positive correlation between the number of black aspergilli isolated from grapes and temperature was found (Table 3). This correlation was mainly due to *A. niger* aggregate. It is known that optimum temperatures for growth of *A. niger* aggregate *in vitro* are between 30–37 °C; meanwhile the optimum for *A. carbonarius* and

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

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

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

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

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

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

uniseriate strains are between 25–30 °C (Mitchell et al., 2003; Bellí et al., 2004b). In addition, water activity (a_w) has been demonstrated to have an effect on *in vitro* growth of strains of black aspergilli, with the highest levels (0.98–0.995 a_w), similar to that of grapes, being the optimum in most cases (Bellí et al., 2004a).

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

Percentages of uniseriate isolates, *A. niger* aggregate and *A. carbonarius* (21, 60 and 19%, respectively) found in a survey of Italian grapes in 1999–2000 (Battilani et al., 2003), were very similar to those found in the present study. High incidence of *A. niger* aggregate was also found by Da Rocha Rosa et al. (2002) in a mycofloral survey of wine grapes from Argentina and Brazil. Less *A. carbonarius* were found, but 25% of these were OTA producers (18–234 μg g^{-1} on CYA). OTA was not detected in any of the must samples analysed, although in 2001, 15% of the musts contained low amounts of OTA: five samples contained 0.091–0.293 ng ml^{-1} and one 0.813 ng ml^{-1} (Bellí et al., 2004b). Similar results for OTA in some years but not others has also emerged from an Italian study of OTA content in grapes, which concluded that temperature, rain and relative humidity are the main factors that influenced OTA production in grapes (Battilani and Pietri, 2002). Due to the absence of OTA in the musts analysed, no correlation between the incidence of OTA-producing strains in grapes and OTA in musts could be established from the present study. In contrast, Sage et al. (2002) found a strong correlation between these factors, as eight of eleven must samples were found to be contaminated with OTA (10–461 ng l^{-1}) and a significant number of *A. carbonarius* strains were previously isolated from grapes.

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

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Ecology and control of ochratoxin in grapes and dried vine fruits

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ABSTRACT

The population dynamics of mould contamination of Corinth currants and sultanas during drying was investigated in Greece in 2004. This showed that the total fungal populations and frequency of isolation was changed with time during the 10-14 day drying period. Altitude above sea level affected the final contamination levels, especially with the mycotoxigenic *Aspergillus carbonarius*. The main species isolated were *Aspergillus* section *Nigri* and *A.carbonarius*, yeasts, *Penicillium* species and occasionally *Cladosporium*, *Alternaria* and *Botrytis* species. The highest frequency of isolation and of ochratoxin (OTA) was isolated from currants/sultanas at sea level and the lowest concentration at the highest altitude (600-1000m). Potential for control of *A.carbonarius* and OTA production by isolates from currants/sultanas using Sodium metabisulphite and controlled atmospheres were evaluated. This showed that potential for control of growth required 750-1000 ppm *in vitro* on a grape juice medium, regardless of environmental conditions. However, >500 ppm was effective for OTA control.

INTRODUCTION

The production of raisins is a traditional cultivation in Greece and mainly two types of raisins are cultivated, the golden raisins called sultanas and the black Corinth raisins (currants). The latter have taken their name of the region of Corinth where this type first grew, the name currant, also, is probably a corruption of the word Corinth. There is a big industry of dried fruits aiming both at the internal and most importantly at the external markets.

Currants are made from the black Corinth grapes. The grapes are harvesting by hand in August and September and placed in the sun to dry. The drying process lasts for about 7-14 days depending on the weather and from time to time they are turned and swept into heaps, until completely dried, the growers then sort out the dried product (Dekanea, 2005). For the sultanas the process is similar except that often potassium carbonate is added during drying for enhancing the desiccation process. At the end of drying the currants/sultanas are taken to industry, quality levels are measured and, if necessary, insect infestation is controlled using methyl bromide or in some cases CO_2 and then the product is stored before processing. The raisins are usually stored in areas with humidity not >60% and a temperature of 12-18°C. Before package the raisins are checked for ochratoxin (OTA). If they are free of OTA and

have the quality levels that are desired the final product is packaged in paper cartons or polyethylene bags.

The highest OTA content, among grapes and its derivatives, has been measured in dried vine fruits (MAFF, 1997) with > 40 μg/kg. Macdonald *et al.* (1999) found a maximum level of 53.6 μg/kg in black dried vine fruits (currants). Magnoli *et al.* (2004) did a survey of black and white dried vine fruits from Argentinian markets. OTA was detected in 67.7% of the black and 84.2% of the white dried vine fruits with mean levels of 6.3 and 4.42 ng/g, respectively. The highest concentration was found in a black dried vine fruit sample (14 ng/g). Another recent survey took place in Sweden for two years (Möller and Nyberg, 2003) found a range of < 0.1-19.0 $\mu g\ kg^{-1}$ in 1999/2000, with a median concentration of 0.9 μg/kg. In 2001/2002 the range was <0.1-34.6 μg/kg with a median of 0.2 μg/kg. In the second study, which was based on nearly the same brand and products, the median OTA was much lower. The results from the first study were similar to those found in the UK by MacDonald *et al.* (1999) and in Greece by Stefanaki *et al.* (2003).

There is little knowledge of the fungal population dynamics and diversity during the drying process. The conditions which allow preferential dominance of the mycotoxigenic *A.carbonarius* have not been previously identified. The objectives of this study were to examine the effect of natural drying regimes on the ecological changes in fungal community structure at different altitudes in drying raisins in two areas of Greece (Peleponisa and Crete). The potential of using sodium metabisulphite to control growth and OTA production was also examined *in vitro*.

MATERIALS AND METHODS

Sampling area

Samples of drying currants were obtained from the region of Aeghio in northeast Peloponnese, Greece, a characteristic production region of black raisins (Corinth currants) with the distinctive name of "Vostizza". Sultanas were obtained from different areas in Crete. Three replicate vineyards from different drying areas were chosen during the 2004 season to represent three different altitudes, sea level, 300-500 m, and 600-1000 m. Just prior to harvest, and after 4, 8 and just prior to storage 1-2 kg samples were taken at random.

Mycological analysis of grapes and currants

To determine the mycoflora on grapes before and during sun drying of raisins two identification media were used: Malt extract agar (MEA, Oxoid Ltd) that was made up as directed and MEA + glycerol a modified water activity level of 0.95 (MEA95). In both media a small amount of chloramphenicol was added prior to sterilization at 121°C for 15 minutes, to inhibit bacterial growth. From each sample 10 g were taken and suspended in 90 ml diluent (sterile-distilled water + 0.5 g agar + 0.005% Tween 80) and homogenized in a Colworth Stomacher 200 (Seword Ltd) for 10 min. The mixture was then serially diluted. From each dilution, 0.2 ml was spread using a sterile glass spreader onto the surface of both media (four replicates of each). All transfers were made with an automatic pipette and disposable sterile tips. After 7 days the numbers of colonies in the serial dilution method were counted and reported as colony-forming units (CFU)/gram of sample.

Ten berries per bunch were randomly selected and directly plated onto MEA and MEA95. The berries were aseptically cut in half before plating in Petri plates. All the Petri plates were incubated at 25°C. Fungal identification of black *Aspergilli* was carried out to species level only for *A. carbonarius*, while the others isolates were grouped in the *A. niger* aggregate. The aim was to identify and differentiate the percentage *A. carbonarius* out of the *A. niger* aggregate and the total mycoflora

OTA production ability of isolates of *A. carbonarius*

Representative strains of *A. carbonarius* were single point inoculated on 50% coconut cream agar CCA (Dyer and McCammon, 1994). The plates were incubated for 7 days at 25°C. The reverse side of each plate were observed under long-wave ultraviolet (UV) light (365nm) for the characteristic blue-green fluorescence produced by ochratoxin A. After 7 days incubation the plates were destructively examined by exposure to 25% ammonia. The plates were left for 1 h and then under UV light. OTA has an intense violet fluorescence.

Ochratoxin analysis of grapes and dried vine fruits

The detection of OTA concentration in grapes and currants was performed by HPLC, following the methodology proposed by Zimmerli and Dick (1996). Injections of 50 μl standard or samples were injected into an HPLC system consisting of a Perkin Elmer 200, equipped with an ISS 200 sampling system and a Perkin Elmer LC 420 fluorescence detector set at 333nm excitation and 470 nm emission. A Spherisorb Excel ODS2 (250x4.6mm; 5μm) column was employed with a mobile phase of acetonitrile-water-acetic acid (57:41:2) at a flow rate of 1.00 ml min^{-1}. The limit of detection was estimated as 0.2 μg/kg.

In vitro* ecological studies on isolates of *A. carbonarius

Six isolates of *A. carbonarius* were used in this study. Three from currants/sultanas respectively, based on those showing very high fluorescence in the screening assay. For comparison an isolate (isolate DM) from wine grapes with known OTA producing ability was also used (Mitchell *et al.*, 2004).

Effect of a_w and sodium metabisulphite (NaMBS) on growth and ochratoxin production

Grape juice agar medium (GJM) was used as the basal medium throughout the in vitro studies. The medium was prepared by mixing 25% (v/v) supermarket long life red grape juice and 2.5% agar (Oxoid, UK technical agar no.3) in distilled water. The medium after the water activity and NaMBS adjustment was sterilized at 121°C for 15 min, cooled to approximately 50°C and poured into sterile 90 mm diameter sterile plastic Petri plates.

Adjustment of the medium a_w to the required treatment was done by adding D(+) glucose (Sigma) to the basal medium. The a_w of this basic medium was 0.985, determined with a Humidat Sprint IC II thermoconstanter (Novasina, Zurich, Switzerland). Two more a_w treatments was used: 0.965 and 0.93. Those were obtained by adding 18.73 and 50.35g D(+) glucose 100/g medium, respectively. The pH of all treatments was modified to 4.2 using a buffer solution. A 100ml solution of this buffer was made by mixing 55.90 ml of 0.1 M citric acid $C_6H_8O_7{\cdot}H_2O$ (AnalaR®) and 44.10 of 0.2 M sodium phosphate Na_2HPO_4 (Sigma). Sodium metabisulphite ($Na_2S_2O_5$; NaMBS; BDH Chemicals Ltd, Poole, England) was used as

source for the SO_2. Six concentrations were used: 0, 100, 250, 500, 750 and 1000 ppm. The Petri plates of each treatment were centrally inoculated with a small loop of spore suspension (10^6 spores/ml). All experiments were carried out with 4 replicates per treatment and incubated at 25°C.

RESULTS

Population dynamics of fungi on drying vine fruits

Figure 1 shows an example of the changes in fungal populations in a field site for currants at sea level for the four main component species on media at 0.95 a_w. This shows that just prior to harvesting and drying there were differences between dominant populations. At sea level yeasts, *Penicillium* spp. and *Aspergillus* section *Nigri* were isolated in about $\log_{10}$ 3 CFUs/g grapes. At the other two altitudes no *A. carbonarius* populations were isolated from the pre-harvest grapes (data not shown). Just prior to harvest, at the low altitude, *A.carbonarius* and *A. niger* group represented about 50% each with Log_{10} 4-5 CFUs/g of the final fungal load. At higher altitudes this was about 30% as *Penicillium* spp. were also present. Figure 2 shows the effect of altitude on the mean frequency of isolation of different species from sultanas during drying based on direct plating onto agar medium modified to 0.95 a_w. This shows that there were a higher frequency of *A.carbonarius* isolated from drying sultanas at sea level than at higher altitude. Contamination with OTA was higher at sea level than at higher altitude with the range being 2.83-11.38 and 1.81-1.62 µg/kg currants respectively (Dekanea, 2005).

Representative isolates *A. carbonarius* were monitored under UV light for the characteristic fluorescence that ochratoxin A produce, using the coconut cream agar and NH_4 solution. Out of the 43 isolates examined from currants 32 (74.4%) produced characteristic fluorescence. For sultanas a total of 45 isolates were examined and 58% were found to be OTA producers.

In vitro control using Sodium metabisulphite

Figure 3 shows the effect of treatments on the growth rate (mm/day) of some of the isolates examined in this study. The use of NaMBS significantly affected the growth rate of all isolates although high concentrations were required for control. Figure 3 shows the efficacy of NaMBS concentrations required for controlling growth of an isolate from sultanas. For more than 50% inhibition concentrations at least 500 ppm NaMBS was required. For complete inhibition, up to 1000 ppm was required for all isolates (6) examined, regardless of the a_w level over the experimental period. For all *A. carbonarius* isolates growth was stimulated by 100-250 ppm NaMBS. All strains had an optimal a_w for growth at 0.985 in the presence of 100 ppm NaMBS. However, there was little difference in relative growth rates at 0.985 and 0.965 a_w. Comparisons between the isolates showed that similar growth patterns occurred under the treatment tested. There was also little difference between isolates from drying vine fruits and from grapes for wine production.

The effect of NaMBS x a_w interactions showed that between 500-750 ppm was required for effective control of OTA production by isolates, regardless of whether from currants or sultanas. Table 1 shows the LD_{50} and LD_{90} values for control of OTA production using NaMBS. This shows that at 0.985 aw at least 650-700 is required for 90% inhibition of OTA. However at the lower a_w levels 400-600 was adequate for 90% inhibition of OTA production.

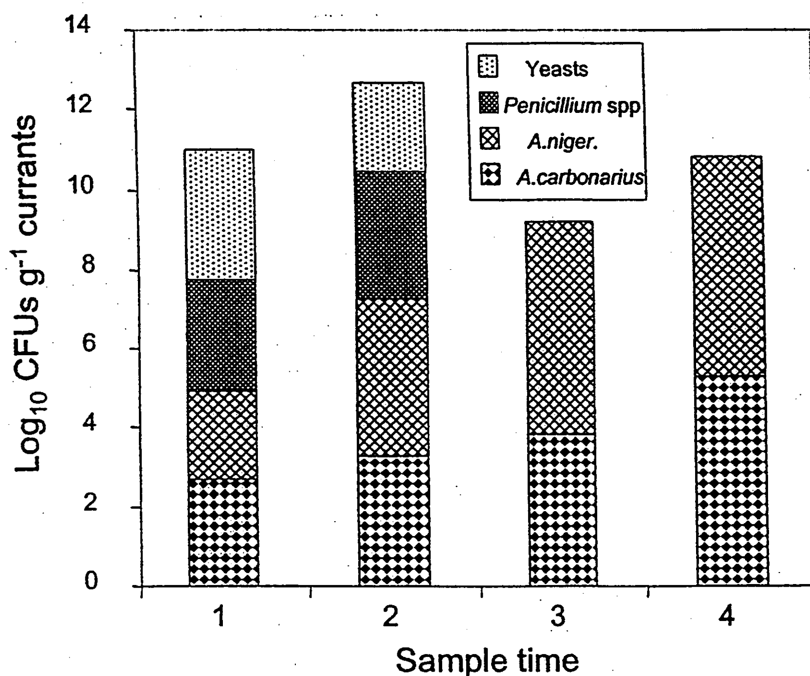

Figure 1. Changes in dominant fungal species/genera on currants prior to harvest to storage at sea level on malt extract agar (0.95 a_w). Key: 1, pre-harvest; 2, 4 days of drying; 3, 8 days drying; 4, end of drying, prior to storage.

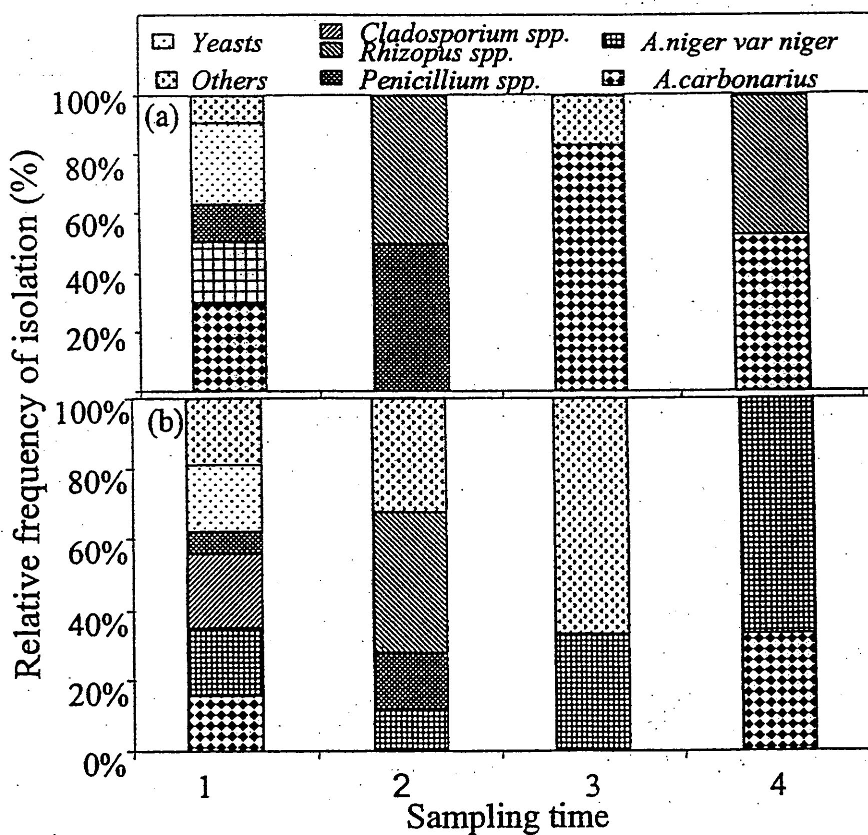

Figure 2. Relative frequency of isolation of different species from direct plated drying sultanas from (a) sea level and (b) 600-800 meters above sea level.

Key: 1, pre-harvest; 2, 4 days of drying; 3, 8 days of drying; 4, end of drying, prior to storage.

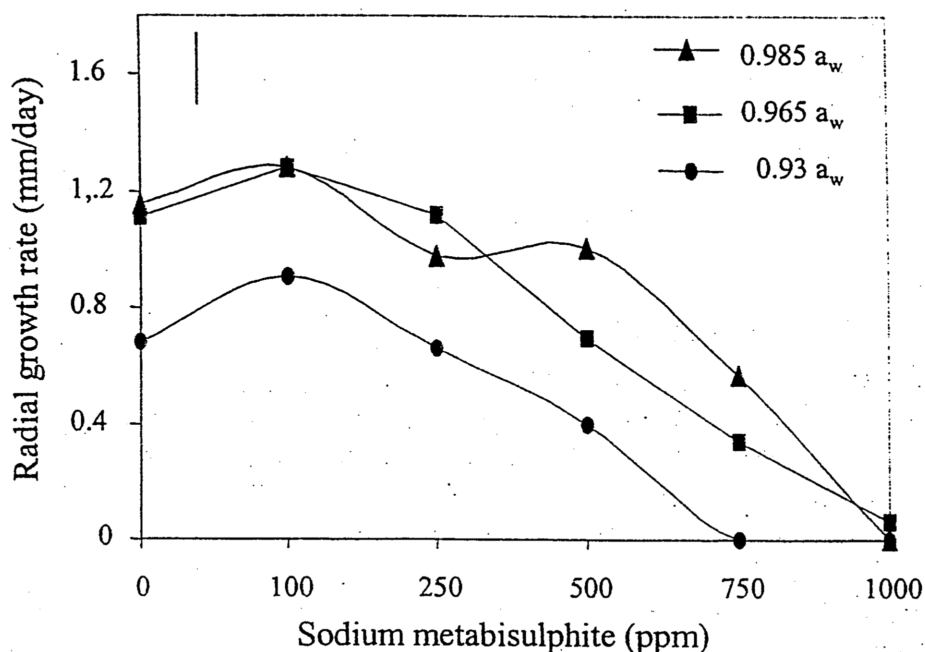

Figure 3. Effect of sodium metabisulphite (ppm) on mycelial growth of a isolate of *A.carbonarius* from sultanas at three different water activity levels at 25oC on a white grape juice medium. Bar indicates LSD (P=0.05).

Table 2. The LD_{50} and LD_{90} concentrations of sodium metabisulphite required for inhibiting ochratoxin A production by three isolates of *A.carbonarius* isolated from currants and grown on red grape juice medium at 25°C.

	NaMBS (ppm)	
	LD_{50}	LD_{90}
Water activity	**Strain DM**	
0.985	600	690
0.965	170	620
0.93	420	480
	Strain 119	
0.985	345	640
0.965	200	465
0.93	400	450
	Strain 127	
0.985	550	735
0.965	185	430
0.93	235	400

DISCUSSION

This study has shown that the incidence of *A. carbonarius*, which is considered to play the major role in ochratoxin A contamination of grapes and dried vine fruits although other black

Aspergilli are also an important component. Generally, the final *A.carbonarius* and *A.niger* aggregate species were present at up to log 3-4 CFUs/gram currants/sultanas at the end of the drying period, especially in fields at sea level. It is known that the spores of black *Aspergilli* are present in vineyard soil in high concentrations (Kazi *et al.*, 2003). Moreover, Battilani *et al.* (2003) demonstrated that *Aspergilli* section *Nigri* are present on wine grapes early in the growing season and that their population increased during later grape maturity. The present study shows that the population of section *Nigri* does gradually increase during drying of grapes for raisin production resulting in almost all being contaminated just prior to storage. Currants and sultanas have a high sugar content which becomes more concentrated as they dry. Thus they may provide a selective nutritional substrate which is conducive to growth of black *Aspergilli* and other such xerotolerant spoilage fungi. There were differences which depended on altitude above sea level for both currants and sultanas. There are certain differences in the meteorology between fields at sea level and those at high altitude. The latter, has cooler mean temperatures and lower relative humidities.

The increase in the populations of *A. carbonarius* during drying could be due to the fact that the drying area is usually on the ground and the currants/sultanas are more exposed to contamination for quite a long period. This coupled with the sugar content could predispose them to colonisation. Furthermore, contamination can be worse if growers do not remove any heavily mouldy bunches. No previous studies have shown the progress of black *Aspergilli* populations and colonisations during drying of raisins. In Australia, Leong *et al.* (2004) studied the black *Aspergilli* populations during drying and demonstrated an increase during the initial stages of drying with a slight decrease in the latter stage. However, in their study rainfall was an important factor. In the present study there was no rainfall during the sampling period.

Although *A. carbonarius* was present on fresh grapes no ochratoxin was detect in these samples. The occurrence of ochratoxigenic fungi does not necessarily mean OTA production occurs (Battilani *et al.*, 2003). Besides, it is known that the optimum conditions for OTA production are different than those for *Aspergilli* growth, at least under *in vitro* conditions (Mitchell *et al.,* 2004). Similarly, OTA producing fungi from the *A.niger* group have not always been isolated when OTA has been found to contaminate samples (Sage *et al.*, 2002). It is well established from previous studies that OTA naturally occurs on grapes and raisins. However, it is difficult to compare our results with those from the literature because previous studies have used raisins purchased from the market and so have been industrially processed and packaged. They thus often have a lower black *Aspergilli* population. However, MacDonald *et al.* (1999) reported higher OTA concentration (53.6 μg/kg) in such samples while others reported lower concentrations (Stefanaki *et al.*, 2003; Magnoli *et al.*, 2004).

The present study examined the efficacy of sodium metabisulpite to control germination, growth and ochratoxin A production of *A. carbonarius* strains. For complete mycelial growth inhibition a concentration of between 750-1000 ppm was required regardless of the a_w level used. The ochratoxin production appeared to be inhibited by up to 750 ppm. At 0.95 a_w the concentration required for inhibition was lower (500 ppm). However, at lower concentrations (100, 250 ppm) *A. carbonarius* growth was stimulated. In the literature there are few studies to examine the effect of SO_2 on the growth of other fungi but to our knowledge this is the first studies where *A. carbonarius* strains have been considered. However, *A.niger* was effectively controlled in onions by SO_2 at 1% (v/v) exposure for 72 hrs (Tamizharasi and Narasimham, 1992).

Generally, the information collected from previous studies suggests that the threshold SO_2 concentration may vary considerably between fungi. Those differences could be due to the fact that several factors influence the efficacy of SO_2. Particularly, the tolerance of *Penicillium* species to high concentrations of SO_2 has been suggested to be due to their ability to actively transport the SO_2 into the mycelia. In the present study it is possible that a percentage of SO_2 was absorbed and bound to the glucose substance reducing its antifungal activity.

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