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PhD Thesis

ECOLOGY OF *A. carbonarius* AND OCHRATOXIN A PRODUCTION IN VINE FRUITS AND CONTROL IN THE PRODUCTION CHAIN

Marianthi G. Pateraki

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Marianthi G. Pateraki

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Local-supervisor: *Prof.* Dimitrios Lydakis

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

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ABSTRACT

This study examined black aspergilli, especially *A. carbonarius* and *A. niger* and ochratoxin A (OTA) contamination of grapes, during drying and industrial processing of dried vine fruits. This was complemented by studies on potential control using preservatives and physical factors such as modified atmospheres. Fungal population kinetics were determined in relation to grapes at harvest, and during drying at three different altitudes (sea level: 0-200 m; medium level: 250-500 m; high level: >500 m) in two seasons. At pre-harvest, *A. niger* aggregate species were the predominant fungal species while *A. carbonarius* was occasionally isolated, in both years studied. Both altitude and bunch position affected black aspergilli population dynamics. Overall, they were increased during drying. However, both black aspergilli groups were mostly isolated, at low and medium altitudes (<500 m). OTA contamination was influenced by bunch position, although altitude did not significantly influenced amounts. The fungal biodiversity was decreased during sun-drying of sultanas. The widest diversity of species occurred at the sea level. However, *A. niger* aggregate, were dominant during drying. Time of drying and altitude significantly influenced fungal loads of black aspergilli. In contrast, OTA production (*ca* 0.001 - 0.0025 μg g^{-1}) was not significantly influenced by altitude and drying time. Shannon Index of Biodiversity (H), for pre-harvest and pos-harvest studies, was determined for the first time.

A. niger aggregate (*ca* 5.0 Log_{10} CFUs g^{-1}) was predominant during industrial processing, while *A. carbonarius* was only isolated at low levels (1.5-2.0 Log_{10} CFUs g^{-1}). Heat treatment (up to 90^{o} C) appeared to be the key-procedure for the elimination of fungal populations. In the contrary, SO_2 treatment did not statistically alter fungal population dynamics. OTA contamination was not significantly affected by industrial processing.

In *vitro* studies conducted on both White Grape Juice Medium (WGJM) and in sultanas with strains of *A. carbonarius* originated from Cretan sultanas and compared

with a strain isolated from Italian wine grapes. They examined the impact of sodium metabisulphite (NaMBS), elevated CO_2 (up to 50%) concentrations and a_w levels, on black aspergilli spore germination, growth and OTA production. Moreover, fungal interactions *in vitro* and *in situ* were also investigated.

In general, spore germination occurred over a wide range of sodium metabisulphite concentrations, although germ tube extension was significantly controlled. At ≥ 750 mg L^{-1} NaMBS, no spore germination was observed while both mycelial growth and OTA production were completely inhibited. Medium concentrations of NaMBS (≤ 250 mg L^{-1}) enabled optimum spore germination, growth and OTA production (x 0.965 a_w). The efficacy of controlled atmospheres x a_w showed that there was very little inhibitory effect on spore germination. However, both germ tube extension and fungal growth were inhibited by 50% CO_2. After 10 days, growth was not as effectively controlled. A_w had a bigger effect on OTA production than modified atmospheres. In *situ* experiments on sultanas confirmed these results. Competition and dominance of *A. carbonarius* over other fungal species showed that a_w and temperature influenced Indices of Dominance and OTA production. In *vitro* and in *situ,* OTA production by *A. carbonarius* was significantly influenced by the fungal competitor used.

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Abréviations

EU	:	European Union
EC	:	European Commission
Apprx.	:	approximately
a_w	:	Water activity
HPLC	:	High Performance Liquid Chromatography
OTA	:	Ochratoxin A
µg	:	microgrammars
µL	:	microliter
mL	:	milliliter
ppm	:	Parts per million
ng	:	nanogrammes
CFU	:	Colony forming Unit
SGM	:	Synthetic grape juice medium
CCA	:	Coconut Cream Agar
MEA	:	Malt Extract Agar medium (a_w=0.985)
MEA97	:	Modified Malt Extract Agar medium (a_w=0.971)
MEA95	:	Modified Malt Extract Agar medium (a_w=0.954)
MEA87	:	Modified Malt Extract Agar medium (a_w=0.87)
MEA83	:	Modified Malt Extract Agar medium (a_w=0.83)
WGJM	:	White Grape Juice Medium
mins	:	minutes
NaMBS	:	Sodium Metabisulphite
PBS	:	Phospate Buffer Saline
v/v	:	Volume/volume
M	:	Molarity
MC	:	Moisture Content
hrs	:	hours
UV light	:	Ultra Violet light

Chapter One

GENERAL INTRODUCTION – LITERATURE REVIEW - OBJECTIVES

1.1. GENERAL INTRODUCTION

There is significant concern amongst consumers for food free of chemical residue and natural toxins. Thus, there is interest in minimising the potential exposure of consumers by effective food safety and prevention systems and quality assurance programmes. Consequently, plant pathogens of staple foods have received attention in relation to their effects on nutritional and calorific value.

Food spoilage fungi have become prominent in recent years because of the associated natural contamination by toxins in a wide range of food products from contaminated raw commodities, bakery products, cheese and meat, spices, fruit juices, fruits, and their beverages. Many spoilage fungi are saprophytes and grow on dead or drying food. Some genera, especially *Aspergillus, Penicillium* and *Fusarium* have the ability to synthesize toxic secondary metabolites. Although some of these metabolites may not directly influence the growth of the producing organism, they can be acutely toxic in mammalian systems.

Diseases produced by exposure to toxic fungal metabolites are collectively called mycotoxicoses (Bennett *et al.,* 2003). Mycotoxin**s** are metabolites produced by fungi that have toxic effects on vertebrates and other animals in low concentrations. So far, more than 300 fungal compounds have been recognized as mycotoxins (Ringot *et al.*, 2006). Approximately a dozen groups are suspected as having a threat to human and animal health (Bennett *et al.,* 2003; Magan & Olsen, 2004). Interestingly, not all fungal metabolites have a negative impact on living organisms. For instance, a significant number have antimicrobial action (e.g. penicillin) and many have been developed as pharmaceutical products including the statin group used as a cholesterol

lowering drug. On the other head, many people are affected by mycoses and mycotoxicoses annually (Bennett *et al.,* 2003).

In order to minimize the impact of mycotoxin contamination in food, research is being focussed on the whole food chain approach to minimise contamination with these toxic secondary metabolites. Indeed, the food industries have to work to the legislative limits which have been set in the European Union (EU) and world wide for a number of the key mycotoxins (e.g. aflatoxins, ochratoxin A, fumonisins and trichothecenes).

The present study deals with the colonization and contamination of grapes and dried vine fruits (sultanas), originated from the Cretan ecosystem, by black aspergilli, *Aspergillus carbonarius* and *A. niger* aggregate in particular, and the production of ochratoxin A (group 2B; IARC, 1999). No information is available on the population diversity and temporal fungal populations in fresh grapes of Sultanina and those being dried for the production of vine fruits (sultanas). There are critical points in this chain, especially during the drying phase and during post-harvest and industrial processing, where minimisation or prevention of OTA contamination could be implemented.

1.2. THE GENUS *ASPERGILLUS*

The genus *Aspergillus* is a group of filamentous ascomycete fungi which consists of a large number of species (approximately 137 known species by Kozakiewicz, 1989). They mostly live on dead organic matters and they are major agents for decomposition and food decay. Many of *Aspergillus* species may have desirable or

useful properties and thus they find applications in medical and food industry. For example, food industry widely uses *A. niger* aggregate in large-scale biotechnological products such as organic acids (it is the main source of citric acid) and enzymes (i.e. glucose oxidase, lysozyme) (Bigelis & Lausure, 1987). Furthermore, the *A. niger* group facilitates potential biocontrol inoculants in tomato by enhancing nematicidal synthesis while *A. quadrilineatus* inhibits bacterial growth through their secondary metabolities (Siddiqui *et al.,* 2004). The United States's Food and Drug Administration has characterized the *A. niger* group as GRAS (Generally Regarded as Safe). Other *Aspergilli* important in commercial microbial fermentations are *A. oryzae,* which is involved in sacharification process (they convert the rice starch to sugars), *A. nidulans* and *A. fumigatus* which are widely used in the research as their genome has been sequenced.

Aspergillus species and its teleomorphs *Emericella* and *Eurotium* are usually reported as the primary cause of spoilage due to their ability to grow over a wide range of temperatures and water activity levels (Magan *et al*., 2007). Some of the consequences of *Aspergillus* contamination are: loss in germinability of seeds, discoloration, heating, tainting, mustiness, caking and finally decay of the product (Kozakiewicz, 1989). *Aspergillus* is regarded as the most important genus containing mycotoxin-producing fungi. Some mycotoxins produced by *Aspergillus* include ochratoxin A, aflatoxins, cyclopiazonic acid and sterigmatocystin.

Most of the methods for fungal identification are based on the morphology of the ascospore (they are produced by teleomoporphs) but mainly on the physical appearance of the anomorph colony that produces conidia. More specifically, they are

based on colour, shape, size, ornamentation of the conidia and the length of the conidiophore. The colour of the aerial growth of colonies is the main characteristic on which the species groups are based. According to Kozakiewicz (1989), there are four main colours: yellow, white, green and black of which the most common colour is green. Apart from this, group divisions are based on shades of the same colour. The shape of the conidial head seen in microscopic slide preparations or observed under the stereoscope microscope is the second visual character. Although the form of the head varies with age, taxonomists have referred to four basic shapes (Figure 1.1).

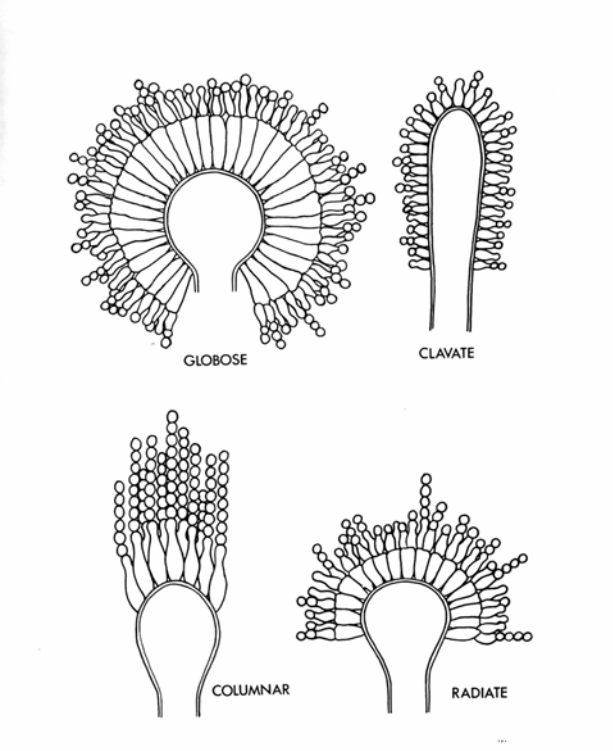


Figure 1.1. Conidial head shape variation commonly encountered in the *Aspergilli spp.*

In addition, a very important character for species identification is the structural differences of the conidiogenous cells (= all cells that actually produce the conidia) (Kozakiewicz, 1989). Any anamorphic spore is called a "conidium" while the term "sterigmata" is used to describe conidiogenous cells. There is a distinction based on primary and secondary sterigmata in *Aspergilli* species. Thus, those conidiogenous cells that have primary sterigmata are termed "uniseriate" and those with primary and secondary sterigmata are termed "biseriate" (Figure 1.2). It is not easy to classify *Aspergilli* into taxa using the structural differences of the conidiogenous cells, because biseriate heads are invariable uniseriate when they are young (Kozakiewicz, 1989).

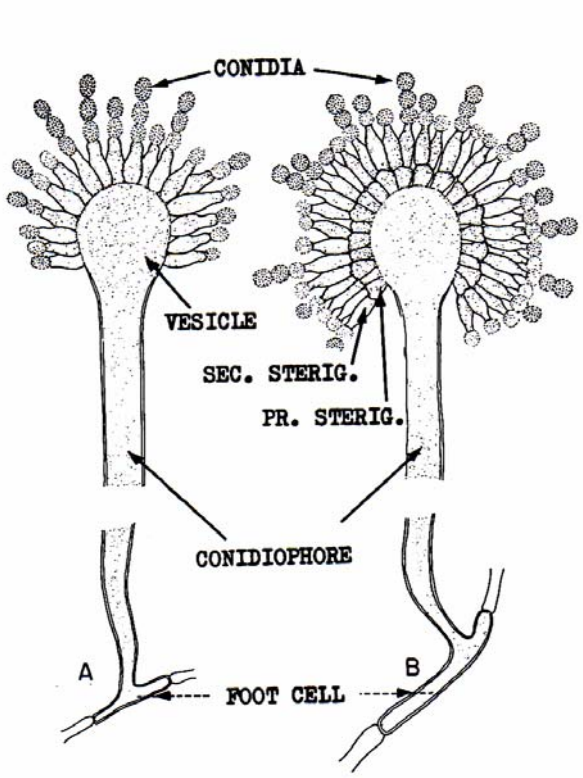


Figure 1.2. Conidial head showing, from the left to right, the uniseriate and biseriate nature of the sterigmata (from Raper & Fennel, 1965).

1.2.1. *ASPERGILLUS NIGER* AGGREGATE (=BLACK ASPERGILLI OR *ASPERGILLUS* SECTION *NIGRI*)

Despite their industrial importance, the taxonomy of black aspergilli has the highest degree of variation. Many efforts have been made in order to find suitable taxonomic criteria since most of the black aspergilli are morphologically similar, thus their classification can be easily confused. According to their morphological characteristics, black *Aspergilli* have been classified into thirty five (Mosseray, 1934), twelve (Rapel and Fennel, 1965), seventeen (Kozakiewicz, 1989) or seven (Al-Musallam, 1980) and eight species (Parenicova *et al.*, 1997). Differences between classification systems indicate that there is a difficulty in distinguishing the taxa included in this group. However, the colour of the conidial heads (some shade of black) remains their unifying feature. Kozakiewicz (1989) subdivided the 17 proposed species into two main groups according to their conidial ornamentation: (a) in the echinulate (conidiophores are roughened) and (b) the verrucose (conidiophores are interpreted as smooth) taxa.

It is notable that growth conditions can influence phenotype of fungal strains (Samson, 1994). Modern molecular analyses such as Restriction Fragment Length Polymorphism method (RFLP), Random Amplified Polymorphic DNA method (RAPD), Polymerase chain reaction (PCR), DNA fingerprinting and nucleotide sequencing, which are more accurate techniques, have attempted to offer a more reliable solution to this problem. However, several reclassifications of black *Aspergilli* have taken place in the last decade or more (i.e. Kusters-van Someren *et al.*, 1991). Abarca *et al.*, (2004) suggest a simplified classification based only on the

primary and secondary sterigmata so that black aspergilli are mainly grouped into "uniseriate" or "biseriate" species.

Studies by Vries *et al*. (2005) added one new species in the group of black aspergilli based on taxonomy suggested by Parenicova *et al.* (1997); *Aspergillus vadensis*. Identification was carried out by combining molecular, biochemical and morphological properties of the new species. However, more surveys are needed to ensure the above outcome. A possible new, non-toxigenic species, called *A. ibericus* which has a similar morphology to *A. carbonarius* (conidia size: 5–7 μm) was suggested by Serra *et al.* (2006). Later studies on *Aspergillus* section *Nigri* isolated from grapes from Italy, France, Spain, Portugal, Greece and Israel, indicated that black Aspergilli could be classified into four taxa; (a) *A. carbonarius,* (b) *A. tubingensis*, (c) *A. "uniseriate"* and finally (d) *A. niger* "like". This identification based on Applied Fragment Length Polymorphisms (AFLP) analysis (Perrone *et al.*, 2006). Finally, a new novel species belonging to *Aspergillus.* section *Nigri* is *Aspergillus brasiliensis* sp. nov., which has been suggested by Varga *et al.,* (2007).

1.2.2. *ASPERGILLUS CARBONARIUS*

Species such as *A. carbonarius* and uniseriate species are easy identifiable. *A. carbonarius* is one of the five members of the echinulate group (Kozakiewicz, 1989). Its conidiogenous cells are characterised as biseriate with long brittle conidiophores (5-6 mm long). The conidial heads are radiate or split into poor columns. The colour of the colony is carbon black (Kozakiewicz, 1989).

A further distinguishing character of *A. carbonarius,* which is considered a useful "tool" for its distinction, is the size of the conidia. These are extremely large (> 5-7 µm (Kozakiewicz, 1989; Serra *et al*., 2006). *A. carbonarius* is a xerophilic filamentous fungus that occupies a wide spectrum of habitats in the environment (Kozakiewicz, 1989). It is the key species responsible for OTA contamination on grapes, wine and dried vine fruits in tropical and sub-tropical regions. Table 1.1 shows the taxonomy of *A. carbonarius.*

Table 1.1. Taxonomy of *Aspergillus carbonarius*

Kingdom:	Fungi
Class:	Deuteromycetes
Order:	Moniliales
Family:	Moniliaceae
Genus:	*Aspergillus*
Species:	*A. carbonarius*

1.3. MAJOR MYCOTOXINS

As indicated previously, mycotoxins can cause significant problems to human health. The cost for controlling mycotoxin hazards is estimated at $1.4B in the United States of America (Russel *et al.,* 2006). There is a wide range of mycotoxins produced by fungi. Table 1.2 summarizes the most important ones and the fungal species that produce them.

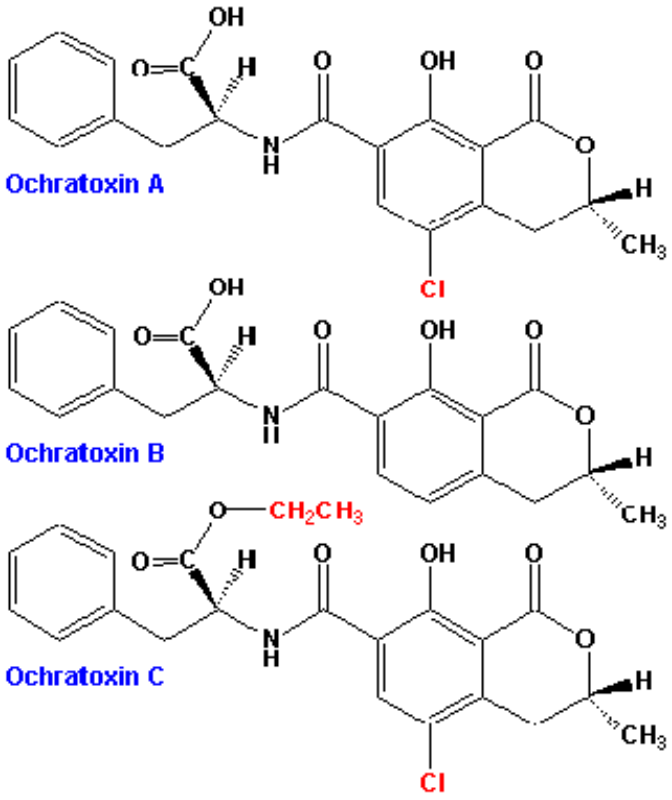
Table 1.2. The major mycotoxins and the principal toxigenic fungi.

Mycotoxins	**Fungal species**
Ochratoxin A	*Aspergillus ochraceus, Penicillium verrucosum,* blac*k Aspergilli*
Aflatoxins	*A. flavus, A. parasiticus*
T-2 toxin, HT-2	*Fusarium sporotrichioides, F. poae, F. equiseti, F. acuminatum*
Zearalenone	*F. culmorum, F. graminearum, F. sporotrichioides*
Deoxynivalenol	*F. culmorum, F. graminearum, F. sporotrichioides*
Patulin	*P. expansum, Byssochlamus fulva, B. nivea*
Citrinin	*P. expansum,P. verrucosum*
Cyclopiazonic acid	*A. flavus, Penicillium sp.*
Diacetoxyscirpenol	*F. sporotrichioides, F. graminearum, F. poae*
Sterigmatocystin	*A. versicolor*
Fumonisins	*F.moniliforme, F. proliferatum*
Ergopeptine alkaloids	*Acremonium coenophialum*
Lolitrem alkaloids	*A. lolii*
Phomopsins	*Phomopsis leptostromiformis*
Nivalenol	*F.graminearum*
Sporidesmins	*Pithomyces chartarum*

1.4. OCHRATOXINS

Ochratoxins are extracellular mycotoxins (secondary metabolites) produced by several species including *A.ochraceus*, *A. niger* section *Nigri* group and *Penicillium verrucosum*. There are three forms of ochratoxin; A, B and C (Figure 1.3 show the differences in structure between the three forms of ochratoxin).

Figure 1.3. Differences in structure of ochratoxins A, B, C (Petzinger *et al.*, 2000).



Ochratoxins B and C are much less toxic than the A form. Furthermore, the B and C forms do not inhibit protein biosynthesis as the A form does (Dirheiner & Creppy, 1991). Consequently, ochratoxin A (OTA) has recently received the most attention. The inhibiting protein synthesis "function" of OTA is variable, according to the exact form of OTA (see Table 1.4). For example, although alanine-ochratoxin A influences protein biosynthesis, the presence of proline-ochratoxin A has no impact (Petzinger *et*

al., 2000). Apparently, this chemical structure offers stability to both temperature and hydrolysis. However, according to Petzinger (1999), bacteria in the rumen of ruminants are capable of splitting this bond to some extent.

1.4.1. OCHRATOXIN A (OTA)

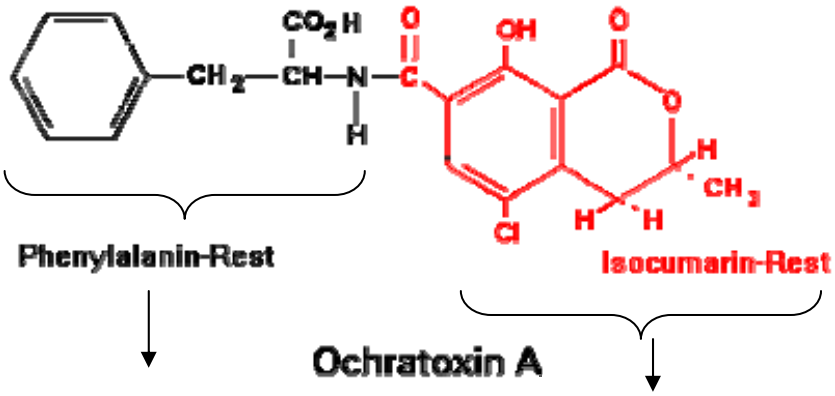
OTA was discovered in 1965 (Van der Merwe *et al.,* 1965) during a large program designed to identify new mycotoxins. At first, it was isolated from *Aspergillus ochraceus* as secondary metabolite. It was mainly associated with species included in section *Circumdati*. Later, it was also isolated from a commercial corn sample in the Unated States of America (USA). Not all *Aspergillus* species are OTA producers. Some well-known ochratoxigenic genera are *A. ochraceus, A. niger* and *A. carbonarius*. Early reports mentioned that some species of *Penicillium* are also implicated in OTA production but recent findings have shown that only *P. verrucosum* produces OTA (Pitt *et al.,* 2000; Bennett *et al.,* 2003).

OTA producing ability of fungal species is included as a character, a usefull "tool", for taxonomical purposes in fungal classification and identification (Cabanes *et al*., 2008). Table 1.3 shows the OTA producing *Aspergillus* species, according to Varga *et al.* (2001).

Table 1.3. OTA-producing *Aspergillus* species (modified from Varga *et al.*, 2001).

Species	Section	References
A. glaucus	*Aspergillus*	Chelkowski *et al*. (1987)
A. repens	*Aspergillus*	El-Kady *et al.* (1994)
A. sydowii	*Aspergillus*	Ueno *et al.* (1990)
A. albertensis	*Flavi*	Varga *et al.* (1996); Peterson (2000)
A. alliaceus	*Flavi*	Ciegler (1972); Doster *et a*l. (1996); Peterson (2000); Bayman *et al.* (2002)
A. flavus	*Flavi*	Atalla and El-Din (1993)
A. auricomus	*Circumdati*	Varga *et al*. (1996)
A. melleus	*Circumdati*	Ciegler (1972)
A. muricatus	*Circumdati*	Frisvad and Samson (2000)
A. ochraceus	*Circumdati*	van der Merwe *et al*. (1965); Krivobok *et al*. Mühlencoert *et al.* (1995); Varga *et al.* (1996).
A. ostianus	*Circumdati*	Ciegler (1972)
A. petrakii	*Circumdati*	Ciegler (1972)
A. sclerotiorum	*Circumdati*	Ciegler (1972); Moss (1996); Varga *et al*. (1996)
A. sulphureus	*Circumdati*	Ciegler (1972); Madhyasta *et al*. (1990)
A. clavatus	*Clavati*	Atalla and El-Din (1993)
A. wentii	*Cremei*	Varga *et a*l. (1996)
A. fumigatus	*Fumigati*	Abarca *et al.* (1997); Atalla and El-Din (1993): Varga *et al*. (2000)
A. awamori	*Nigri*	Ono *et al*. (1995); Téren *et al*. (1996); Accensi *et al*. (2001
A. carbonarius	*Nigri*	Horie (1995); Téren *et al.* (1996); Wicklow *et al.* (1996); Heenan *et al.* (1998); Joosten *et al.* (2001)
A. foetidus	*Nigri*	Ueno *et al*. (1991); Téren *et al.* (1996); Magnoli *et al*. (2003)
A. japonicus	*Nigri*	Dalcero *et al.* (2002); Battilani *et a*l.(2003)
A. lacticoffeatus	*Nigri*	Samson *et a*l. (2004)
A. niger	*Nigri*	Abarca *et al.* (1994); Ono *et al*. (1995); Téren (1996); Nakajima *et al.* (1997); Heenan *et al*. (1998)
A. sclerotioniger	*Nigri*	Samson *et al*. (2004)
A. tubingensis	*Nigri*	Medina *et al*. (2005)
A. usamii	*Nigri*	Ono *et al*. (1995); Accensi *et al.* (2001)
A. vadensis	*Nigri*	De Vries *et al*. (2004)
A. terreus	*Terrei*	Ueno *et al*. (1991)
A. ustus	*Usti*	Ueno *et al*. (1991)
A. versicolor	*Versicolores*	Abarca *et al.* (1997)

Notice: Some other workers have found *A. japonicus* and *A. aculeatus* as non-toxigenic fungi (Abarca *et al., 1994*; Teren *et al*., 1996; Varga *et al.*, 1996).



Molecular weight = 403.82.

The empirical formula of OTA = $C_{20}H_{18}O_6NCL$

pK_a=4.2-4.4 pK_a=7.0-7.3

Figure. 1.4. Chemical structure, molecular weight and empirical formula of OTA.

OTA is a colourless, crystalline compound that belongs to a group of closely related derivatives of isocoumarin linked to L-phenylalanine and classified as pentaketide (see Figure 1.4). .Although it is highly soluble in polar organic solvents, it is not soluble in water. It can easily dissolve in a hydrogen carbonate solution with water. Under ultra violet light OTA can exhibit (λ_{max}^{MeOH}, nm; ε=333) blue fluorescence (Poland *et al.,* 1982). However, steps in OTA biosynthesis have not been clearly defined. A polyketide synthetase (PKS) mediates to the isocumarin polyketide synthesis, which is the first step for OTA biosyntesis (Ringot *et al.,* 2006; Russel *et al.,* 2006) (see Figure. 1.5).

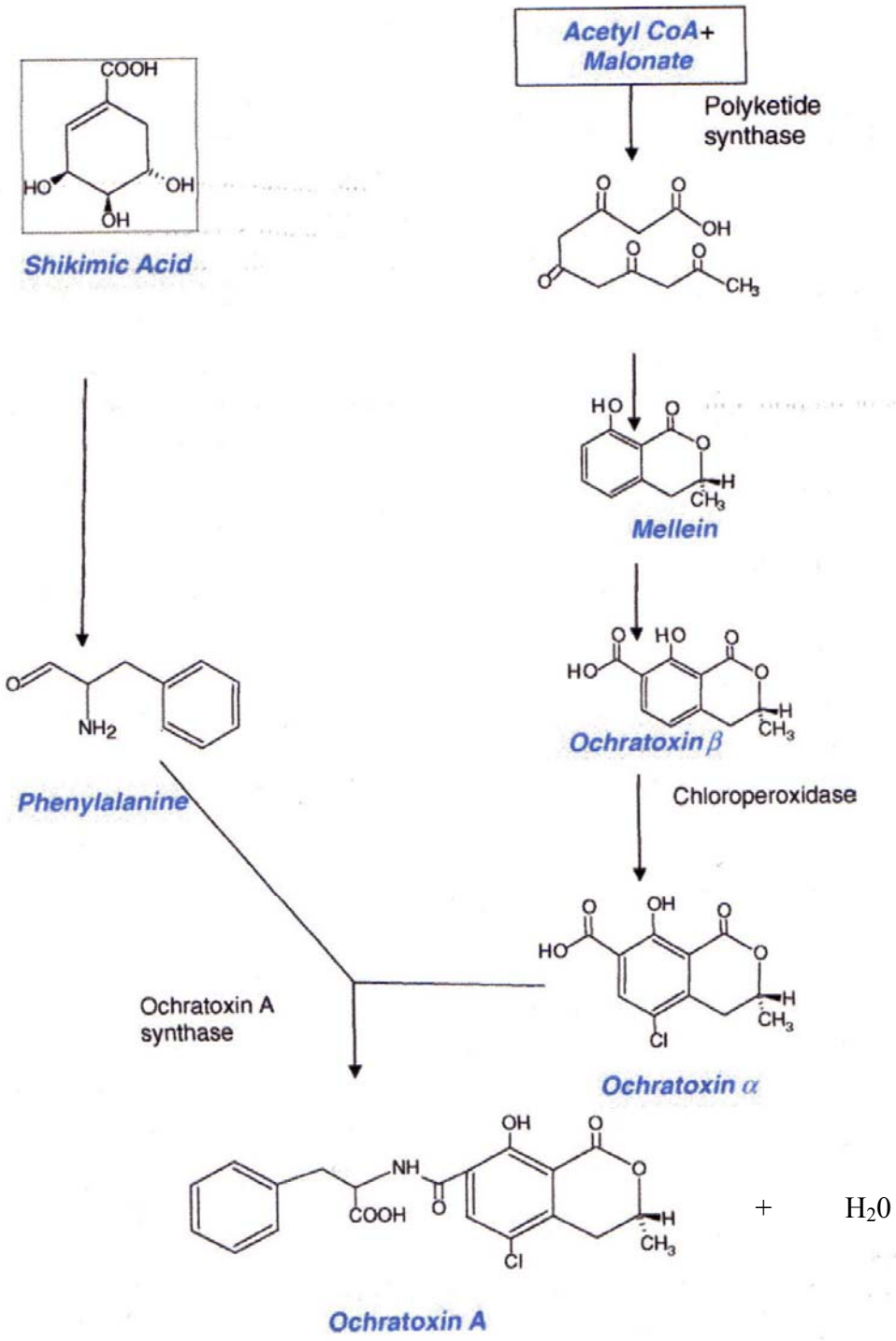


Figure 1.5. Ochratoxin A biosynthesis (source: Ringot *et al.*, 2006).

Studies on *A. ochraceus* indicate that the polyketide synthetase gene is expressed under certain conditions, "*OTA permissive conditions*", and only according to the early steps of OTA biosynthesis. However, there is little knowledge about the expression of the aforementioned gene associated with others ochratoxigenic fungi such as *A. carbonarius.* Some abiotic parameters, like pH, are crucial factors for OTA absorption. For example, OTA (fully protonised form) is favoured in conditions similar to the ones presented in the gastrointestinal track (Ringot *et al.,* 2006).

There is a significant amount of knowledge on the presence of OTA in combination with other mycotoxins (see Table 1.4). It is also almost impossible to avoid OTA dietary intake due to the range of products in which it is found. As it has been already referred to above, OTA is a secondary metabolite which is synthesized by some *Aspergillu*s and *Penicillium* spp. when they are grown under certain environmental conditions. The current study investigated some abiotic and biotic factors which influence OTA biosynthesis by *A. carbonarius* in sultanas.

Table 1.4. Effects of OTA combinations with other mycotoxins (Ringot *et al.*, 2006)

OTA combination with…	Interaction	Potential mechanism
Ochratoxin B (OTB)	Antagonistic	Common biosynthetic pathway
Citrinine (CIT)	Synergistic	CIT potentiates OTA carcinogenicity
Penicillic acid	Synergistic	Penicillic acid inhibits carboxypetidase A thus, the degree of hydrolysis (OTA→Ota) is reduced
Aflatoxin B1 (AFB1)	No interaction	
Fumonisin B1 (FB1)	Combined, Synergistic	Similar toxic events induced by both toxins
Zeralenone (ZEA)	Antagonistic	The mechanism still remains unknown
Tricothecenes (T_2)	additive	Some workers found antagonistic effects or no interaction.
Deoxynivalenol (DON)	No interaction	

1.4.2. OCHRATOXIN A DETECTION METHODS

Two main analytical methods have been described in the literature for mycotoxins analysis and OTA detection: (a) chromatographic methods and (b) immunologic ones. In chromatographic methods, the compound is extracted and cleaned-up (immunoaffinity columns are more often used; clean-up tandem assay columns suggested by Lobeau *et al.* (2005). The compound is then measured using a number of detectors (i.e. fluorescence, photodiode array, mass spectrometry). Overall, chromatographic detection is more complicated and time-consuming. Other methods for OTA detection mentioned in the literature are Liquid chromatography–Mass spectrometry (Lau *et al.*, 2000), capillary electrophoresis (Bohs *et al.,* 1995; Corneli & Maragos, 1993), ion exchange chromatography (Breihtoltzemanuelssson *et al.,* 1998), electrospray mass spectrometry (ESMS: Jornet *et al.,* 2000) and Thin Layer Chromatography (Larson & Moller, 1996; Valente, 1998; Shermna, 2000). According to Soleas *et al.* (2001), the Gas Chromatography–Mass spectrometry (GC-MS) method has "poor sensitivity, recovery and precision". Finally, the Reversed-phase High-Performance Liquid Chromatography with fluorometric detection (HPLC-FLD) is the most commonly used method world-wide for OTA analysis. Fluoremetric detection is implicated as a highly sensitive technique since OTA has natural fluorescence. HPLC-FLD is suggested by Commission Directive 2002/26/EC, (13 March 2002), as an official method for OTA detection in food products.

The immunologic analysis is based on the Enzyme Linked Immuno Sorbent Assay (ELISA) principle; it is popular thanks to both its simplicity and to "its availability of polyclonal and monoclonal antibodies against OTA" (Lobeau *et al,* 2005). However,

findings show that the detection limits reported by ELISA are usually higher than the chromatographic techniques.

Modern techniques which achieve very low detection levels of OTA are in development. Molecularly imprinted polymers and biosensors are new-promising methods (Magan, 2006). These methods are currently employed for the detection of mycological quality and mycotoxin potential in several foodstuffs such as barley and malt.

1.4.3. RECENT EUROPEAN LEGISLATIONS AND DETECTION LIMITS OF OTA FOR SEVERAL AGRICULTURE PRODUCTS.

The European Commission (EC) established maximum levels for OTA concentration in some of the most important OTA food sources; for cereals, dried vines fruits, coffee, wine and infant foods (Commission of the European Communities, 2002, 2004, 2005). Main foodstuff products with their permitted maximum OTA accumulation, as EC has recently set, are represented in Table 1.5.

Table 1.5. Summary of the European Commission regulation (EC) No 1881/2006 of 19 December 2006 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 (μg kg^{-1})
Unprocessed cereals (including rice and buckwheat) and derived cereal products	5.0
Raw cereal grains (including raw rice and buckwheat)	
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	Not set
cocoa products, liqueur wines, meat products, spices and liquorice.	

1.4.4. NATURAL OCCURRENCE OF OTA

Humans can be exposed to OTA contamination either by direct consumption of contaminated food products or by consumption of meat or derived products from "contaminated" animals. Thus, OTA has been extensively documented as a global contaminant of a diverse range of foods in the normal diet, including cereals (Solfrizzo *et al.,* 1998) and cereal-based foods, barley (Haggbloom, 1982), green coffee beans (Trucksess *et al.,* 1999; Joosten *et al.*, 2001; Taniwaki *et al.,* 2003) and roasted coffee (Studer-Rohr *et al.,* 1995; Van der Stegen *et al.,* 1997; Jorgensen, 1998; Otteneder and Majerus, 2001; Lombaert, 2002). Processing of coffee beans appears to reduce but not eliminate OTA (Tsubouchi *et al.,* 1987; Micco *et al.,* 1989; Heilmann *et al.,* 1999).

Moreover, a study carried out in Germany showed that 50-75% of roasted coffee contained between 0.3-0.6 μg OTA Kg^{-1} coffee (Petzinger, 2002). The same study reported that although black tea is free from OTA, 42% of childrens' herbal tea was found to have high levels of OTA (up to 10μg kg^{-1}). OTA has been detected in the final coffee brew as prepared by commonly used methods (Leoni *et al.,* 2000; Lombaert *et al.,* 2002). It has also been detected in cocoa and cocoa products (Matissek and Raters, 2000), figs, groundnuts, spices, chilli peppers (Thirunala *et al.,* 2000), and beverages such as beers and wine. Foodstuffs of animal origin, mainly poultry eggs, pork and milk, including human breast milk, have also been found contaminated with OTA (Walker, 1999). Furthermore, significant contamination of wine has been reported (Zimmerli and Dick, 1996; Visconti *et al.,* 1999). Wine has been suggested as an important possible source of OTA in human blood (Ueno *et al.,* 1998).

1.4.5. OCHRATOXIN A IN GRAPES AND IN THEIR DERIVATIVES (WINE, GRAPE JUICES AND VINEGAR)

Wine is one of the main (second) sources of OTA (15% of OTA intake) in human blood (Ueno, 1998), after cereals, due to its daily consumption by humans (CAC, 1998). In 1998, the Codex Committee on Food Additives and Contaminants pointed out that traces of OTA were found in Swiss table wines for the first time in 1995 (Zimmerli and Dick, 1996). Since then, several surveys in different European countries as well as in Morocco, Japan and Australia have confirmed the frequent presence of OTA in grapes and wine (Ueno *et al.*, 1998; Burdaspal and Legarda, 1999; MAFF, 1999; Festas *et al.,* 2000; Filali *et al.,* 2001; Markaki *et al.,* 2001; Pietri *et al.,* 2001, reviewed by Belli *et al.,* 2002; Serra *et al*., 2003). Table 1.6 shows findings of previous studies concerning OTA contamination in wine. Many workers pointed out that red wines have more potential for OTA hazard (Visconti *et al.*, 1999; Filali *et al.*, 2001; Belli *et al.*, 2004a).

Table 1.6. OTA contamination in wines.

Origin	Kind of wine	Year	Percentage of samples contamination	OTA concentration	Reference
Spain	red	1997	85%	>0.05 ng ml^{-1}	Lopez de Certain (2002)
	white	1998	15%	>0.05 ng ml^{-1}	
_	red		54%	25-1153 ng l^{-1}	Otteneder and Majerus (2000)
	rosé		40%	119 ng l^{-1}	Review
	white		25%	12-108 ng l^{-1}	
Greece	red dry (n=104)		no stat.significant	median=0.09 µg l^{-1}	Stefanaki *et al,*. (2003)
	white (n=118)		no stat.significant	median=0.06 µg l^{-1}	
	rosé (n=20)		no stat.significant	median=0.09 µg l^{-1}	
	dessert wine			0.33 µg l^{-1}	
	retsina			0.27 µg l^{-1}	
Sweden	_	1999/00		< 0.1-19.0 µg kg^{-1}	Moller and Nyberg, 2003
	_	1999/00		< 0.1 – 34.6 µg Kg^{-1}	
South Africa	Wine (n=24),			0.04 and 0.39 µg L^{-1}	Shephard *et al,*. (2003)
Italy	Wine (n=8)			> 0.5 µg Kg^{-1}	
Rio de Janeiro, Brazil	grape and frozen pulps			positive for OTA	Da Rocha Rosa *et al..*, 2004

Several studies in grape juices confirmed OTA presence. Zimmerli and Dick (1996) working with commercial grape juices found that red juices contained more OTA concentration (<3 to 311 ng L^{-1}, mean of 188 ng L^{-1}) than white ones (<5 ng L^{-1}). Other studies indicated ochratoxin A accumulation in grape juices in a range of 15 to 102 ng L^{-1} (Burdaspal and Leganda, 1999) or in <20 to 2,050 ng L^{-1} (Ministry of Agriculture, Fisheries and Food, 1999). Regarding to red grape juices, in the same study, only 8 out of 73 samples had no OTA contamination while the toxin was ranged from <10 to 5,300 ng L^{-1}, in the rest of the samples (Wolff *et al.,* 2000). The same year, OTA was detected in vinegar (*ca* 220 ng L^{-1}) by Majerus *et al.* (2000) while samples of balsamic vinegar were the most OTA contaminated (*ca* 3110 ng). This is in agreement with a Greek survey (Markaki *et al.,* 2001) where 15 vinegar samples were analyzed. The findings showed that OTA accumulation was ranged between 8-46 ng L^{-1} in 12 samples while three of balsamic vinegars have OTA in excess of those amounts (102-252 ng L^{-1}).

In addition, black asperiglli and most specific ochratoxigenic species of *A. carbonarius* and *A. niger* aggregate have been isolated from grapes in Europe (Sage *et al.*, 2002; Abarca *et al.*, 2003; Batillani *et al.*, 2003b; Belli *et al*., 2004; Tzamos *et al.*, 2004; Dekanea, 2005; Mitchell, 2006). Simultaneously, black aspergilli have been found in grapes from other parts of the world like Australia (Leong *et al.*, 2004) and South America (Da Rocha Rosa *et al.*, 2002). The above demonstrates the world-wide character of black aspergilli and OTA contamination in grapes. Many studies pointed out black aspergilli is one of the dominant fungal genera of grapes since they appears to increase just before harvesting (Battilani *et al.,* 2003a; Serra *et al.*, 2003; Belli *et al*., 2004, Dekanea, 2005; Leong *et al.,* 2006).

1.4.6. FUNGAL CONTAMINATION AND OTA IN DRIED VINE FRUITS

Black *Aspergilli* and more specifically, *A. carbonarius,* are primarily responsible for OTA contamination in dried vine fruits (Battilani *et al.,* 2004). The method of removing water from grapes, during drying, probably influences the type of fungal community on the grapes. Methods that usually use enclosed high temperature and strong sunlight generally create conditions not conducive to fungi. However, black *Aspergilli,* including *A. carbonarius,* are tolerant of such conditions. Black spores may provide protection from UV light and high temperatures (Abarca *et al.,* 2003).

The highest OTA content, among grapes and its derivatives, has been measured in dried vine fruits (MAFF, 1997) with >40 μg kg^{-1}. MacDonald *et al.* (1999) found a maximum level of 53.6 μg Kg^{-1} in black dried vine fruits (currants). In Greece, Stefanaki *et al.,* (2003) analysed 81 samples of raisins from the 1998/99 and 1999/2000 vine crops. According to these findings, OTA was detected in excess of 0.5 μg Kg^{-1} in 87% (average of 2.8 μg Kg^{-1}) and 63% (average of 2.1 μg Kg^{-1}) of the currants and sultanas, respectively. OTA was detected in 67.7% of the black and 84.2% of the white dried vine fruits from Argentinean markets with mean levels of 6.3 and 4.42 ng g^{-1}, respectively (Magnoli *et al.,* 2004). The highest concentration was found in black dried vine fruit samples (14 ng g^{-1}). However, there have been practically no detailed studies of the contamination by *A. carbonarius* between harvest and the final dried vine fruit products. There is also little knowledge of the colonisation patterns and its relationship with moisture conditions and OTA contamination.

1.5. COMMON FUNGI ON GRAPES AND DRIED VINE FRUITS

Tournas *et al.* (2005) examined various types of grapes (red seedless, red seeded, green seedless, black seedless and black seeded) purchased from local markets in Washington. They isolated *Aspergillus carbonarius,* yeasts*, Fusarium spp., Botrytis cinerea, Alternaria spp, Cladosporium spp, Penicillium spp. and Rhizopus spp*. Similarly, a survey in Spanish wine grapes (Bau *et al.,* 2005) using direct plating isolation method, pointed out that the predominant mycobiota, isolated at the last development stage of the berries, was predominantly *Alternaria* spp. (75,6% frequency of isolation), *Cladosporium* spp. (22,5%), black *Aspergillus* spp. (17,3%), *A. carbonarius* (3,6%), *A. niger* aggregate (16,9%) and *Penicilium* spp. (13.8%-18.5%) but no *P. verruccosum*.

Medina *et al.* (2005) conducted a similar survey on Spanish varieties (44 red and 8 white grape ones), during harvesting. Eight fungal genera were identified; *Alternaria, Cladosporium, Aspergillus, Acremonium, Penicillium* excluding *P. verruccosum, Fusarium, Rhizopus* and *Phoma* with the first two genera predominant. This is in agreement with a study from Argentina that showed clear dominance of *Alternaria* spp. (Magnoli *et al.,* 2003) while some other workers have not isolated *Aspergillus carbonarius* at all (Abrunhosa *et al.*, 2001). Although the majority of the reports on grapes indicates the dominance of *Aspergillus niger* aggregate among *Aspergillus* species, Da Rocha Rosa *et al.* (2002) and Belli *et al.* (2004c) referred to *A. ochraceus* as the most frequently isolated fungus from grapes originated from Brazil and Spain, respectively.

In dried vine fruits black Aspergilli are referred to as the dominant fungal genera. Abarca *et al.* (2003) worked on sultanas, currants and raisins. Using Dichoran Rose Bengal Cloramphenicol agar (DRBC), they isolated *A. niger* aggregate (79.8%), *A. carbonarius* (28.5%) and *Mucorales* (23%). Magnoli *et al.* (2004) worked on 31 black and 19 white samples of dried vine fruits which indicated that black aspergilli were the predominant fungi; *A. niger* var *niger* (75%), *A.niger* var *awamori* (80%) and *A. carbonarius* (45%). A recent study conducted by Dekanea (2005) is in agreement with the above, and showed that black *Aspergilli* were the predominant fungal species on Greek currants. While many studies have been conducted of retail samples, practically none have followed the dynamics of fungal populations, diversity and relative frequency of isolation from harvest, during drying to industrial processing of sultanas.

1.6. RAISINS (DRIED VINE FRUITS)

Raisin is the name of the product that is derived from the vine grapes after drying. The main grape varieties used for raisin production worldwide are all of *Vitis vinifera* cultivars. In Greece, two vine cultivars are used for drying and thus, two types of raisins are produced; (a) small dark seedless Black Corinth or Zante type grape that gives currants raisins and (b) Sultanina grapes are used to produce "sultanas" or "sultana type raisins". This white seedless cultivar (otherwise known as Thomson Seedless) is also used in many other countries (Australia, Turkey, Iran etc) to produce sultanas or other types of raisins.

Sultana raisins are produced from sun-dried Sultanina grapes. Grapes are hand picked when ripe with 20-24 % sugar content. The grapes are usually dipped in or sprayed

with 5-7% K_2CO_3 and 0.5% olive oil or 2.5% K_2CO_3 and 2% ethyl oleate before drying. This pre-treatment accelerates the drying rate of the grapes resulting in a better quality product. The grapes are placed on wire-racks and are covered by plastic films. After drying, (water content <16% w/w), raisins are separated from the main stalks and are stored in plastic bins.

During industrial processing, Sultana raisins are thoroughly washed, sometimes sprayed with sulphur dioxide and dried in a convective air drier at 60-80^{o} C to a water content of 14-15% (w/w). May then be given a light coating of special vegetable oil to prevent stickiness and facilitate pedicle removal. Air blowers and sieves are used to remove foreign matter (vegetative parts, hollow fruits, small stones etc.) and magnets to trap metallic objects. Raisins are then sorted either by hand or using automatic systems to remove defective berries (dark coloured, diseased or infested etc.) and are classified into different types according to their size and colour. Finally, raisins are packaged in small laminated bags (200-1,000 g) or in polyethylene bags (5-15 Kg) and placed in carton boxes. The content of each package must be uniform and contain raisins of the same size, production year and quality.

It is crucial to keep a high standard of hygiene. The chemical residues and natural contaminants in the final product (sultanas) should be within the established European levels. Moreover, both substrate, on which the molds grow, and the presence of competitive microflora interacts to influence the level of mycotoxin produced (Bennett *et al.,* 2003; Magan and Aldred, 2007). The composition of mycoflora is also influenced by climatic conditions and cultural practices such as irrigations, pruning regime, pesticides or fungicides, harvest time, winemaking process and

different conditions of storage (Heilman *et al.,* 1999; Batillani *et al.,* 2004; Leong *et al.*, 2007).

1.7. ABIOTIC AND BIOTIC FACTORS THAT THEY INFLUENCE OTA ACCUMULATION AND FUNGAL GROWTH

It is well known that in nature, a range of both spatially and temporally abiotic and biotic factors influence fungal activity (Magan, 1997) and consequently, mycotoxin accumulation in foodstuffs during storage, or even before harvest time. The list of these factors includes contaminant moulds and their respiratory action, water content, temperature, gas composition, substrate pH and nutrients, insects and mites (Magan *et al.,* 2003; 2004). There is no doubt that growth rate and thus OTA biosynthesis is influenced by the interaction of those abiotic and biotic agents, but the question that has been arisen is how and in which degree these factors influence growth of *A. carbonarius* and OTA production.

1.7.1. WATER ACTIVITY (A_W)

Water constitutes the most critical agent for every kind of life. Numerous detailed reviews have established the main role of water for fungal germination, growth and development. Water activity (a_w) is a measure of the amount of water available for microbial growth and hydration of materials (see Figure 1.6). It is an accurate "tool" for controlling microbial growth and expanding shelf-life of food products.

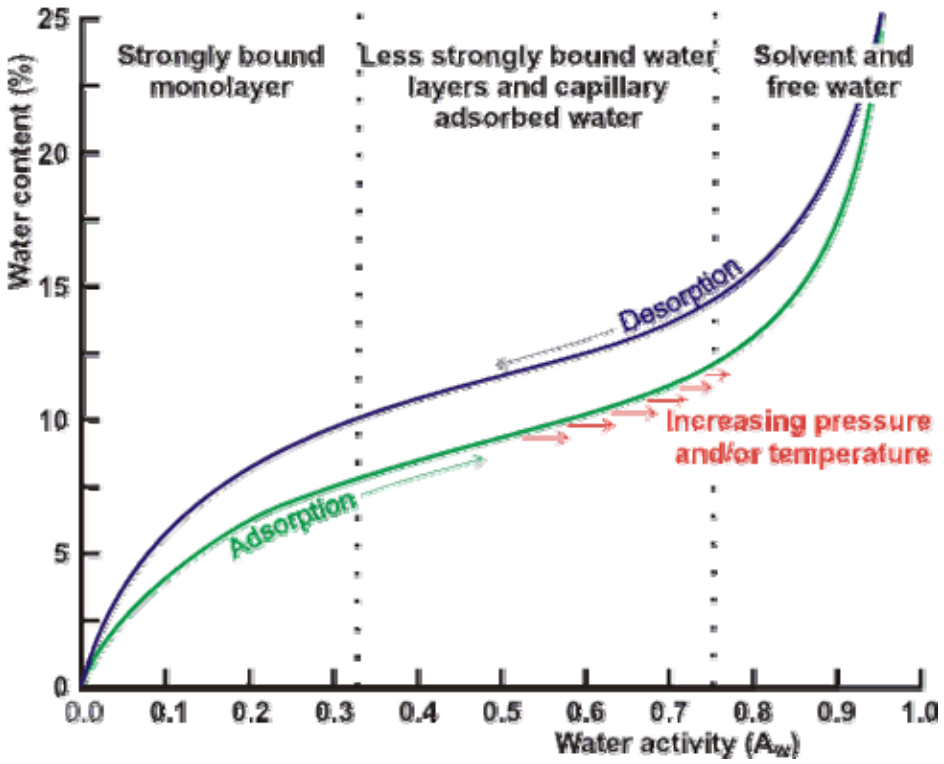


Figure. 1.6. An indicative water activity isotherm displaying the hysteresis often encountered depending on whether the water is being added to the dry material or removed (drying) from the wet material (Seiler, 1976).

It 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) (see Table 1.7).

$$Aw = p/p_o = ERH\ (\%)/\ 100 \quad \text{(Vos and Labuza, 1974)}$$

Aw : Water activity

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.

Table 1.7. Water activity, equilibrium relative humidity (E.R.H) and water potential at 25°C

Water activity	E.R.H. (%)	Water Potential (-MPa)
1.00	100	0
0.99	99	1.38
0.98	98	2.78
0.97	97	4.19
0.96	96	5.62
0.95	95	7.06
0.90	90	14.50
0.85	85	22.40
0.80	80	30.70
0.75	75	39.60
0.70	70	40.10
0.65	65	59.30
0.60	60	70.30

(Source: Naresh Magan, 1997. Fungi in extreme environments)

Blandamer *et al.* (2005) recently reviewed a_w and pointed out particular relevance in food chemistry and presentation methods (see Table 1.8). According to Burrington (Food Product Design, 1998): "Development of many baked products involves maximizing moisture content to produce the best possible eating qualities while minimizing a_w**.;** lowering the a_w increases product stability in terms of susceptibility to microbial growth**.**" To be more specific, water activity influences several stages of fungal development such as germination, growth, sporulation and mycotoxin production. Each toxigenic fungus has a different moisture and temperature requirements for these phases (Lacey and Magan, 1991; Magan *et al.* 2004). Moreover, low levels of pH (<4) at lower water activities (<0.90 a_w) could efficiently result in fungal inhibiting and thus in a safe storage of food. Leake (2006) showed that at a_w levels below 0.92, only pH values > 4.2 could present potential microbiological hazards in food products without any heat treatment.

Table 1.8. Several levels of water activity which result in microorganism's inhibition (adapted from Beuchat, 1984).

a_w levels	Microbes inhibition by aw at this point	Examples of foods within water activity
0.950	*Pseudomonas, Escherichia, Proteus, Shigella, Klebsiella, Bacillus, Clostridium perfringens,* some yeasts	Highly perishable foods (fresh and canned fruits, vegetables, meat, fish) and milk; cooked sausages and breads; foods containing up to 4oz (w/w) sucrose or 7%NaCl
0.910	*Salmonella, Vibrio parahaemolyticus, C. botulinum, Serratia, Lactobacillus, Pediococcus,* some molds, *Rhodotorula, Pichia*	Some cheese (Cheddar, Swiss, Muenster, Fermented sausage (salami); sponge cakes; dry cheese; margaring; foods containing 65% (w/w) sucrose (saturated) or 15%NaClProvolone); cured meat (ham); some fruit juice concentrates; foods containing 55% (w/w) sucrose or 12%NaCl
0.870	Most molds (mycotoxigenic penicillia), *Staphylococcus aureus,* most *Saccharomyces (baillii) spp., Debaryomyces*	Most fruit juice concentrates; sweetened condensed milk; chocolate syrup; maple and fruit syrups; flour; rice; pulses containing 15-17% moisture; fruit cake; country style ham; fondants; high-sugar cakes
0.750	Most halophilic bacteria, mycotoxigenic aspergilli	Jam, marmalade; marzipan; galcé fruits; some marshmallows
0.650	Xerophilic molds (*Aspergillus chevalieri, A. Candidus, Wallemia sebi*), *Saccharomyces bisporus*	Rolled oats containing ~10% moisture; grained nougats; fudge marshmallows; jelly; molasses; raw cane sugar; some dried fruits; nuts
0.600	Osmophilic yeasts (*Saccharomyces rouxii*), few molds (*Aspergillus echinulatus, Monascus bisporus*)	Dried fruits containing 15-20% moisture; some toffees and caramels; honey
0.500	No microbial proliferation	Noodles, spaghetti, etc. containing ~12% moisture; spices containing ~10% moisture
0.400		Whole egg powder containing ~5% moisture
0.300		Cookies, crackers, bread crusts, etc. containing 3-5% moisture
0.030		Whole mild powder containing 2-3% moisture; dried vegetables containing ~5% moisture; corn flakes containing ~5% moisture; dehydrated soups; some cookies and crackers

The influence of a_w on growth and OTA accumulation by contamination of *A. carbonarius* and the *A.* section *Nigri* has been studied on grapes. For example, Belli *et al.* (2004) conducted experiments on synthetic grape juice medium and found that the optimum incubation time for OTA accumulation of *A. niger* aggregate isolates depended on a_w (0.98–0.995 a_w) and was between 5-13 days, respectively. In contrast, the maximum OTA accumulation by *A. carbonarius* was recorded at a_w levels of 0.96 combined with an incubation period of 5 days (Belli *et al.,* 2004).

Mitchell *et al.* (2004) used strains of *A. carbonarius* from grapes from a range of southern European countries and determined the lag phase prior to growth, growth rates and OTA production on synthetic grape juice medium. According to these findings, the optimum conditions for OTA production were different from those for growth. More specifically, the lag phase increased from <1 day at 25-35^{o} C and 0.985 to 0.95 a_w to >20 days at marginal temperatures and water availabilities (0.93-0.88 a_w). The optimum a_w for growth varied from 0.97-0.987 which is similar to the a_w of grapes in the field, depending on the strain. Interestingly, *A. carbonarius* growth rate was significantly lower than those of *A. niger* aggregate and uniseriate isolates, with the *A. niger* aggregate having the faster growth rates (Mitchell *et al,.* 2004). Esteban *et al.* (2006) showed that water activity levels (0.86-0.99) significantly affected growth and OTA biosynthesis for several *A. niger* species. However, very little knowledge is available, in relation to the comparison of fungal strains isolated from vine fruits and currants or sultanas, to determine whether they are ecologically similar or different.

1.7.2. SULPHUR DIOXIDE (SO_2) AS A PRESERVATIVE

Sulphur dioxide (SO_2) is one of the oldest food additives. It was well known to the ancient civilizations such as the Greeks, Romans and Egyptians for its disinfectant properties. In these civilizations, SO_2 was obtained by burning the elemental sulphur and used the remaining ashes. In the wine industry, SO_2 have also been used as the main additive for wine preservation. Sulphur dioxide is a pungent, colourless gas, soluble in water and it remains a very important food additive due to its versatility. In general, salts of sulphurous acid (i.e. sodium and potassium metabisulphite, sodium and potassium bisulphate and sodium sulphite, (see Table 1.9) are usually used by the food manufacturing industry as antimicrobial agents, antioxidants, inhibitor of enzymatic and non-enzymatic browning reactions and finally, as bleaching agents (Kim, 1995) since they stabilize vitamin C. Their antimicrobial action is based on their ability to prevent growth of spoilage fungi or to inhibit spore germination, germ tube extension and to restrict mycelial growth (Magan & McLeod, 1986). The tolerance of fungi to high concentrations of SO_2 might due to their ability to transport SO_2 into their mycelia (Tweedie and Segel, 1970). However, it is not entirely clear what the relationship is between SO_2 depositions and microbial activity and why some microorganisms are very tolerant of SO_2 while some others are not.

Furthermore, several parameters such as the water activity, formulation of SO_2 (for example gaseous treatments), duration of the exposure, temperature, pH of the substrate and of course, interactions between each factor, influence efficacy of SO_2 application. In general, the antibiotic properties of all sulphites work better at pH of <4.5 while at higher pH levels, they effectively hind bacterial growth (Clark and Takacs, 1980). It has been shown that lowered pH alone may significantly reduce fungal germination of deuteromycetes (Magan & Lacey, 1984).

Table 1.9. Numbering of sulfites according to International Numbering System for food additives (INS), Codex Alimentarius Committee (CAC/GL 36-1989, Last amendment 2007).

INS. No	Name of Food Additive	Technical Function(s)
E220	Sulphur dioxide	Preservative, Antioxidant
E221	Sodium sulphite	Preservative, Antioxidant
E222	Sodium bisulphite (sodium hydrogen sulphite)	Preservative, Antioxidant
E223	Sodium metabisulphite	Preservative, Antioxidant, Bleaching agent
E224	Potassium metabisulphite	Preservative, Antioxidant
E225	Potassium sulphite	Preservative, Antioxidant
E226	Calcium sulphite	Preservative, Antioxidant
E227	Calcium hydrogen sulphite	Preservative, Antioxidant
E228	Potassium hydrogen sulphite	Preservative, Antioxidant

Appart from their desirable properties, sulphites have been found to cause various physiological problems (i.e. bone marrow atrophy, polyneuritis, damages to nervous system, reproductive organs). They are also implicated as initiators of asthmatic reactions (Congressional Hearing on Sulfites, 1985). In 2000, World Health Organization (WHO), in the frame of a world-wide discussion, set the acceptable daily intake (ADI) for sulphur dioxide at 0.7 mg Kg^{-1} of body weight (bwi). The European Parliament decided that food products with sulphites concentrations of >10 g Kg^{-1} should refer this on their labels (OJEV, 2003). However, in the United States, sulphite salts are characterized as "Generally Accepted As Safe" (GRAS; FDA/CFSAN, 1992) although they are not permitted in foods with thiamine (vitamin

B), like raw fruits or vegetables, because they destroy it. On the other hand, they can be used in fruit juices, dried fruits and wines. Overall, SO_2 is occasionally used during industrial processing of sultanas (dried vine fruits) mostly for colour lightening. Little information is available on the relationship between SO_2, germination and growth of *A. carbonarius* strains and their effect on OTA production.

1.7.3. MODIFIED CONTROLLED ATMOSPHERES STORAGE

Gas composition of atmosphere may influence the rate of fungal spoilage and thus the biosynthesis of several mycotoxins (Magan *et al.*, 2003). With this in mind, one can control the gas atmosphere surrounding a foodstuff (controlled atmospheres), at different stages of production or in storage, in order to inhibit growth of spoilage fungi and consequently, to decrease contamination with mycotoxins. For example, changing the proportion of atmospheric gases in an environment can influence the efficacy or the rate of spore germination of a fungus.

Paster *et al.* (1983) working with *A. ochraceus* on an agar-based media found that OTA production was completely inhibited by > 30% CO_2 after 14 days. Studies with *A. flavus* on groundnuts showed that aflatoxin production was effected by < 5% O_2 or by exposure to 20% and 25% CO_2, while exposure to 80% CO_2 resulted in a complete toxin production inhibition. The same study showed that a combination of a_w (0.86) and exposure to higher levels of CO_2 (40-60%) resulted in an almost complete control of aflatoxin in groundnuts at 5-10^o C.

Findings from a recent study with *P. verrucosum* on wheat grain (Cairns-Fuller *et al.*, 2005) showed that there is an interaction between a_w and CO_2 concentration which

affects growth and OTA production. Moreover, studies have also showed that germ tube length is significantly inhibited by 50% CO_2, especially at 0.90-0.95 a_w while spore germination is not affected (Cairns-Fuller, 2004). Fungal growth and OTA accumulation were respectively decreased by 25 and 50% CO_2 regardless of the a_w level tested.

Production of zearalenone by *Fusarium equiseti* was almost completely inhibited by >20% CO_2 with either 20% or 5% O_2 in grain (Paster *et al.,* 1991). Earlier *in vitro* studies with *Fusarium sporotrichioides* showed that T-2 toxin production could be reduced by 80% by exposure to 50% CO_2/20% O_2, growth was not affected by <60% CO_2 (Paster *et al.,* 1986; Paster and Menasherov, 1988). Generally, it seems that composition of a_w and CO_2 causes an enhanced inhibitory effect, although this is not synergistic (Cairns-Fuller *et al.,* 2005). No studies have examined the relationship between *A. carbonarius* growth, OTA production and controlled atmospheres and their interactions on sultanas.

1.7.4. TEMPERATURE, INCUBATION TIME AND pH

Preliminary surveys highlighted that temperature, incubation time and pH of the substrate, all play an important role on fungal growth and OTA production (Mitchell *et al.,* 2004; Belli *et al.,* 2004b,c). More specifically, fungi have the ability to grow over a wide range of temperature. As a result, fungi have been classified into four groups according to their behavior under several levels of temperature: psychrophiles, mesophiles, thermotolerant or true thermophiles (Magan, 1997). For instance, a thermophilic fungus can grow in a range of 20^o C (minimum) to 50^o C (maximum) temperature with a range of 40-50^o C as optimum temperature (Magan, 1997).

A. niger agreggate is able to synthesize OTA in a wide range of temperatures (10-37^{o} C) (Belli *et al.*, 2004) while the temperature range for OTA ability of *A. carbonarius* (key-fungus for OTA production on grapes) is more restricted, 15-20^{o} C (Mitchell *et al.*, 2004). Similarity, *A. niger* may grow at relatively high temperatures (maximum at 45–47^{o} C) while its optimal temperature is about 35-37^{o} C. On the other hand, *A. carbonarius* grows optimally at 30^{o} C (Belli *et al*., 2005) or 25-35^{o} C depending on the isolate (Leong *et al.*, 2004; Mitchell *et al.*, 2004). Some other workers pointed out that it can slightly grow at 35^{o} C, while at 41^{o} C no growth occurs (Palacios-Cabrera *et al.*, 2005). Significant growth reduction occurs at 42^{o} C (Leong *et al.*, 2004) and no growth at <15^{o} C or > 45^{o} C was observed (Mitchell *et al.*, 2004). Overall, the range of temperatures where *A. carbonarius* can grow is between 15-37^{o} C and the majority of *A. carbonarius* strains grew optimally at 25-30^{o} C, depending on the isolate. Moreover, there is an influence between abiotic parameters. For example, if a high level of Relative Humidity (RH) is combined with high temperatures in the field, the possibility for OTA synthesis in grapes is greater (Belli *et al.,* 2004).

Marin *et al.* (2006) examined the effect of incubation time (up to 10 days) and temperature (7-42^{o} C) on *A. carbonarius* growth and OTA accumulation on a grape-like medium. OTA production was higher at 20^{o} C after 10 days incubation, while at a higher temperature (30^{o} C) OTA was synthesized after 6-8 days. These findings indicate that maximum mean OTA-producing capacity was found earlier with increasing incubation temperatures. Esteban *et al.* (2006) demonstrated that, as the incubation time increased, OTA accumulation is decreased due to the fact that strains might remove and assimilate the phenylalanine moiety from the OTA molecule to use as a nitrogen source. According to Belli *et al. (*2005) an incubation period of seven

days is enough to allow OTA detection on a grape-like medium, produced by *A. carbonarius,* while only 5 days are needed for a maximum OTA accumulation on a synthetic nutrient medium.

In general, fungi can grow over a range of pH 4.0-8.5 but most filamentous xerophilic fungi favoured a range of pH 6.5-6.8 (Beuchat and Hocking, 1990). Moreover, most bacteria prefer near neutral pH levels (Tournas *et al.,* 2005). Gock *et al.* (2003) demonstrated that the optimum pH for all examined fungi was pH 4.5-5.5, regardless of temperature (25^{o} C, 30^{o} C, and 37^{o} C). Some *Aspergillus* and *Penicillium* can grow at lower levels of pH, i.e. pH=2.0 (Deacon, 2005). Studies conducted on some *Aspergillus* and *Penicillium* spp showed that the effects of potassium sorbate (a food preservative) is closely related with the pH levels; fungal growth inhibition was successful at pH = 6.0 (Guynot *et al.,* 2002). Recent findings have shown that *Aspergillus carbonarius* is able to synthize OTA in a wide range of pH levels, 2.0<pH<10.0 (Esteban *et al.*, 2005).

1.7.5. NUTRITIONAL COMPOSITION OF SUBSTRATES

Earlier studies have shown that nutritional source partitioning, specifically on the surface of the plant/habitat, could mediate microbial co-existence (Wilson and Lindow, 1994a,b). *A. ochraceus, A. carbonarius* and *A. niger* seem to grow differently on three culture media: Czapeck Yeast Extract Agar (CYA), Dichloran 18% Glycerol Agar (DG18) and Malt Yeast Extract 40% Glucose Agar (MY40G), indicating that the substrate may affect the fungal thermotrophic behavior (Palacios-Cabrera *et al.*, 2005). Complementary, the same study shows that *A. carbonarius* grew better at MY40G, as this has higher sugar content in comparison to the other two

media. Moreover, Esteban *et al.* (2006) demonstrated that the Czapek yeast autolysate (CYA) agar is a better culture medium than the Yeast Extract Sucrose (YES) agar for OTA production by *A. carbonarius.*

Overall, nutrient status should be taken under consideration since it could be used to determine Niche Overlap Indices (NOI) (Wilson *et al.,* 1994a). The NOI is a ratio of the number of similar C-sources utilized and those unique to an individual isolate or species. Thus, using NOI, a value of "0" to "1" is obtained. NOI of <0.9 indicates that there is a co-existence between species in an ecological niche while NOI>0.9 shows an occupation on separate niches. As a result, the composition of microbial population may change the fungal dominance. According to the latest findings, rate growth, biosynthesis of mycotoxin, niche occupation and finally competitiveness by mycotoxigenic species are impacted by the presence of other spoilage fungi (Magan, 2003).

1.7.6. CLIMATE – GEOGRAPHICAL REGIONAL CONDITIONS

Climatic conditions and different geographical regions may affect considerably mould contamination and thus, OTA biosynthesis (Zimmerli and Dick, 1996; Pietri *et al.,* 2001; Lopez de Cerain *et al.*, 2002; Stefanaki *et al,* 2003). Based on previous studies, the growing-zone (distinct climatic conditions) influences OTA contamination in wine, resulting in different OTA occurrence from northern to southern regions, especially in red varieties due to its winemaking process (Otteneder and Majerus, 2000). In overall, *P. verrucosum* is the key fungus for OTA production in temperate and cold climates while *A. ochraceus* in cereals, and *A. carbonarius* in grapes, are

more commonly associated with tropical and sub-tropical regions, like the Mediterranean basin (Dekanea, 2005; Bau *et al.,* 2005).

Battilani *et al.* (2006) conducted a survey on Italian grapes originated in several parts of Italy. The above study has showed that the highest levels of ochratoxin A were detected in samples from the South region, at harvest time. On the other hand, the populations of black aspergilli had decreased at the ripening stage, due to the high level of yeast growth. Meteorological conditions as well as the year, the cropping system, the age of the plants, time of harvesting and use of fungicides seem to play an important role to fungal colonization and OTA contamination. According to Batillani, *et al.* (2006), there is a significant correlation of berries colonized by *A. carbonarius* at harvesting, positive with the summation of degree-day and negative with the summation of rain between early veraison and ripening.

1.7.7. FUNGAL INTERACTIONS

Fungi usually occur as a mixed consortium of several microorganisms (i.e. yeasts, filamentous fungi, bacteria) or inspects and therefore inter-specific and intra-specific interactions take place depending on the prevailing environmental conditions or nutritional status of the habitat. Subsequent interactions between spoilage fungi result in combat, antagonism and niche overlap which all influence secondary resource capture (Magan *et al.*, 2003). Cooke and Whipps (1993) demonstrated that fungi have several methods of occupying a niche, which are the followings; combative (C-selected), ruderal (R-selected), stress (S-selected) or merged secondary strategies (C-R, S-R, C-S, C-R-S).

Magan and Lacey (1984b, 1985) developed an Index of Dominance (Table 1.10) According to this Index, a numerical score indicates the fungal capability of dominance *in vitro*. This score is obtained by adding the scores of each species. It has been shown that this Index might be significantly influenced by some abiotic parameters like levels of a_w, temperature or substrate. For example, *A. ochraceus* is dominant fungal species against A. *flavus* and *A. candidus* at 18^o C *in situ* but it loses its dominance against the aforementioned fungi when the temperature rises to 30^o C. Moreover, several studies *in vitro* and *in situ* indicate that fungal interactions markedly influence mycotoxin accumulation by inhibiting or stimulating its production (Marin *et al.,* 1998b; Lee and Magan, 2006b).

Table 1.10. Score of Index of Dominance (I_D) indicating fungal overlap, as it is suggested by Magan and Lacey (1984b).

Score	Kind of fungal overlap/ interaction
1:1	Mutual intermingling
2:2	Mutual antagonism on contact
3:3	Mutual antagonism at a distance
4:0	First species dominant over the other on contact
5:0	First species dominant at a distance over the other

1.8. OBJECTIVES

The objective of this study is to investigate the accumulation of Ochratoxin A (OTA) production in dried vine fruits (sultanas). OTA accumulation in the field and in the processing stage will also be studied. *A. carbonarius,* which is the key-fungus for OTA production in grapes and in their derivatives, such as sultanas, in sub-tropical

and tropical regions like Greece, is the main examined fungus under *in vitro* and *in situ* conditions. Other black aspergilli (*A. niger* aggregate) were also studied in some trials. This study attempted to identify the parameters both at pre-harvest and post-harvest phases that influence OTA production by the mentioned ochratoxigenic fungi. Overall, *A. carbonarius* ecology, which is the main ochratoxigenic fungal species in grapes and sultanas, as well as the role of some environmental factors in OTA biosynthesis were examined. This study can be used by decision makers to develop potential control approaches for minimal OTA contamination in sultanas.

Objectives of this project were:

- To determine the temporal changes in the fungal mycoflora a) in fresh grapes, just prior to harvest time (b) in sultanas, during drying period and (c) in sultanas during industrial process.

- To investigate the effect of altitude, of bunch position on the plant and of water activity (a_w) of the habitat on fungal populations dynamics in grapes, just prior to harvesting.

- To evaluate the effect of both altitude and bunch position on the plant on ochratoxin A accumulation in grapes, just prior to harvest time.

- To investigate both the temporal and the altitude effect on fungal diversity in sultanas during sun-drying period, in the fields.

- To understand both the temporal and the altitude effects on OTA contamination in sultanas during the sun-drying process.

- To determine whether *A. carbonarius* is the most dominant ochratoxigenic fungus in fresh grapes and sultanas originating from Cretan vineyards and generally from Mediterranean regions.

- To examine the effects of SO_2 and a_w on lag phase prior to germination, on germination, mycelial growth and OTA production.

- To examine the effects of gas atmospheres and a_w on lag phase prior to germination, on germination, on mycelial growth and on OTA production.

- To investigate the effect of fungal competition on *A. carbonarius* growth using the Index of Dominance and OTA production.

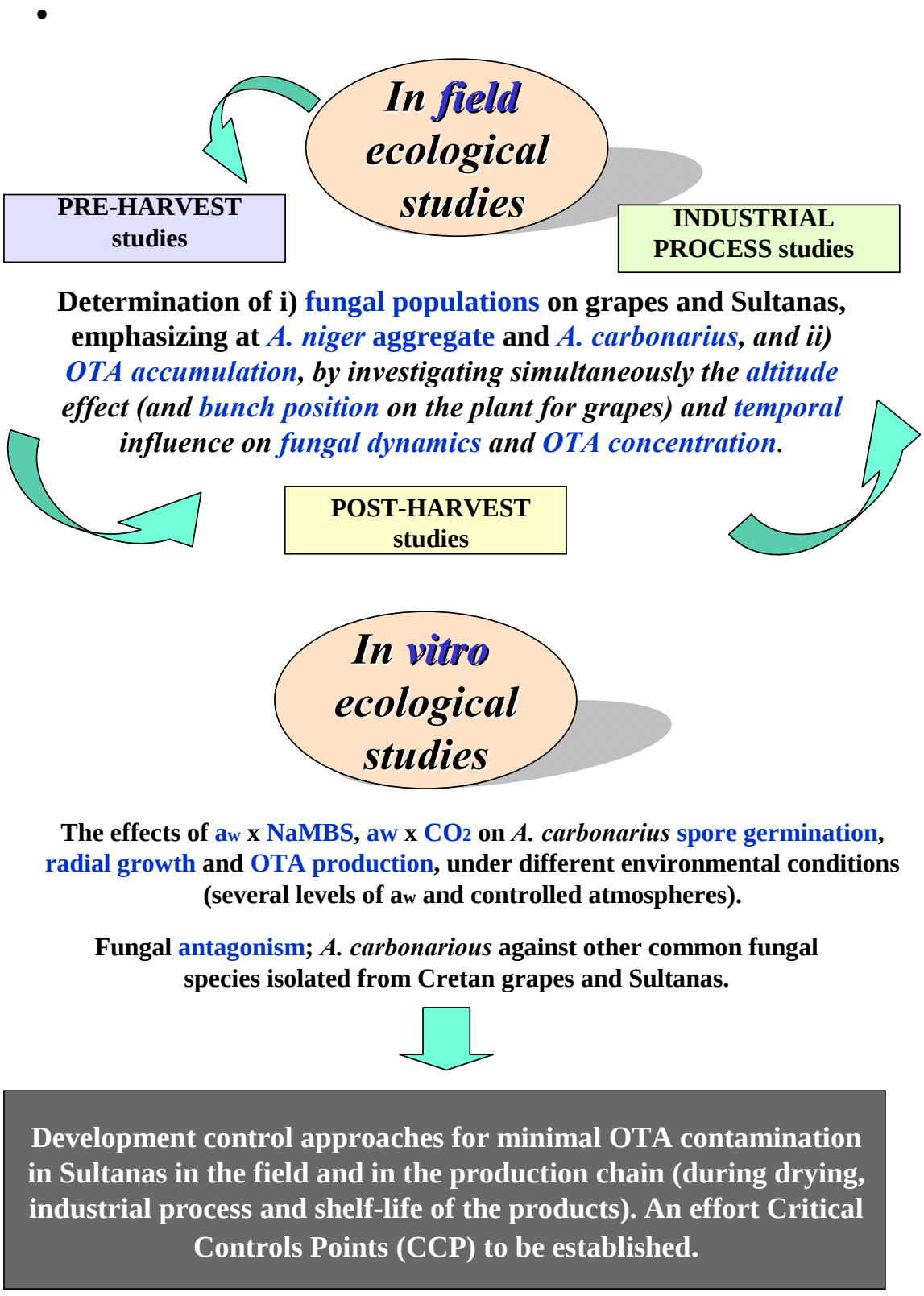


Figure 1.7. Flow chart of different components of thesis studies.

Chapter Two

FIELD STUDIES

2.1. INTRODUCTION AND OBJECTIVES

There has been interest in the development of approaches to minimize ochratoxin A (OTA) contamination of numerous agricultural products in the field before industrial procedures take place. These could result in the protection of the consumer's health by decreasing the toxin accumulation with the aid of integrated management of production and processing in general.

Several studies have been conducted with the aim of assessing the risk of ochratoxin A (OTA) in grapes and in their beverages (see Int. J. Food Microbiol., Special Issue, 2005). However, there is little knowledge of the populations of ochratoxigenic fungi associated with grapes at harvest time and during drying and production of dried vine fruits.

The aim of this field work study was to determine some of the Critical Control Points (CCP) for OTA biosynthesis under field conditions and during the vine fruit drying process. This would enable the identification of the most dominant fungal populations on grapes and on drying sultanas, with an emphasis on the population dynamics of black aspergilli (*A. niger* aggregate and *A. carbonarius*). Furthermore, the effect of some abiotic parameters (i.e altitude) on OTA production was also assessed. This is the first study to determine the fungal population dynamics of both Sultanina and drying sultanas, and OTA production, in association with altitude and bunch position, in the area of Crete.

2.2. MATERIALS AND METHODS

2.2.1. SAMPLING REGIMES USED

2.2.1.1. Pre-harvest sampling

All samples originated from Cretan vineyards, with the Sultanina (*Vitis vinifera L.)* cultivars. Samples were selected from the region of Iraklion situated in the northern part of Crete, a characteristic production region of sultanas, in two consecutive cultural periods, 2004/05 and 2005/06. Crete is an island at the south part of Greece (Plate 2.1. in APENDIX A). On the piloted trials of the first year, three vineyards with different environmental properties but with the same cultural treatments were chosen according to their position. Three altitudes were used: (a) Ag. Thomas's hillsides, *ca* 700 m.; (b), Asites: 500 m., and (c) Karteros at sea level (Plate 2.2 in APENDIX A).

In the second cultural season (2005/06), the experimental plots were modified. As a result, nine representative vineyards with the same cultural treatments were chosen according to their position. Three of the vineyards were near the sea (sea level), three of them were situated in fields at intermediate altitudes (*ca* 250 m .- 500 m above sea level) and the final treatment was at higher altitudes (*ca* 510 m – 680 m above the sea level) (Plate 2.2 in APENDIX A).

In both years, sampling took place five days prior to harvest time. Each vineyard was divided into three blocks (see Figure 2.1). From each block, three sorts of sub-samples of *ca* 1 Kg were randomly collected (Plate 2.1):

(i) Bunches in the sunshine and usually at the top parts of the plants.

(ii) Bunches that were in the shadow and chiefly situated at the middle and lower parts of the plants.

(iii) Bunches touching the soil.

Nine sub-samples in total were taken from each sampling area. A total of eighty-one samples were examined. Moreover, the total sample was obtained from different points (approximately 100 points) in the block area with an attempt to cover the whole sampling area. All samples were stored at -20^{o} C until all analyses to be occured.

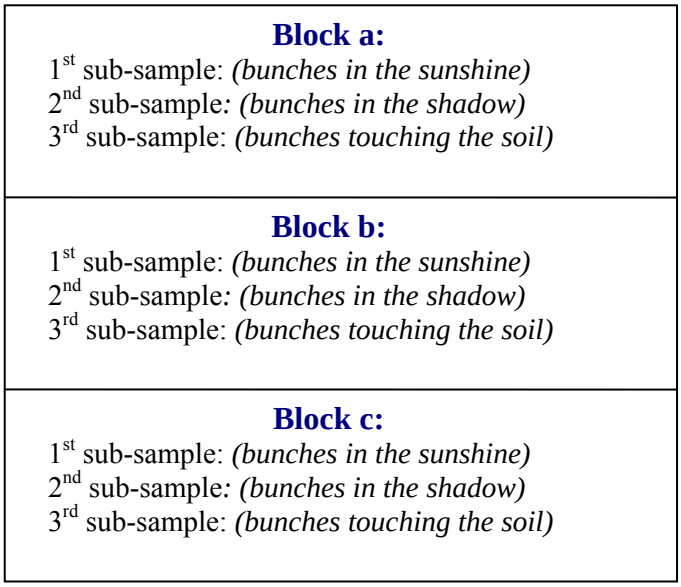


Figure 2.1. Replication and sampling pattern at each sampled vineyard.

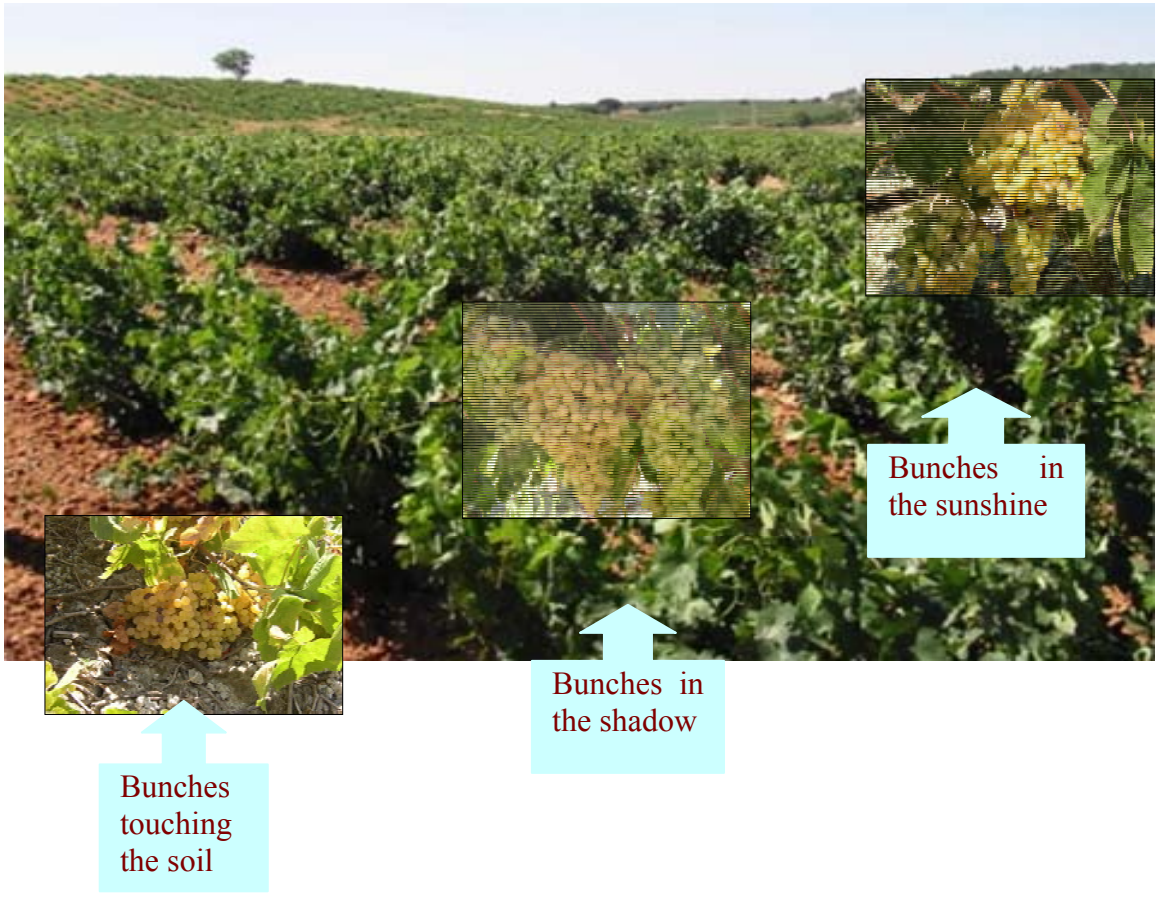


Plate 2.1. Sub-sampling pattern of each block. Bunches were selected according to their distance from the ground and to their exposure to the direct sunlight.

2.2.1.2. Post-harvest sampling

There is interest in the population dynamics of black *Aspergilli* with an emphasis on *A. carbonarius* during drying; hence sampling was carried out at three stages during the drying process; at harvesting = day 0, at the 5^{th} and 10^{th} day of sun-drying. Drying was usually completed on the 12^{th} – 14^{th} days. Representative post-harvest samples (*ca* 1 Kg) were obtained from several points (approximately 10 points) of sultanas originating from the same vineyards as the pre-harvest ones (Plate 2.2). All samples were stored at -20^{o} C until analyses. The same sampling pattern was performed in both field trials (2004/05 and 2005/06).

Plate 2.2. A drying area where sultanas were sun-dried.

2.2.2. MICROBIAL ASSESSMENT

2.2.2.1. Mycological analysis of grapes

Fungal identification of black *Aspergilli* was carried out at a species level only for *A. carbonarius*, while the others isolates were grouped as the *A. niger* aggregate. Identification was based mainly on the physical appearance of the anomorph colony (section 1.2.) by using coplimentary stereoscope and optical microscope. Conidial size and ornamentation were some of the most important characters for *A. carbonarius* identification. The aim was to identify the percentage of *A. carbonarius* out of the *A. niger* group and the total fungal mycoflora before sun-drying of the grapes (Plate 2.3).

To determine the mycoflora on grapes before sun-drying, two identification media were used: Malt Extract Agar (MEA, Oxoid Ltd) that was made as directed with a water activity 0.985 a_w (MEA98) and that modified with glycerol to 0.954 a_w (MEA95) (see Table 2.1. in APPENDIX A). In both media a small amount of

chloramphenicol was added prior to sterilization at 121° C for 15 minutes, to inhibit bacterial growth.

Plate 2.3. Sultanina contaminated by black aspergilli.

Each sub-sample (*ca* 1 Kg) was analysed twice; 30 g sub-samples were taken and suspended in 270 ml diluent (sterile-distilled water + 0.5 g agar + 0.005% Tween 80) and homogenized in a Seward 200 Stomacher for ~ 5 min. The mixture was then serially diluted. From each dilution, 0.2 mL was spread-plated using a sterile glass spreader onto the surface of both media (three replicates of each). All transfers were made with an automatic pipette and disposable sterile tips.

Complementary to serial dilution, fifteen berries per bunch were randomly selected and direct plated onto MEA98 and MEA95. The berries were aseptically cut in half before plating in Petri plates (Plate 2.4). All the Petri plates were incubated at 25º C. After 7 days the number of colonies in the serial dilution method were counted and reported as Colony-Forming Units (CFU) g^{-1} of grape.

Plate 2.4. Direct plating isolation method on MEA97. Berries aseptically cut in half.

2.2.2.2. Mycological analysis of drying sultanas

Fungal identification of black aspergilli was carried out to species level only for *A. carbonarius*. The others isolates were grouped in the *A. niger* aggregate. Fungal biodiversity, population loads and relative frequency of isolated fungal species, emphasizing to black aspergilli, during production of sultanas, were investigated. Biodiversity was determined using Shannon (H) Index. This index is commonly used to measure species diversity. It accounts for both abundance (evenness) and richness of the species present. The index is increased either by having additional unique species or by having greater species evenness. It can be calculated by the following formula:

$$H = -\sum_{i=1}^{S} p_i \, \mathrm{Ln} p_i$$

H : Shannon Index

n_i : Abundance of each species (the number of individuals in each species)

S : Richness of each species (the total number of species)

p_i : The relative abundance of each species, calculated as the proportion of individuals of the given species to the total number of individuals in the community (N): (n_i/N)

On harvesting, the same identification media was used as described at pre-harvest studies: Malt extract agar (MEA98) and modified medium to 0.954 a_w (MEA95). In addition, Malt extract agar media modified to 0.87 a_w (MEA87) and 0.83 a_w (MEA83) were also used for fungal assessments at the 5^{th} and 10^{th} day of drying, respectively. They were prepared by adding the appropriate glycerol concentrations. This was to determine the fungal population dynamics during drying process in the field. In these studies six replications of each treatment were used. Media preparation and methods of inoculation and incubation were performed as described in §2.2.2.1.

2.3. OCHRATOXIN A PRODUCTION ABILITY OF ISOLATES OF *A. CARBONARIUS*

In both years, representative strains of *A. carbonarius* were single point inoculated on 50% Coconut Cream Agar CCA (Dyer and McCammon, 1994). For medium preparation, creamed coconut purchased from a supermarket was used (Bart, UK.). The plates were incubated for 7 days at 25°C. During the incubation time, they were examined for OTA production, according to Heenan *et al*. (1998). The reverse side of each plate was observed under long-wave ultraviolet (UV) light (365 nm) for the characteristic blue-green fluorescence produced by OTA. After one week incubation, the plates were destructively examined using another complementary method. In this method the plates were exposed to ammonia before checking under UV light. The plates were put without lids in an airtight glass tank (desiccators) in which a small beaker with ammonia was present. They were left in the tank for an hour and checked under UV light. In this method OTA had an intense violet fluorescence.

2.4. OCHRATOXIN A ANALYSIS

2.4.1. GRAPE SAMPLE PREPARATION AND IMMUNOAFFINITY COLUMN CLEAN-UP (IAC)

The method for grapes and dried vine fruits was a modified method of Stefanaki *et al.* (2003). A direct clean-up method (IAC) using an immunoaffinity column was applied. A 100 g portion of each sample was blended, with 40 mL of a water solution containing 2% sodium bicarbonate ($NaHCO_3$) and 0.5% PEG 8000, at high speed for 5 minutes to give an homogeneous paste, after their homogenization in a Seward 200 Stomacher for ~ 2 min. 50 g of this paste (which contained 35.7 g grapes and 14.3 g water solution) were mixed with another 90 mL of the aforementioned water solution. The mixture was homogenised again for 5 min in the Stomacher and filtered through Whatman filter paper. The next step was to add 10 mL PBS to 10 mL of the filtrate and this solution was passed through an immunoaffinity column (Ochraprep, Rhone Diagnostics) which was placed on a vacuum manifold. It was allowed to pass through either by gravity or at a flow rate of 1 mL min^{-1}. After that, the column was washed with 10 mL of HPLC grade water at a flow rate of about 1-2 mL min^{-1} and dried with air. OTA was eluted by passing 1.5 mL of a mixture of methanol-acetic acid (98:2) through the immunoffinity column (Ochraprep®, R-Biopharm Rhône Ltd, Scotland) by gravity, followed by 1.5 mL HPLC grade water. During the elution, back flushing (or reversing the direction of flow) of desorption solution was used to ensure complete elution of the ochratoxin A. When all the amount of the eluate was collected, air was pushed using the syringe to collect the last drops of eluate. The eluate (3 mL) was collected in a graduated, dark coloured vial and stored at 4^{o} C until analysed.

2.4.2. DRYING VINE FRUITS PREPARATION AND IMMUNOAFFINITY COLUMN CLEAN-UP (IAC)

Sultanas samples were prepared and directly cleaned-up using the methodology suggested by Stefanaki *et al.* (2003) and Rhone Diagnostics (1999), respectively. A 100 g portion of each sample was blended with 80 mL of a water solution containing 1% sodium bicarbonate ($NaHCO_3$) for 5 minutes in total, to give a homogeneous paste. After their homogenization in a Seward 200 Stomacher for ~ 2 min. 45 g of this paste (which contained 25 g of grapes and 20 g of water solution) were mixed with another 180 mL of the aforementioned water solution. The mixture, after its homogenization for 5 min in the same Stomacher, filtered through Whatman folded filter paper. The clean up method was detailed in §2.4.1.

2.4.3. HIGH-PERFORMANCE-LIQUID-CHROMATOGRAPHY COUPLED WITH FLORESCENCE DETECTOR (HPLC-FLD) METHOD

Ochratoxin A analysis was performed using reverse-phase High-Performance-Liquid Chromatography with fluorometric detection (reverse phase HPLC-FLD). This consisted of a Millipore 712 WISP autosampler, a Millipore Waters 600E System controller, and a Millipore Waters 470 scanning fluorescence detector (Millipore Corporation, MA, USA). The samples were separated using a C18 Luna Spherisorb ODS2 column (150 mm x 4.6 mm, 5 μm, Phenomenex, Macclesfield, UK). The guard column was made of the same material. An excitation wavelength of 333 nm and an emission wavelength at 460 nm were used for UV detection. The flow rate of the mobile phase (acetonitrile: water: acetic acid; 57:41:2) was 1 mL min^{-1}. The standard solution was made in methanol. Concentration was confirmed by using an Ultra Violet spectrophotometer. Treatments were performed in triplicate. Run time

for samples was 12 min with OTA being detected at about 6 min (see Fig. 2.2, in APPENDIX A). The quantification level of 0.2 μg OTA Kg^{-1} was estimated as detection limit, based on a signal to noise ratio of 3:1. Several injections of OTA standard solutions (from 0.1-1.2 μg L^{-1}) were used in order to build a calibration curve for quantitative analysis of unknowns (see Fig. 2.1, in APPENDIX A). All results were analysed on a computer with Kroma Systems 2000 Software (Bio-Tek Instruments, Milan, Italy).

2.5. MEASUREMENT OF WATER AVAILABILITY (A_W) DURING DRYING

Water availability (a_w) of the drying sultanas was measured. Three representative samples from each replicate (vineyards) of the three altitudes were analysed during drying.

2.6. STATISTICAL ANALYSIS

Univariate Analysis of Variance (ANOVA) was used. Duncan$^{(a,b)}$ test was performed to compare means. Significance was determined at the 95%, and 99% confidence level depended on the analysed data. Statistical handling of the data (i.e. data input, data manipulation) was performed by SPSS, version 12.0. Figures were plotted using both Microsoft Excel 2003 and Sigma Plot version 10.0. Data from the serial dilution method were transformed (Log_{10} CFUs g^{-1}) prior to analysis in order variance to be homogenised.

2.7. RESULTS

2.7.1. PRE-HARVEST MYCOFLORA AND OTA CONTAMINATION

A total of 3 (2004/05) and 9 (2005/06) fields at three altitude levels were sampled 5 days prior to harvesting. Figure 2.2 shows the fungal diversity of the most dominant fungal species/genera of 2005/06 crop according to (a) fungal populations (CFUs) and (b) frequency of isolation (%). Although patterns presented are based on MEA95 (0.954 a_w) isolation medium, similar patterns were observed with MEA98 (0.985 a_w), for both years examined.

Figure 2.2a shows that *A. niger* aggregate was the predominant fungal species (3.9 Log_{10} CFUs g^{-1}), followed by yeasts (3.7 Log_{10} CFUs g^{-1}) and *Penicillium* species (3.5 Log_{10} CFUs g^{-1}). No *P. verrucosum* was isolated. *Cladosporium* species were isolated in a range of 1.5-1.8 Log_{10} CFUs g^{-1}. Other fungal genera indentified were *Rhizopus* and *Botrytis,* but they were isolated in very low populations (about 0.3 Log_{10} CFUs g^{-1}). Interestingly, very few populations of *A. carbonarius* were isolated (*ca*. 0.23 Log_{10} CFUs g^{-1}) from pre-harvest samples.

Similar patterns were observed when examining the frequency of isolation (Figure 2.2b). Indeed, *A. niger* aggregate species were frequently isolated from about 40% of plated berries, followed by *Penicillium* spp. and yeasts (15%). *A. carbonarius* was frequently isolated at only about 4%. Other isolated fungal species were from the *Botrytis*, *Rhizopus* and *Cladosporium* genera, but with a low frequency of isolation. The Shannon (H) Index of biodiversity (Table 2.1) gives a more detailed picture of fungal biodiversity in relation to altitude and grape position on the plant, five days prior to harvest time.

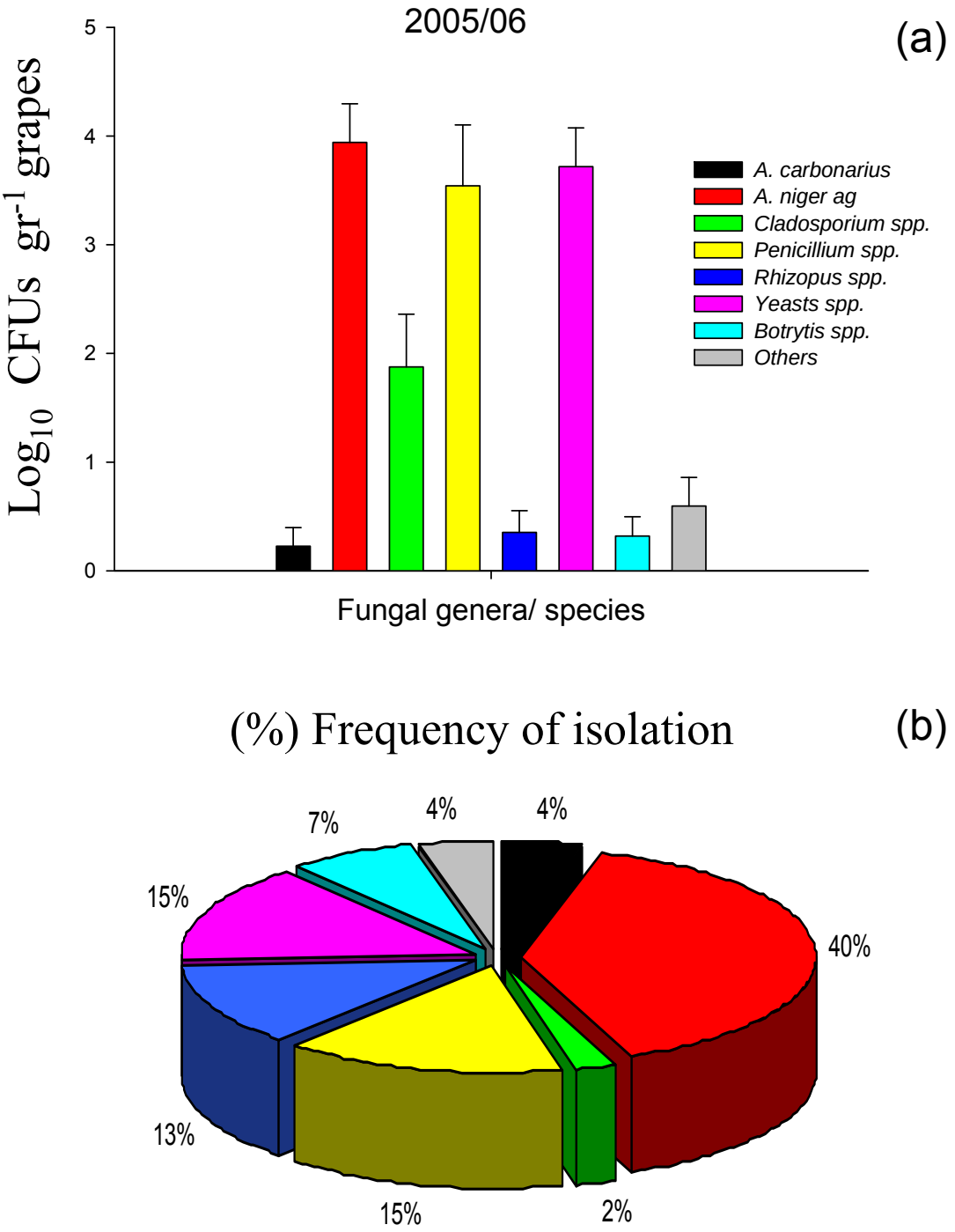


Figure 2.2. Fungal diversity according to (a) fungal populations and (b) percentage (%) of frequency of isolation of the most dominant fungal species/genera on MEA95, isolated from fresh grapes (Cretan Sultanina) 5 days prior harvesting, on 2005/06. Same pattern was observed in the 2004/05 crop (see Fig.2.3-2.4, in APPENDIX A). Values are the total means, regardless of the altitude and bunch's position.

Table 2.1. Shannon Index (H) of biodiversity (CFUs g^{-1}) on (a) 2004/05 and (b) 2005/06 in relation to altitude (sea level, medium level, high level) and to bunch's position on the plant (sun: bunches in the sun; shadow: bunches in the shadow; touching: bunches touching the soil), five days prior harvesting.

(a)

Altitude	Bunch's position	*A.carbonarius*	*A.niger* ag	*Cladosporium* spp.	*Penicillium* spp.	*Rhizopus* spp.	*Yeasts* spp.	Others
Sea Level	Sun	0.000	0.223	0.330	0.009	0.064	0.095	0.009
	Shadow	0.107	0.033	0.001	0.001	0.008	0.007	0.007
	Touching	0.004	0.097	0.044	0.004	0.004	0.045	0.041
Medium	Sun	0.030	0.030	0.030	0.035	0.030	0.030	0.030
Level	Shadow	0.029	0.029	0.029	0.100	0.029	0.029	0.200
	Touching	0.005	0.343	0.001	0.072	0.005	0.223	0.001
High Level	Sun	0.016	0.016	0.016	0.043	0.107	0.016	0.016
	Shadow	0.003	0.003	0.003	0.031	0.003	0.008	0.003
	Touching	0.002	0.030	0.187	0.014	0.002	0.080	0.025
	Total	**0.044**	**0.366**	**0.057**	**0.094**	**0.009**	**0.331**	**0.011**

(b)

Altitude	Bunch's position	*A.carbonarius*	*A.niger* ag	*Cladosporium* spp.	*Penicillium* spp.	*Rhizopus* spp.	*Yeasts* spp	*Botrytis* spp	Others
Sea Level	Sun	0.000	0.332	0.002	0.007	0.000	0.193	0.000	0.000
	Shadow	0.000	0.004	0.001	0.008	0.000	0.018	0.000	0.000
	Touching	0.000	0.177	0.001	0.002	0.000	0.320	0.000	0.000
Medium	Sun	0.001	0.024	0.368	0.365	0.001	0.328	0.001	0.008
Level	Shadow	0.000	0.340	0.359	0.091	0.001	0.028	0.001	0.005
	Touching	0.001	0.100	0.002	0.234	0.000	0.017	0.000	0.000
High Level	Sun	0.000	0.028	0.004	0.043	0.000	0.123	0.001	0.002
	Shadow	0.000	0.026	0.000	0.033	0.000	0.102	0.000	0.000
	Touching	0.000	0.062	0.002	0.312	0.001	0.178	0.000	0.000
	Total	**0.000**	**0.336**	**0.079**	**0.368**	**0.000**	**0.367**	**0.000**	**0.001**

2.7.1.1. Influence of bunch's position and their exposure to direct sunlight on fungal diversity, focusing to black aspergilli species

Figure 2.3 presents the changes of black aspergilli populations (*A. carbonarius* and *A. niger* aggregate) in relation to the position of bunches and, therefore, to their exposure to direct sunlight, in two consecutive years (2004-06). There appears to be a higher risk of contamination by *A. niger* aggregate in bunches touching the soil. *A. niger* aggregate species were isolated from theses bunches in a range of 2.4 - 4.8 Log_{10} CFUs g^{-1}, in 2004/05 and 2005/06, respectively. Lower fungal populations were found in grapes in the shadow (1.5 and 4.2 Log_{10} CFUs g^{-1}, depending on the year) while grapes exposed to the direct sunshine had the lowest populations (1.0 and 3.5 Log_{10} CFUs g^{-1}, from 2004/05 and 2005/06 crops, respectively).

In contrast, patterns for *A. carbonarius* were less clear. In 2004/05, it was isolated predominantly from grapes in the shadow (about 1.0 Log_{10} CFUs g^{-1}) while bunches in the sunshine had no *A. carbonarius*. In 2005/06, when the experiment was repeated and extended to multiple vineyards, grapes touching the soil were more favourable to this species (0.9 Log_{10} CFUs g^{-1}) than bunches in the shadow.

It must be highlighted, that, in both years studied, *A. niger* aggregate species were dominant amongst the black aspergilli, while isolations of *A. carbonarius,* which is the key-fungus for OTA contamination in grapes, were relatively restricted, regardless of the bunch's position.

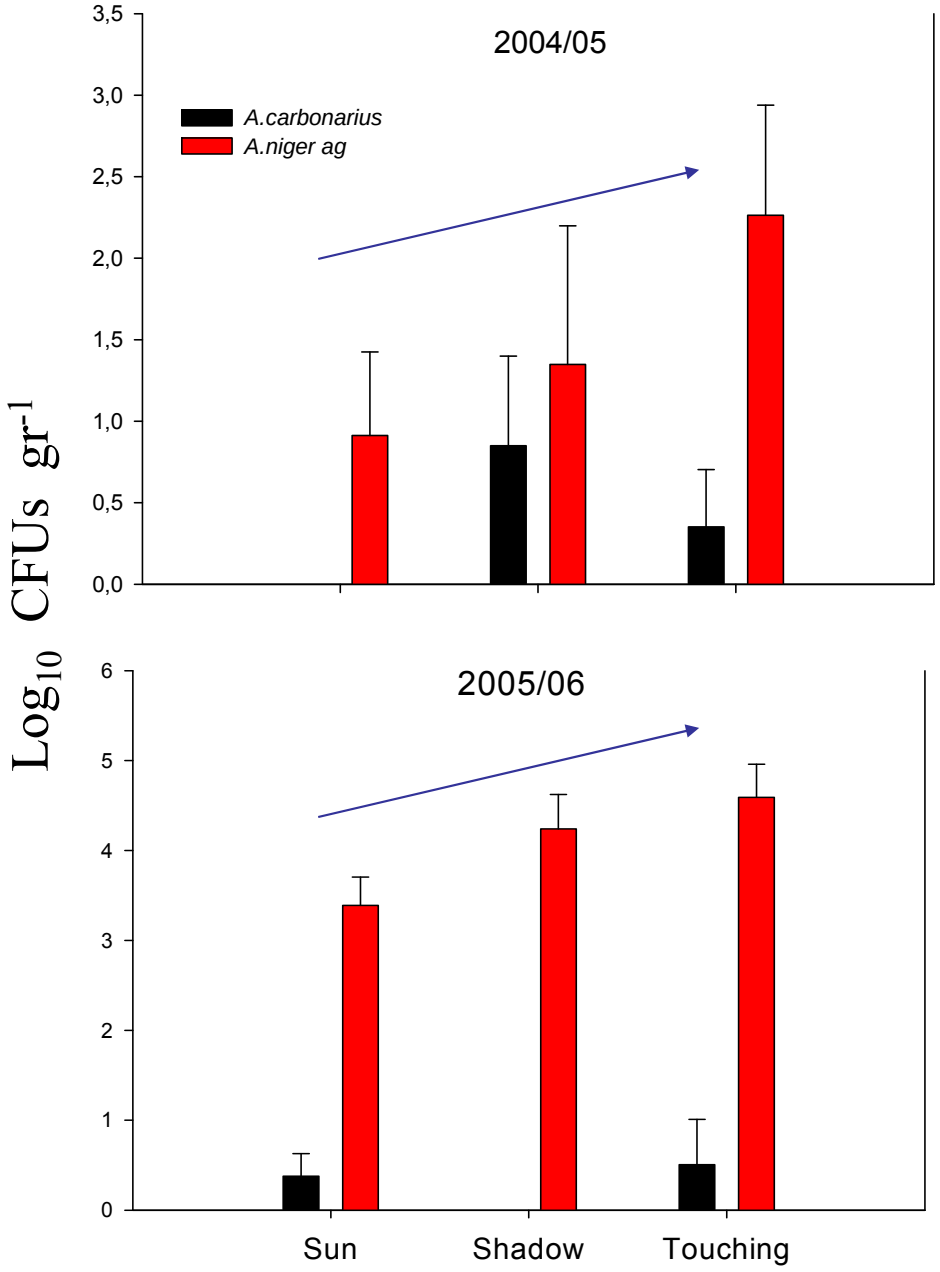


Figure 2.3. Population dynamics of black aspergilli (*A. carbonarius* and *A. niger* aggregate) on fresh grapes, five days prior to harvest time, on MEA95 (0.954 a_w), in relation to their exposure to the direct sunlight (Sun: bunches in the sun; Shadow: bunches in the shadow; Touching: bunches touching the soil). Sampling years: 2004/05 and 2005/06. Error bars indicate standard errors of the mean. Same pattern was observed on MEA98.

In this study, samples were collected five days prior to harvest, in two consecutive years (2004-06). The relative frequency of isolation of black aspergilli on MEA95 isolation medium, according to bunch's position on the plant, is presented in Figure 2.4. Among to black aspergilli, *A. carbonarius* was frequently isolated from bunches touching the soil, in about 38% and 22% in 2004/05 and2005/06, respectively.

Grapes in the shadow were less favourable to black aspergilli, in comparison to those touching the soil or exposed to the sun. Thus, *A. carbonarius* was frequently isolated in about 20% and 14%, in the first and second year examined, respectively. Interestingly, no *A. carbonarius* were frequently isolated from bunches which were exposed to the direct sunlight, in both years of the survey. However, *A. niger* group was the dominant fungal species over to *A. carbonarius.*

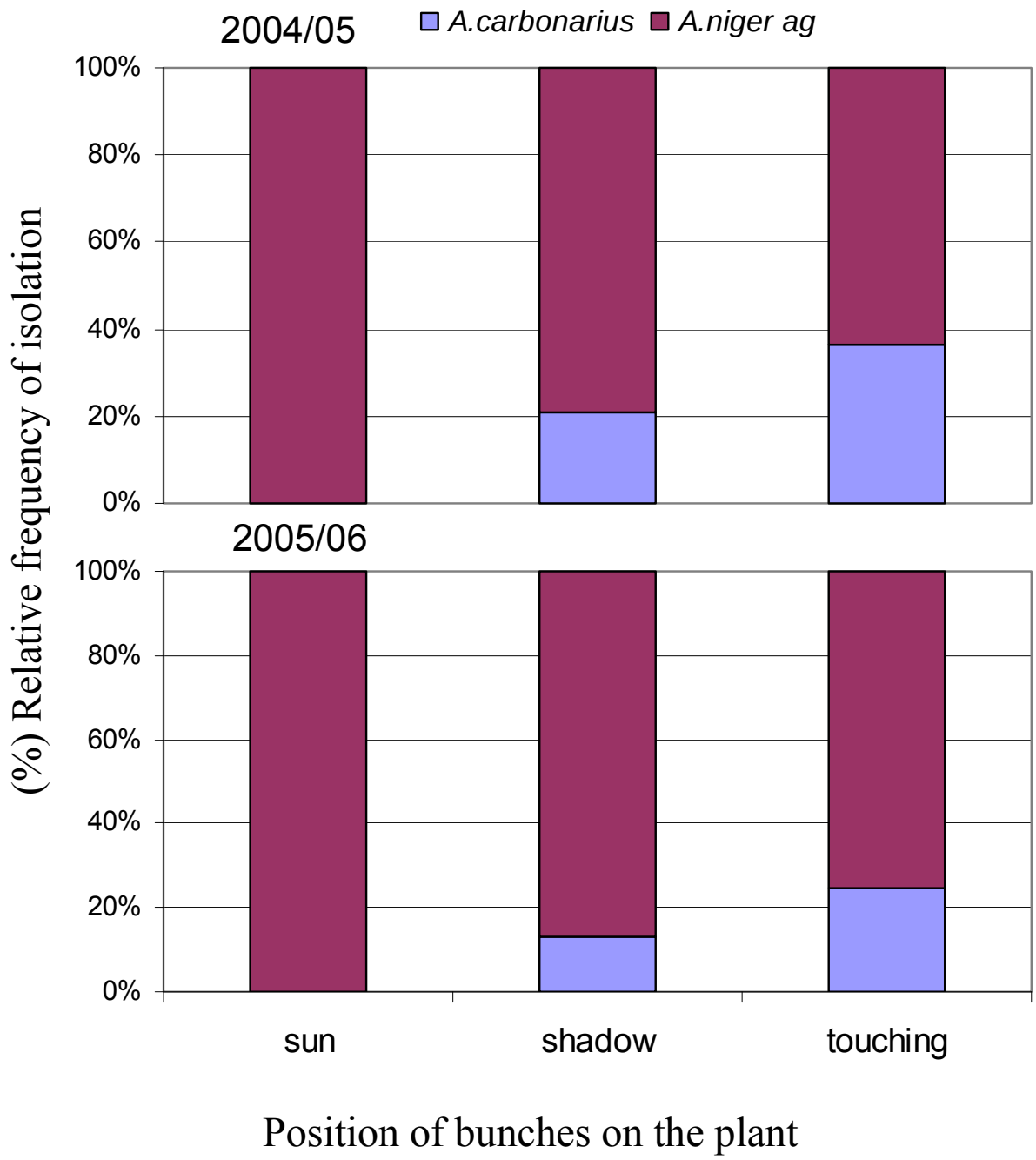


Figure 2.4. Relative frequency of isolation of black aspergilli (*A. carbonarius* and *A. niger* aggregate) on fresh grapes, five days prior to harvest time, on MEA95 (0.954 a_w), at three different bunch's positions on the plant (Sun: bunches in the sun; Shadow: bunches in the shadow; Touching: bunches touching the soil). Sampling years: 2004/05 and 2005/06. The same pattern is observed on MEA98.

2.7.1.2. Effect of altitude on black aspergilli populations

In this study, populations of black aspergilli were investigated in relation to three examined altitudes (sea level: <200 m.; medium level: 200-500 m.; high level: >510 m.) (Figure 2.5). The following key results are summarised. At sea level *A. niger* aggregate populations were 2.7 and 4.8 Log_{10} CFUs g^{-1}in 2004/05 and 2005/06 respectively. *A. carbonarius* populations were lower at 0.8 and 0.2 Log_{10} CFUs g^{-1} in these two seasons.

At intermediate altitudes, the fungal population patterns were different from those at sea level. More specifically, in 2004/05, *A. niger* aggregate populations were half of those at sea level. In 2005/06, a tendency of population decrease was observed, but this was not significant. In contrast, populations of *A. carbonarius* were slightly increased in 2005/06 while, in the previous year, a decrease (*ca* to half) in its dynamics, similar to that of *A. niger* group, was observed.

Finally, at higher altitude, it seemed that populations of both black aspergilli were dramatically reduced. In 2004/05, isolates of *A. carbonarius* were eliminated while those of *A. niger* aggregate were reduced to 0.4 Log_{10} CFUs g^{-1}, in comparison to the other altitude treatments. In 2005/06, a trend of decrease in fungal population was observed. Overalll, the populations of black aspergilli gradually decreased as the altitude increased. However, this pattern was more clear in *A. niger* aggregate populations.

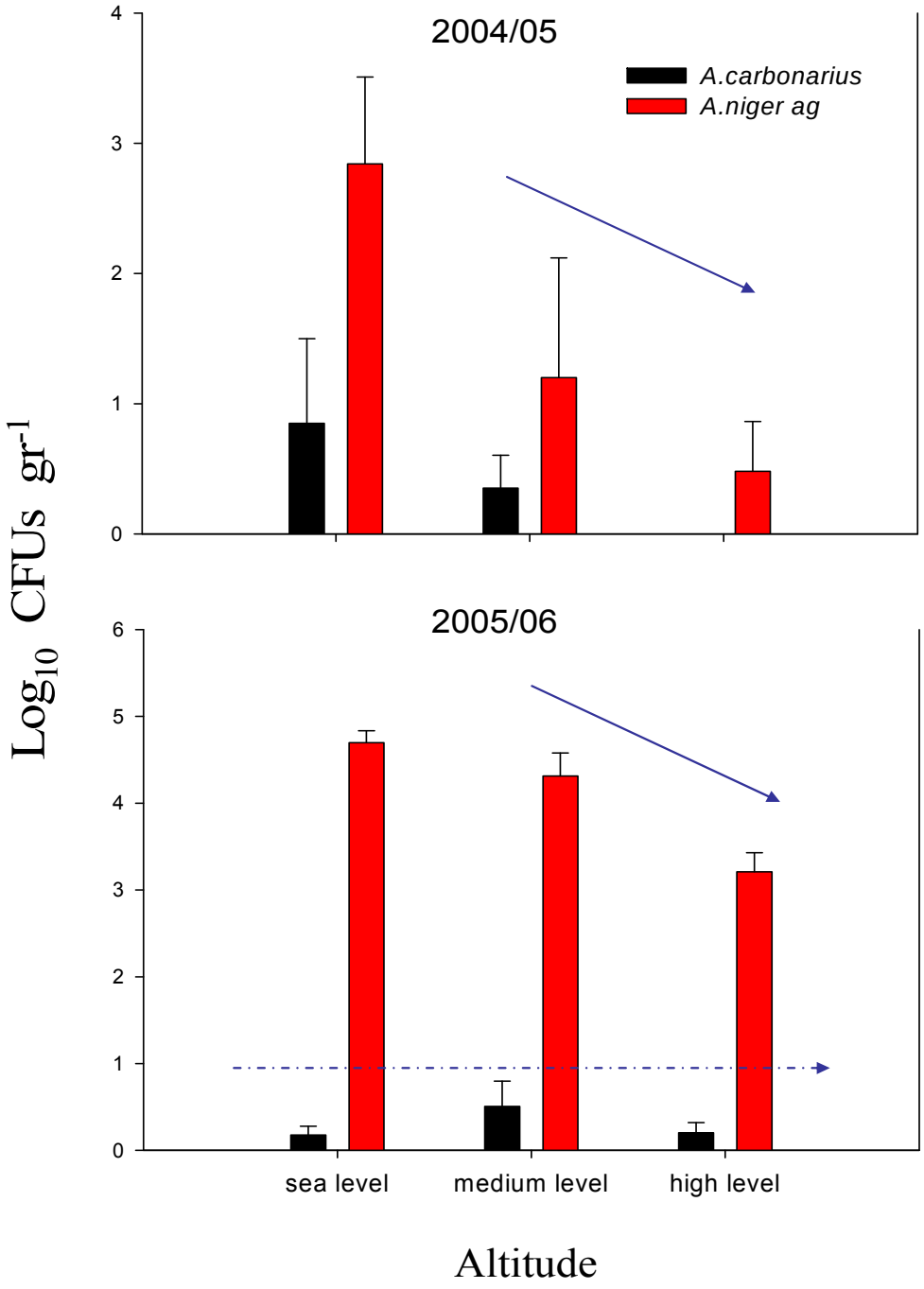


Figure 2.5. Population dynamics of black aspergilli (*A. carbonarius* and *A. niger* aggregate) on fresh grapes, five days prior to harvest time, on MEA95 (0.954 a_w), at three different altitudes (sea level: 0-200 m; medium level: 250-500 m; high level: >500 m). Sampling years: 2004/05 and 2005/06. Same pattern is observed on MEA98.

The effect of the altitude on the relative frequency of isolation of black aspergilli is presented in Figure 2.6. The pattern was completely different from that of isolated populations which was shown previously.

At sea level (< 200 m.), *A. carbonarius* was relatively frequently isolated in about <5% in both examined years (2004-06). In contrast, intermediate altitudes (210-500 m) seem to be more favourable to the fungus. Thus, in 2004/05 *A. carbonarius* was frequently isolated in about 50% while fungus dynamic was more restricted (*ca* 20%), the second year of the survey.

Interestingly, at higher altitudes (> 510 m), the results were sligthy different in the two examined years. In 2004/05, vineyards at higher altitudes seemed to be the second (38%), after medium levels (50%), most favourable environment for *A. carbonarius* isolates. In contrast, while *A. carbonarius* was more frequently isolated at the medium levels, no fungus was isolated at highest altitudes, in the second year of this study.

To sum up, Figure 2.6 shows that *A. carbonarius* was most frequently isolated from samples originated from intermediate altitudes, in both years (2004-06). Less *A. carbonarius* was isolated from grapes originated from vineyards at sea level, both years. Furthermore, it seems that as *A. niger* aggregate populations increase, fungal dynamics of *A. carbonarius* are restricted.

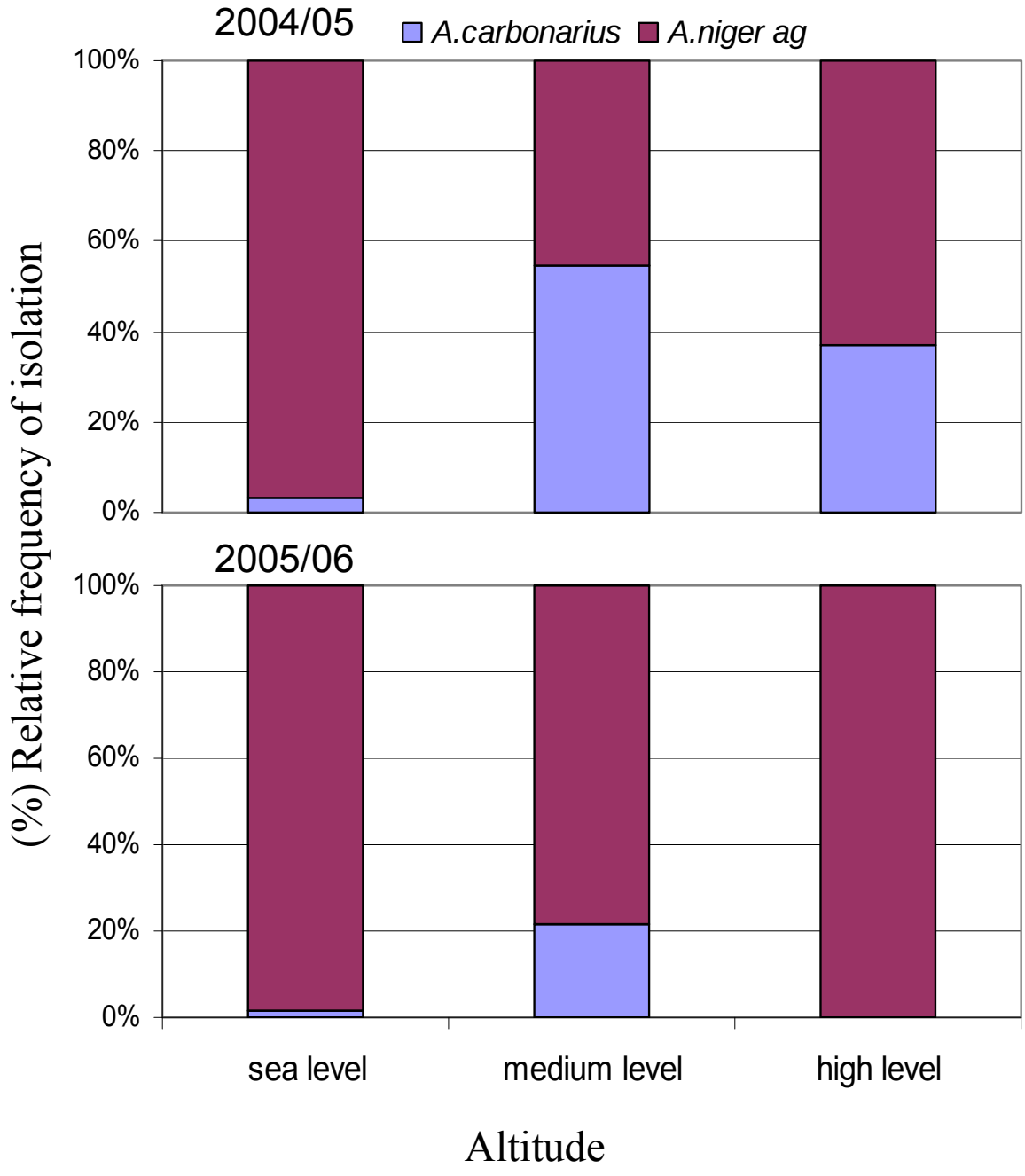


Figure 2.6. Relative frequency of isolation of black aspergilli (*A. carbonarius* and *A. niger* aggregate) on fresh grapes, five days prior to harvest time, on MEA95 (0.954 a_w), at three different altitudes (sea level: 0-200 m; medium level: 250-500 m; high level: >500 m). Sampling years: 2004/05 and 2005/06. Same pattern is observed on MEA98.

♦ *Statistical analysis*

The position of the grape bunches may affect populations of the fungal species examined (Table 2.2). More specifically, grapes touching the soil seem to be more potentially at risk. Concerning to relative frequency of isolation of both black aspergilli groups, as well population dynamics of *A. niger* aggregate, they were significantly affected by both examined parameters (altitude and bunch's position) (see Tables 2.2, 2.3(a,b) - 2.5(a,b)). Overall, more fungal isolates were found in samples originating at lower altitudes (< 500 m) and in grapes touching the soil.

Table 2.2. Homogenous subsets of the effect of the bunch's position on the plant, on *A. carbonarius* populations (Log_{10} CFUs g^{-1}) at pre-harvest sampling (5 days prior harvesting), in both examined years (2004-06).

cos	Bunch position	N	Subset	
			1	2
Duncan(a,b)	touching	42	0.7255	
	shadow	42	0.8618	0.8618
	sun	42		0.9423

Significance level = 0.05

Table 2.3(a,b). Homogenous subsets of the effect (a) the altitude and (b) the bunch's position on the plant, on *A.niger* aggregate populations (Log_{10} CFUs g^{-1}) at pre-harvest sampling (5 days prior harvesting), in both examined years (2004-06).

	(a)				(b)			
	altitude	N	Subset		Bunch 's position	N	Subset	
			1	2			1	2
Tukey HSD(a,b)	high	42	20738.7839		sun	42	22837.6184	
	medium	42	91032.5659		touching	42	217338.9348	217338.9348
	sea	42		97756.043	shadow	42		645505.8065

Significance level = 0.05

Table 2.4(a,b). Homogenous subsets of the effect (a) the altitude and (b) the bunch's position on the plant, on *A. carbonarius* (relative frequency of isolation), at pre-harvest sampling (5 days prior harvesting), in both examined years (2004-06).

(a)

	Altitude	N	Subset		
			1	2	3
Duncan(a,b,c)	sea	195	0.09		
	high	170		0.16	
	medium	180			0.,28

(b

	Bunch position	N	Subset		
			1	2	3
Duncan(a,b,c)	sun	175	0.01		
	shadow	195		0.10	
	touching	175			0.42

Significance level = 0.05

Table 2.5(a,b). Homogenous subsets of the effect (a) the altitude and (b) the bunch's position on the plant, on *A. niger* aggregate (relative frequency of isolation), at pre-harvest sampling (5 days prior harvesting), in both examined years (2004-06).

(a)

	altitude	N	Subset	
			1	2
Duncan(a,b,c)	high	170	0.,36	
	medium	180	0.41	
	sea	195		0.,88

(b)

	micro	N	Subset		
			1	2	3
Duncan(a,b,c)	sun	175	0.45		
	shadow	195		0.56	
	touching	175			0.67

Significance level = 0.05

2.7.1.3. OTA accumulation in grapes, five days prior to harvesting

In this study, ochratoxin A concentration was determined for grapes originating from vineyards at different altitudes (sea level, intermediate and higher altitudes), 5 days prior to harvest time. Simultaneously, the potential effect of the bunch's position on OTA biosynthesis was also examined.

OTA accumulation was influenced by bunch's position (see Table 2.6). More heavily contaminated samples were found from grapes not touching the soil (bunches in the shadow and in the sunlight). Bunches touching the soil had the least toxin. This could be explained by the fact that population dynamics of *A. niger* aggregate was higher on grapes touching the soil; therefore degradation of OTA by *A. niger* species may have occured.

Although impact of the altitude on OTA accumulation was not statistically significant, a trend was observed. Indeed, OTA accumulation was detected in higher concentrations (3 μg Kg^{-1}) at medium levels, in comparison to sea (*ca* 2 μg Kg^{-1}) or higher altitudes (0.5 μg Kg^{-1}). This potential pattern is in agreement with that related to the population development of *A. carbonarius* (see Figure 2.7).

Table 2.6. Homogenous subsets of the effect of bunch's position on the plant and on OTA accumulation (μg g^{-1}), 5 days prior to harvesting.

	micro	N	Subset	
			1	2
Duncan(a,b,c)	touching	8	0.00000000	
	sun	9	0.00062739	0.00062739
	shadow	8		0.00190177

Significance level = 0.05

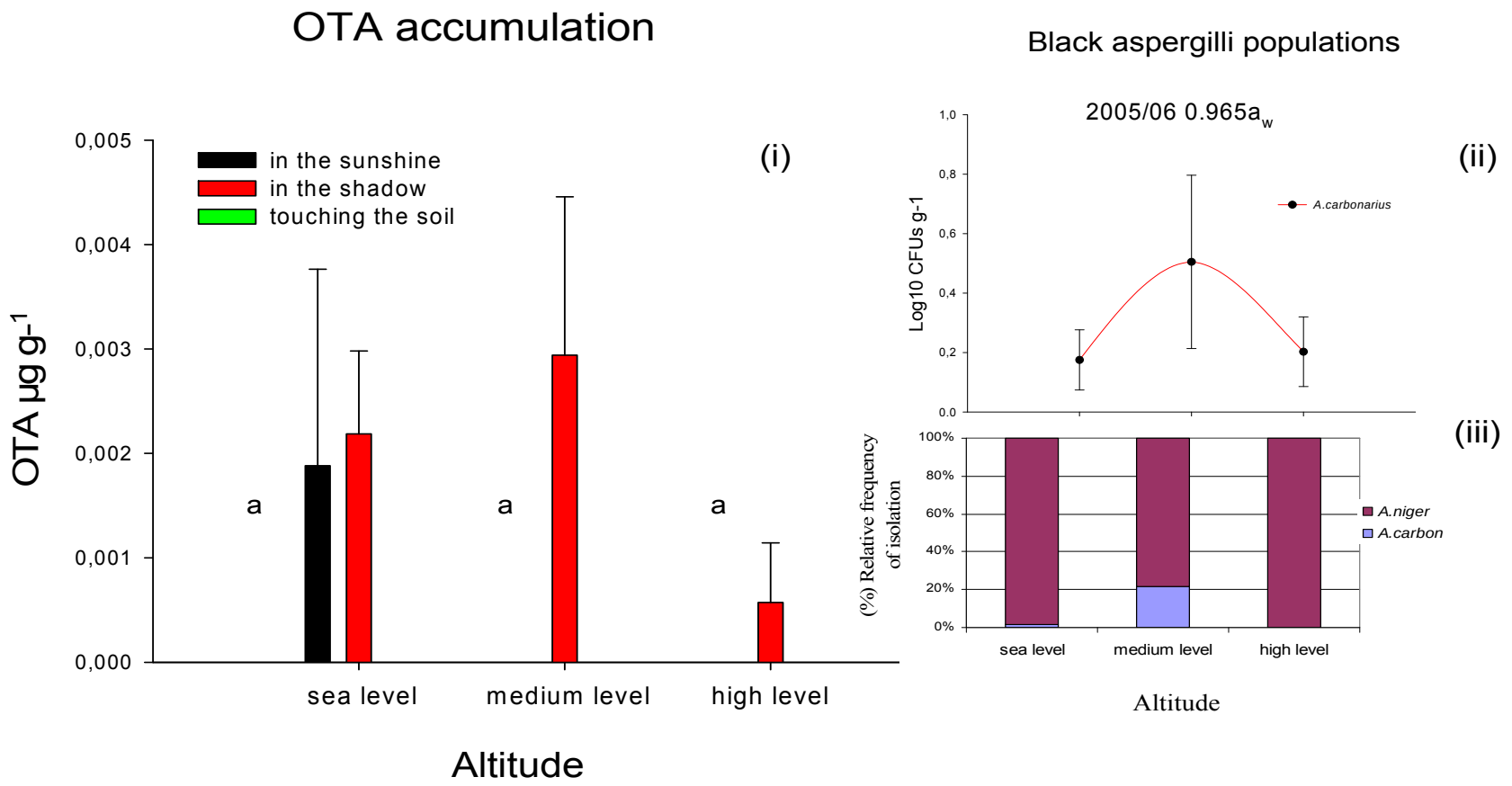


Figure 2.7. Fig.(i): Ochratoxin A contamination of grapes (collected 5 days prior to harvesting) in relation to the altitude (sea, medium, high altitudes) and bunche's position on the plant (bunches in the sunshine, in the shadow and touching the soil). Same letter indicates that there is no difference on OTA accumulation in relation to the altitude. Fig. (ii) and (iii) show isolation and relative frequency of isolation of black aspergilli (altitude).

2.7.2. POST-HARVEST MICROBIAL ASSESSMENT AND OTA CONTAMINATION

2.7.2.1. Temporal changes in water availability (a_w) and fungal biodiversity, during sun-drying of sultanas

In this study, water availability (a_w) of the drying sultanas was measured. More specifically, 3 representative samples from each replicate (3 vineyards) of the 3 altitudes examined were analysed during drying. Thus, samples were collected on harvest time, at 5th and 10th day of drying. Figure 2.8 shows a_w changes during sun-drying of sultanas. The loss of the water volume is higher in the first 5 days. Thus, it may be a window during this period when both *Aspergillus* and OTA contamination is more likely to occur.

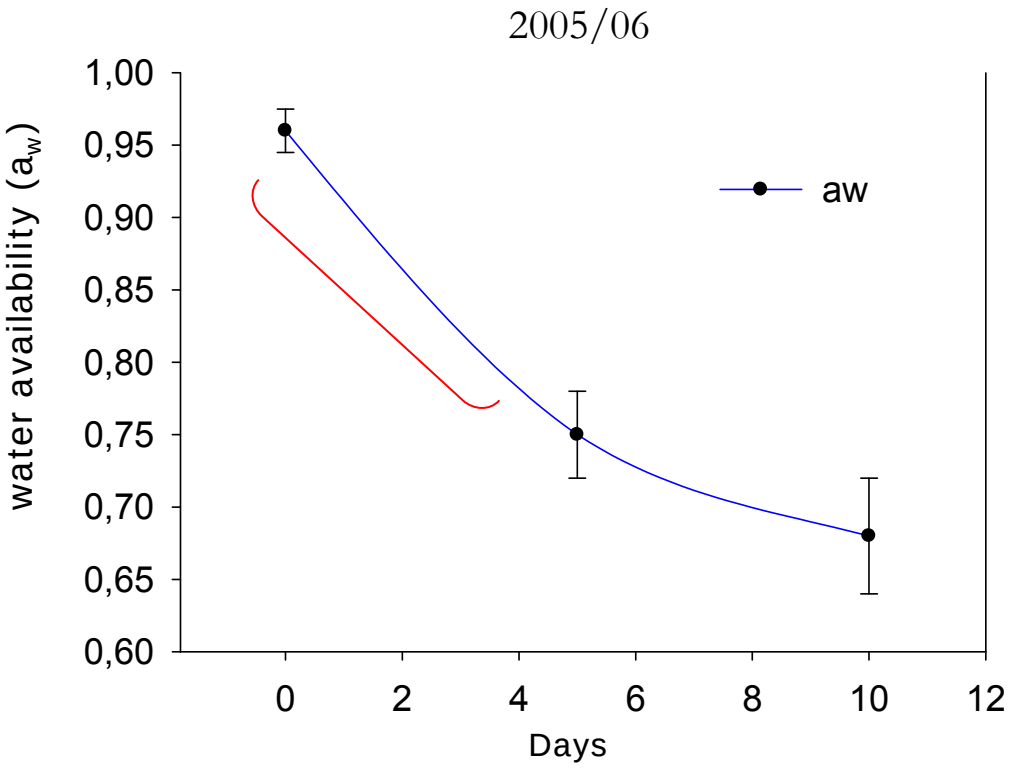


Figure 2.8. Temporal changes in water availability (a_w) of drying sultanas, during drying. The red line indicates the key period of potential *Aspergillus* and OTA contamination, since rapid decrease in a_w occurs.

Figure 2.9 (a,b) presents temporal changes in population dynamics of the most dominant fungal species/genera on MEA95, in the second year examined (2005/06). Samples were collected 5 days prior to harvest and during sun-drying of sultanas (0–10 days of drying). The same pattern was observed in the 2004/05 crop (see Fig. 2.5-2.6, in APPENDIX A).

Fungal biodiversity was high on pre-harvested samples (see section 2.7.1). At least seven fungal genera were isolated and identified, three of which were the most predominant; *A. niger* aggregate, *Penicillium* spp and yeasts. The number of isolated fungal genera actually remained stable during drying. What changed was the population dynamics of each fungal genus. Black aspergilli (chiefly *A. niger* aggregate) kept clear dominance over other isolated fungal species, especially after the first days of drying (see Figure 2.9a).

Temporal changes in frequency of isolation of the most dominant fungal species/genera are presented in Figure 2.9b. Fungal diversity was higher (at least 7 identified genera) on grapes but, during sun-drying it was decreased to 3 genera on the 10^{th} day of drying. Again black aspergilli were the dominat fungal genera. Moreover, an example of fungal biodiversity (Shannon Index) of the Cretan ecosystem is presented on Table 2.7.

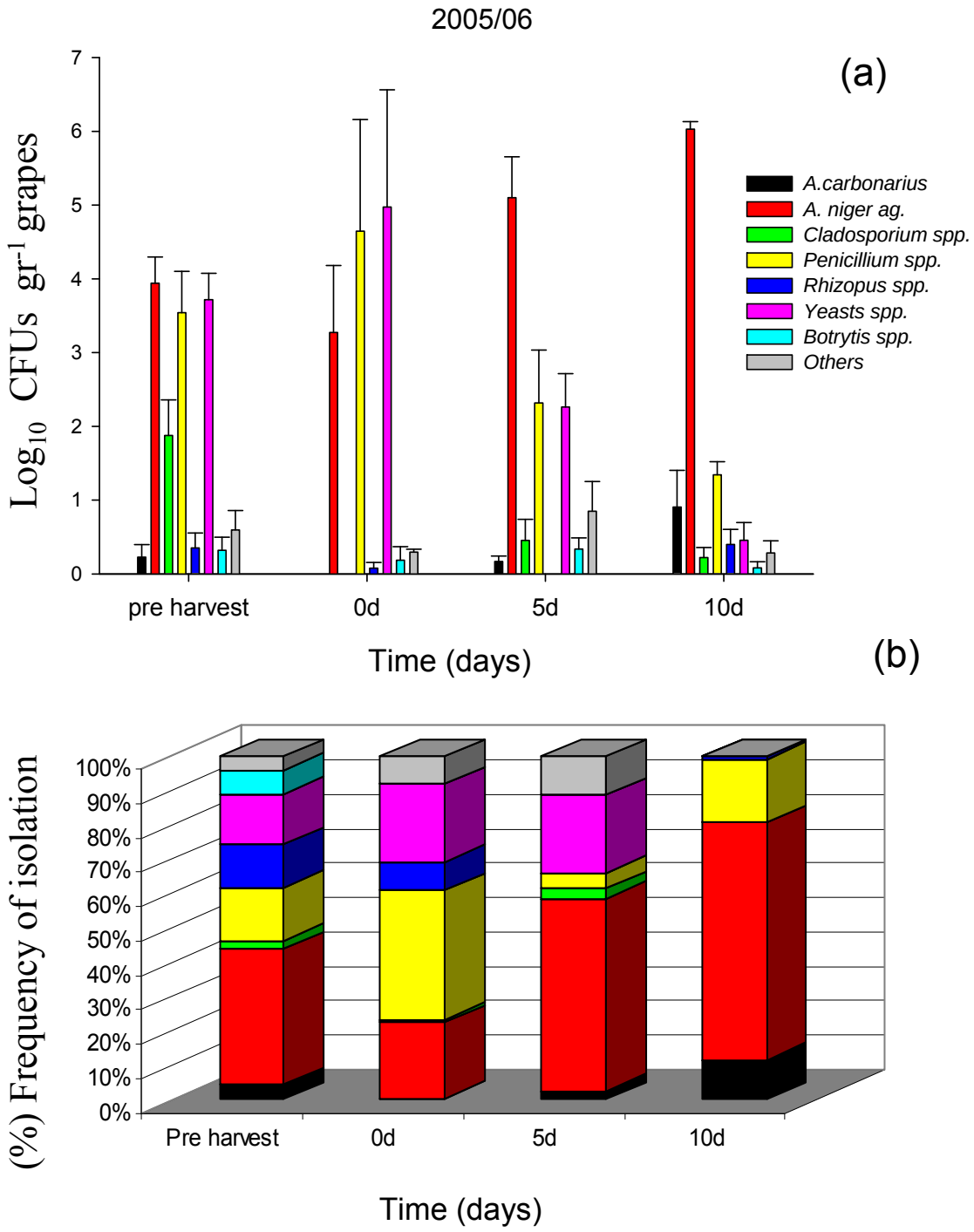


Figure 2.9(a,b). Temporal changes of (a) fungal populations and (b) frequency of isolation of the most dominant fungal species/genera on MEA95, isolated from fresh grapes (Cretan Sultanina) 5 days prior to harvesting and drying sultanas (0d: at harvest day; 5d: 5 days of drying; 10d: 10 days of drying) on 2005/06. The same pattern was observed in the 2004/05 crop.

Table 2.7. Shannon Index (H) of biodiversity (CFUs g^{-1}) on (a) 2004/05 and (b) 2005/06 in relation to altitude (sea level, medium level, high level) and to time of sun-drying (0d: harvest day; 5d: 5 days of drying; 10d: 10 days of sun-drying).

(a)

Altitude	Time	*A.carbonarius*	*A.niger* ag	*Cladosporium* spp.	*Penicillium* spp.	*Rhizopus* spp.	*Yeasts* spp.	Others
Sea Level	0d	0.000	0.000	0.358	0.000	0.000	0.240	0.000
	5d	0.000	0.365	0.000	0.000	0.000	0.264	0.000
	10d	0.000	0.363	0.000	0.000	0.000	0.255	0.000
Medium	0d	0.000	0.000	0.000	0.000	0.000	0.025	0.094
Level	5d	0.000	0.129	0.017	0.000	0.000	0.353	0.353
	10d	0.000	0.366	0.000	0.000	0.000	0.272	0.039
High Level	0d	0.000	0.320	0.000	0.308	0.000	0.294	0.000
	5d	0.000	0.256	0.000	0.000	0.000	0.114	0.000
	10d	0.000	0.357	0.000	0.000	0.000	0.334	0.000
	Total	**0.004**	**0.301**	**0.121**	**0.103**	**0.004**	**0.271**	**0.227**

(b)

Altitude	Time	*A.carbonarius*	*A.niger* ag	*Cladosporium* spp.	*Penicillium* spp.	*Rhizopus* spp.	*Yeasts* spp.	*Botrytis* spp.	Others
Sea Level	0d	0.000	0.001	0.000	0.003	0.000	0.004	0.000	0.000
	5d	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001
	10d	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Medium	0d	0.000	0.000	0.000	0.363	0.000	0.320	0.000	0.000
Level	5d	0.000	0.006	0.000	0.013	0.000	0.020	0.000	0.000
	10d	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000
High Level	0d	0.000	0.002	0.000	0.292	0.000	0.146	0.000	0.000
	5d	0.002	0.230	0.009	0.344	0.001	0.086	0.002	0.007
	10d	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Total	**0.000**	**0.355**	**0.000**	**0.352**	**0.000**	**0.357**	**0.000**	**0.000**

2.7.2.2. Effect of the altitude on fungal diversity, focusing on black aspergilli population dynamics

Figures 2.10 and 2.11 show temporal changes in populations (CFUs) of black aspergilli (*A. carbonarius* and *A. niger* aggregate) in relation to the altitude, in two consecutive years; 2004-05 and 2005-06. More specific, in the first year of this study (2004/05) population dynamics of *A. carbonarius* seem to be eliminated over time, in comparison to the pre-harvest fungal loads (0.5-1.0 Log_{10} CFUs g^{-1}), regardless of the altitude. In contrast, a considerable increase in the population loads of *A. niger* aggregate was observed (4.0-5.0 Log_{10} CFUs g^{-1}), within the first five days of drying, in comparison to the pre-harvest samples (*ca* 1-3 Log_{10} CFUs g^{-1}). Similar well-defined patterns were observed at the three altitudes studied (Fig. 2.10).

At the second year of this study (see Fig. 2.11), when the experiment was extended to 9 vineyards, population loads of *A. carbonarius* increased slightly over time (from 0.2 Log_{10} CFUs g^{-1} at pre-harvest samples up to 1.1 Log_{10} CFUs g^{-1} at 10^{th} day of drying). This change of fungal populations was clearer in samples originating from sea-level regions. Similarly, populations of *A. niger* aggregate also increased over time. Thus, at altitudes 250-500 m (high and medium levels), fungal population dynamics were rapidly arised up to to 5.5 Log_{10} CFUs g^{-1}, within 5 days of sun-drying. After the 5^{th} day of drying, a lower rate of increase took place. At higher altitudes (>500 m) *A. niger* aggregate loads slightly increased (3.4 Log_{10} CFUs g^{-1}) during the first five days of drying, in comparison to pre-harvest samples (3.0 Log_{10} CFUs g^{-1}). On the contrary, the rate of fungal development rapidly increased (5.5 Log_{10} CFUs g^{-1}) after the 5^{th} day of drying.

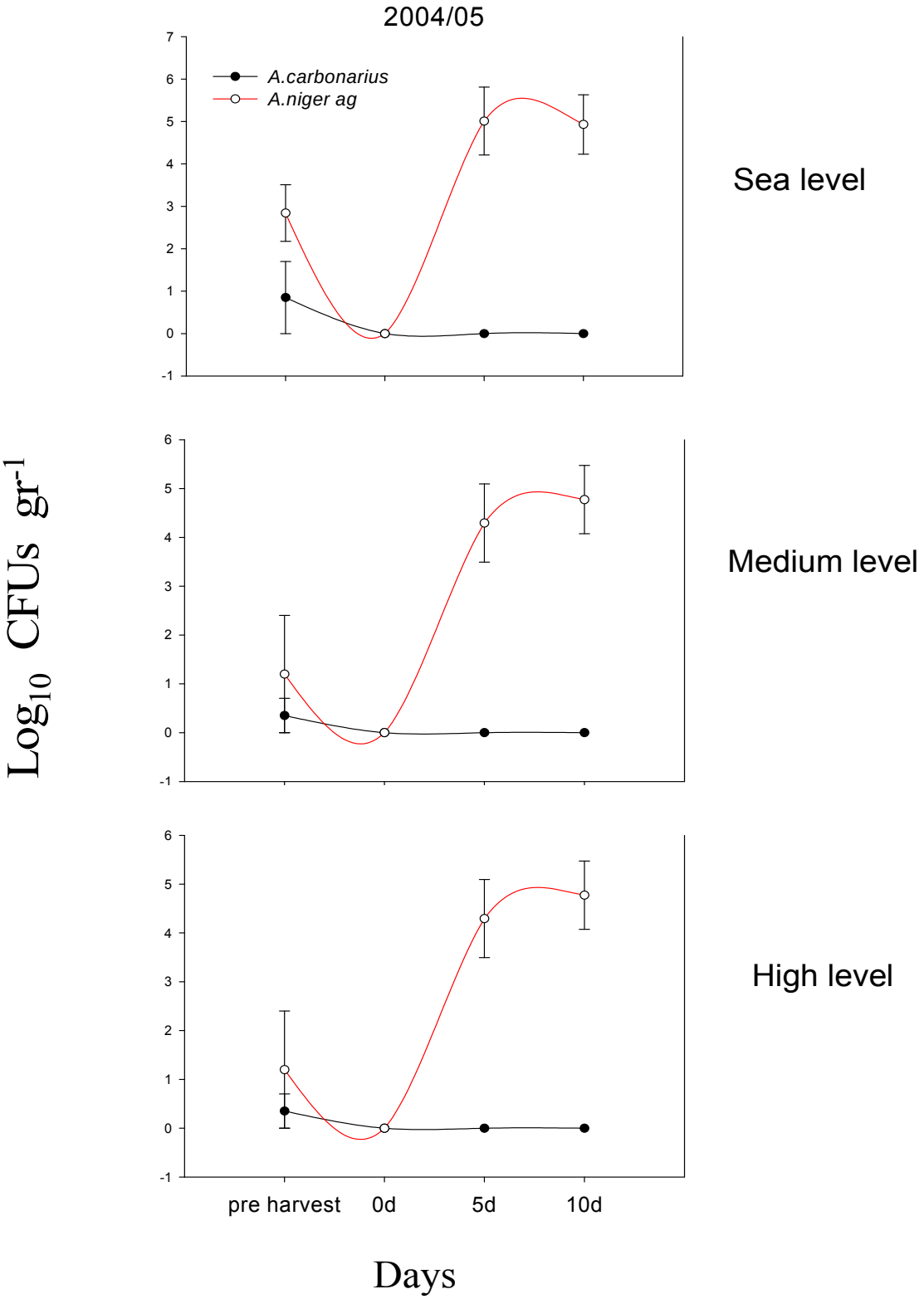


Figure 2.10. Changes in population dynamics of black aspergilli (*A. carbonarius* and *A. niger* aggregate) on drying sultanas (2004/05) over time, in relation to three altitudes (sea level: 0-200 m; medium level: 250-500 m; high level: >500 m). Isolation medium: MEA95. Same pattern was observed on MEA98.

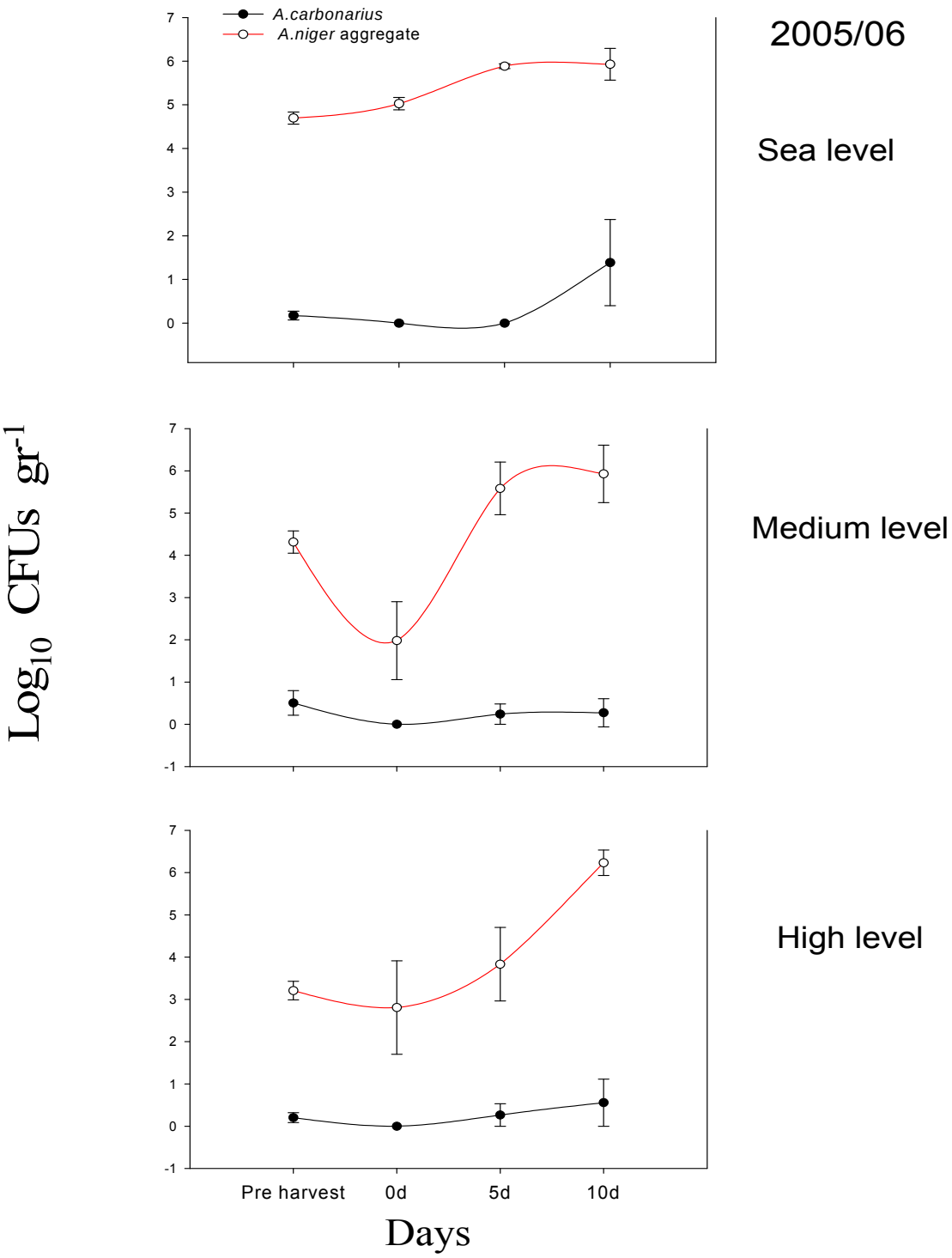


Figure 2.11. Changes in population dynamics of black aspergilli (*A. carbonarius* and *A. niger* aggregate) on drying sultanas (2005/06) over time, in relation to three altitudes (sea level: 0-200 m; medium level: 250-500 m; high level: >500 m). Isolation medium: MEA95. Same pattern was observed on MEA98.

Relative frequency of isolation of black aspergilli is presented in Figure 2.12. Measurements were taken 5 days prior to harvesting (pre harvest), at harvest time (0d), and at the 5th and 10th day of drying (5d and 10d, respectively). Thus, changes in relative frequency of isolation were examined over time. Simultaneously, the effect of the altitude on fungal dynamics of black aspergilli was also investigated.

Overall, results showed a gradual increase in relative frequency of isolation of both groups of black aspergilli, over time. Thus, at sea level, where patterns were more well-defined, *A. carbonarius* (which is the most ochratoxigenic fungal species examined) was relatively frequently isolated in about 25% at the 10th day of drying, while its pre-harvest percentage was about 5%.

At intermediate altitudes, a similar tendency of fungus development was shown. However, although *A. carbonarius* was frequently isolated in about 20% from the grapes, the fungus was isolated in about 16%, at the last day of drying. A slight decrease was occurred. At higher altitudes, the tendency was similar but the fungus was isolated in much lower percentages (*ca* 3%).

To sum up, changes in the frequency of isolation of black aspergilli were observed during drying. Focusing on *A. carbonarius*, a tendency in fungal loads development over time was shown. However, as the altitude increased the relative frequency of fungus isolation decreased. Similar patterns were observed in the two seasons examined and on two isolation media (MEA98 and MEA95).

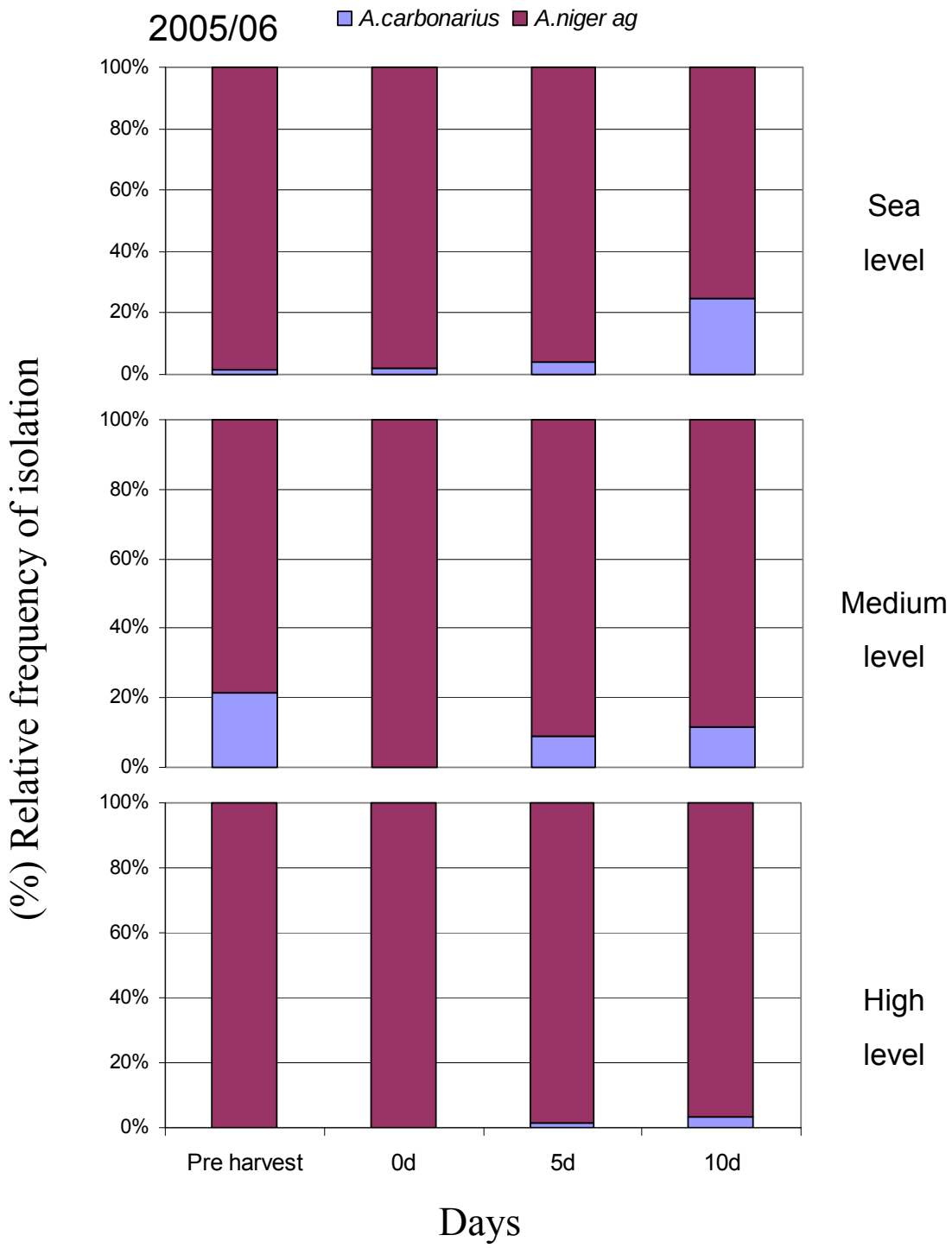


Figure 2.12. Temporal relative frequency of isolation of black aspergilli (*A. carbonarius* and *A. niger* aggregate) on drying sultanas (2005/06), in relation to three altitudes (sea level: 0-200 m; medium level: 250-500 m; high level: >500 m). Isolation medium: MEA95. Same pattern was observed on MEA98.

Statistical analysis

Time of drying significantly affected *A. carbonarius* population dynamics (Table 2.8). Both time of drying and altitude affected to population loads of *A. niger* aggregate (Table 2.9(a,b)). Similar patterns were observed in relation to the relative frequency of isolation of both fungal species (Tales 2.10, 2.11).

Table 2.8. Homogenous subsets of the effect of time (0d: harvest day; 5d: 5 days of drying; 10d: 10 days of drying) on *A. carbonarius* population (Log_{10} CFUs g^{-1}) during drying, in both years examined (2004-06).

	TIME	N	Subset	
			1	2
Duncan(a,b)	0d	60	0.0628	
	5d	60	0.1321	
	10d	60		0.8522

Significance level = 0.01

Table 2.9(a,b). Homogenous subsets of the effect (a) time (days) of sun-drying and (b) the altitude, on *A. niger* aggregate populations (Log_{10} CFUs g^{-1}) during drying, in both years examined (2004-06).

(a)

	Time	N	Subset		
			1	2	3
Duncan(a,b)	0d	57	3.,2380		
	5d	57		5.1021	
	10d	57			5.8479

(b)

	Altitude	N	Subset	
			1	2
Duncan(a,b)	Medium level	57	4.2973	
	High level	57	4.5954	
	Sea level	57		5.2953

Significance level = 0.05

Table 2.10(a,b). Homogenous subsets of the effect of (a) time (days) sun-drying and (b) the altitude, on *A. carbonarius* (relative frequency of isolation), during drying, in both years examined (2004-06).

(a)

	Time	N	Subset 1	Subset 2
Duncan(a,b,c)	0d	165	0.0061	
	5d	135	0.0222	
	10d	121		0.2149

(b)

	Altitude	N	Subset 1	Subset 2
Duncan(a,b,c)	Sea level	151	0.0331	
	Medium level	150	0.0533	
	High level	120		0.1417

Significance level = 0.01

Table 2.11(a,b). Homogenous subsets of the effect of (a) time (days) sun-drying and (b) the altitude, on *A. niger* aggregate (relative frequency of isolation), during drying, in both years examined (2004-06).

(a)

	Time	N	Subset 1	Subset 2
Duncan(a,b,c)	0d	165	0.3939	
	5d	121		0.8926
	10d	135		0.9333

(b)

	Altitude	N	Subset 1	Subset 2
Duncan(a,b,c)	medium level	150	0.5733	
	high level	120	0.6083	
	sea level	151		0.9272

Significance level = 0.01

2.7.2.3. Effect of water availability (a_w) on fungal diversity of drying sultanas

Complementary to the previous work, an effort to investigate the impact of changing a_w levels on fungal biodiversity during drying was made. Figure 2.13a presents the most dominant fungal genera isolated from drying sultanas in relation to the altitude

at the 5th day of drying, on Malt extract agar (MEA90) modified to 0.90 a_w,. At sea level three fungal genera were isolated, using serial dilution isolation method: *A. niger* aggregate, *Penicillium* spp and *Eurotium* spp. Black aspergilli was the prevalent fungal group (3.5 Log_{10} CFUs g^{-1}) followed by *Penicillium* spp (1.5 Log_{10} CFUs g^{-1}). At medium altitudes, only *A. niger* aggregrate species were present (*ca* 5.4 Log_{10} CFUs g^{-1}), while at highest altitudes, two fungal genera were isolated; *A. niger* group (5.7 Log_{10} CFUs g^{-1}) and *Eurotium* spp (2.3 Log_{10} CFUs g^{-1}). It is worth noting that fungal dynamics of *A.niger* aggregate at the highest altitude examined (>500 m) were lower (3,8 Log_{10} CFUs g^{-1}) on MEA95 (see Fig. 2.11) compared with those on MEA90 (5,3 Log_{10} CFUs g^{-1}) (see Fig. 2.13a).

In Figure 2.13b, the pattern is similar with the previous one, shown in Fig. 2.13a. It seemed that fungal richness was higher in vineyards at sea level (data not shown). As altitude increased, fungal diversity was restricted. However, more black aspergilli (*A.niger* group) were frequently isolated at higher altitudes. Predominance of black aspergilli was obvious especially at >200 m altitudes.

2.7.2.4. OTA contamination

In general, ochratoxin A was detected up to 3.5 μg Kg^{-1} sultanas. This level is below to limit established by the European Union (see Table 1.5). Both parameters examined (altitude, time) and their interaction did not significantly affect OTA production during drying (see Fig. 2.14).

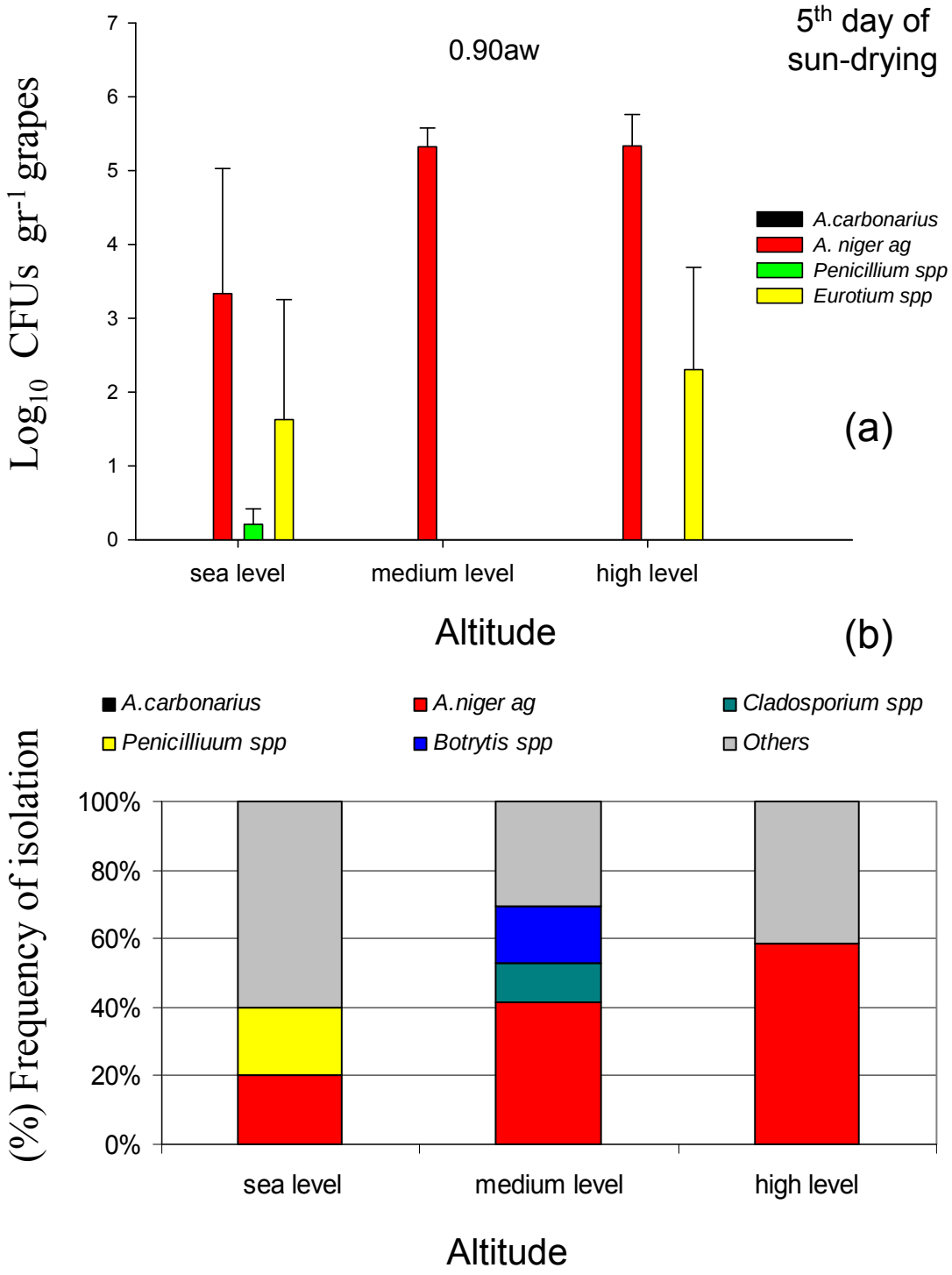


Figure 2.13. Fungal diversity defined by (a) fungal populations and (b) percentage (%) of frequency of isolation of the most dominant fungal genera, isolated from drying sultanas at 5th day of sun-drying, in 2005/06. Isolation medium: MEA90. Others: mostly *Eurotium* spp and *A. flavus*.

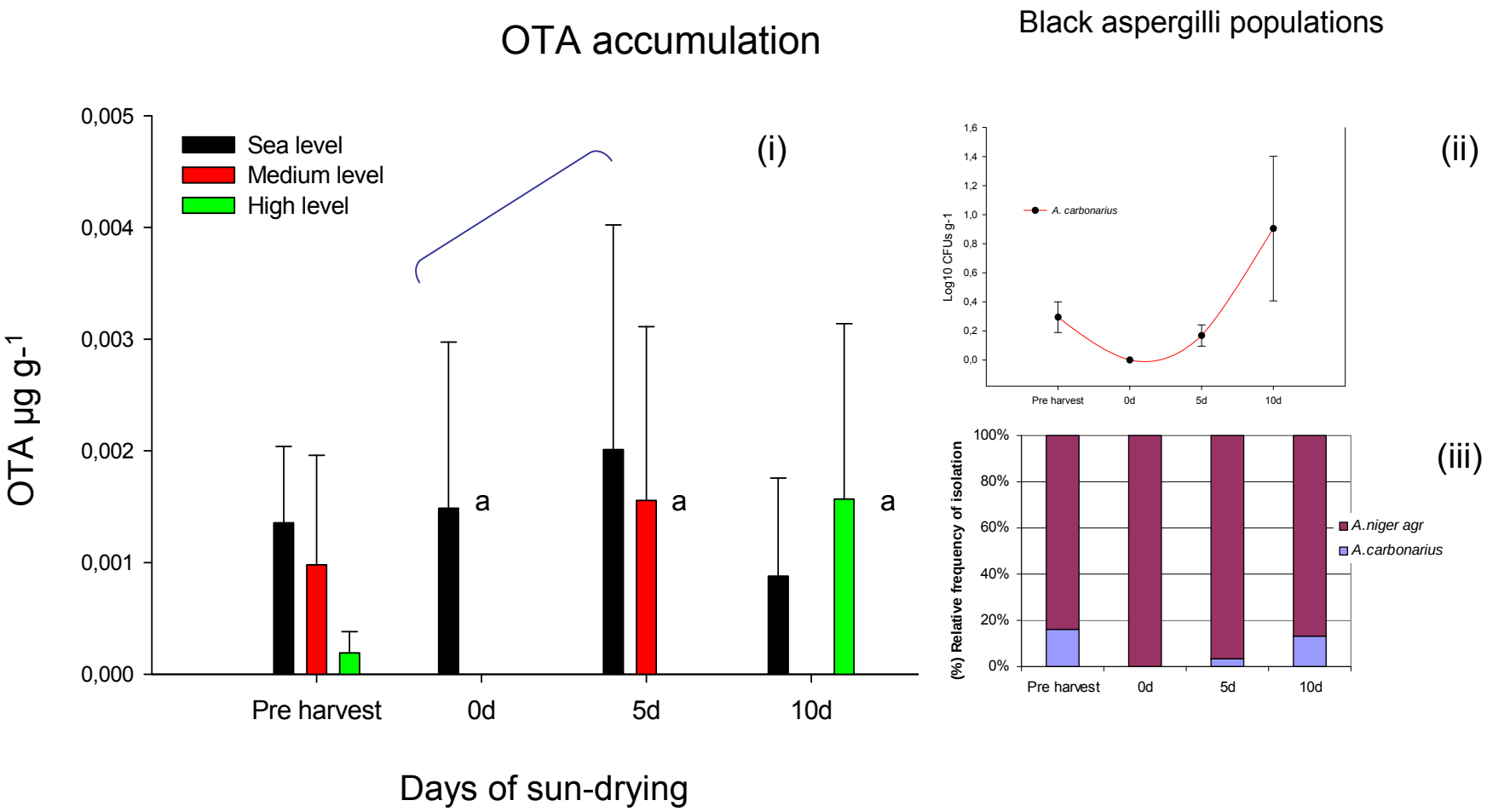


Figure 2.14. Figure (i) shows temporal ochratoxin A contamination in drying sultanas in relation to altitude (sea level, medium level, high level). Same letter indicates that there is no significant difference on OTA accumulation, in relation to time of drying. Figures (ii) and (iii) show isolation (ii) and relative frequency of isolation (iii) of black aspergilli over time, during sun-drying.

2.8. DISCUSSION

2.8.1. EFFECT OF BUNCH'S POSITION AND ALTITUDE ON FUNGAL DIVERSITY, FOCUSING ON BLACK ASPERGILLI, AT PRE-HARVEST STUDIES

Field studies of the current thesis showed that black aspergilli species were an important component of the fungal biodiversity of grapes and drying vine fruits. In order from the lower altitude to the highest one, *A. niger* aggregate were frequently isolated in *ca* 95%, 65% and 13%, respectively, from grapes just prior to harvest. *A carbonarius* was also isolated from pre-harvested samples. In order from the lower altitudes to the higher level, *A. carbonarius* was frequently isolated in about 1.6%-17%, respectively. Other fungal species isolated were *Penicillium* species, yeasts, *Cladosporium, Alteranaria, Rhizopus* and *Botrytis* spp. This is in agreement with previous pre-harvest studies (Abrunhosa *et al*., 2001; Magnoli *et al*., 2003; Belli *et al.,* 2003), where similar fungal biodiversity plus *Epicoccum* spp., *Eurotium* spp. and *Fusarium* spp. were refered as the main natural contaminants in grapes and their derivatives. Moreover, when approaching harvest, sugar content increases and the berry texture softens (MAPA, 1998) therefore grapes are more susceptible to fungal infection. Indeed, black aspergilli are commonly present in vineyards causing a characteristic berry rot, known as *Aspergillus* rot (Snowdon, 1990; Leong *et al.,* 2004; Tzamos *et al.,* 2004). They are the main OTA producers on grapes and dried vine fruits including currants, raisins and sultanas (El Halouat *et al.,* 1997; Giridhar *et al.,* 2001; Abarca *et al.,* 2002; Esteban *et al.,* 2006). This is the main reason why they have been characterized as the most important mycotoxin-producing fungal species (Accensi *et al.,* 2004).

Regarding the first parameter tested (bunch's position on the plant) our findings showed that there were significant differences in the fungal populations of grapes. Overall, findings demonstrated that both black aspergilli groups were mostly isolated from grapes which were touching the soil. This is in accordance with previous studies (Kazi *et al.,* 2003), which pointed out vineyard soil as a potential source of fungal contamination, since it contained black aspergilli in high concentrations. The highest counts of viable spores were observed at surface soil; as the soil depth increased the fungal loads decreased. On the contrary, studies carried out in Italy by Battilani *et al.* (2003b) demonstrated that both position of grapes and their distance from ground level were not significantly important factors to influence fungal presence. In these studies, samples were selected from bunches which were at elevated positions (around 150 cm) on the plants. In the present study, samples were collected from bunches lower than 150 cm from the soil level.

Furthermore, our studies showed that the presence of black aspergilli was significantly restricted on bunches directly exposed to the sun. This is probably due to the fact that bunches in the sunshine have better exposure to the pesticides and fungicides. Tzamos *et al.* (2004) reported that the fungicide Switch (a.i. fludioxonil and cyprodinil) was very effective in controlling the incidence of *Aspergillus* spp. in raisins and wine-producing vineyards. Moreover, although *A. carbonarius* is a xerophilic fungus, its optimum growth condition ranges between 25-35^{o} C (Mitchell *et al.,* 2004, Esteban *et al.*, 2006), in combination with intermediate a_w levels (0.97-0.985), depending on the strain. Grapes in the shadow might have the appropriate temperature and relative humidity for black aspergilli growth and, for *A. carbonarius* in specific, in comparison to those exposed to the direct sunlight. With these

considerations in mind, a training system (i.e pruning, irrigating, chemical application), which determines the height of plants, the type of the canopy and the relative humidity, may influence microclimate of each vine plant and consequently, fungal contamination.

As regard to the second factor examined, altitude seems to affect fungal diversity and populations. Samples originated from lower altitudes have greater diversity and fungal population dynamics, in comparison to those from regions at higher levels. Thus, vine fungi generally, *A. niger* aggregate and *A. carbonarius* in particular, were more frequently isolated at <500 m altitudes. However, between the two groups of black aspergilli, *A. niger* aggregate were the prevalent fungal species. Generally, the majority of *Aspergillus* populations of a vineyard belong to *A. niger* group (Serra *et al.,* 2003; Batillani *et al.,* 2003; Bau *et al*., 2005). *A. niger* aggregate species have been reported as the primary cause of *Aspergillus* rot in grapes before harvest by many reaserchers (Snowdon, 1990; Leong *et al.,* 2004). Previous studies *in vitro* have demonstrated that *A. niger* aggregate strains are capable of more rapid growth at high temperatures (i.e. 37^{o} C) than isolates of *A. carbonarius* (Battilani *et al.* 2003; Leong *et al.,* 2004). The optimum growth rate for *A. niger* aggregate species ranges between 30-37^{o} C, while *A. carbonarius* grows better at lower temperatures (*ca* 30^{o} C) (Belli *et al.,* 2004). The southern regions of Greece (such as Crete) are characterized by both high temperatures and humidity. Under these conditions, growth of OTA-producing fungi and more specifically, of *A. niger* aggregate, is facilitated (Stefanaki *et al.,* 2003). The climatic properties mentioned are also common at lower altitudes (< 500 m). All the above pointed out the crucial role of climatological conditions of the season on fungal contamination. Batillani *et al.*, (2006), showed that there is a

significant correlation of berries colonized by *A. carbonarius* at harvesting, positive with the summation of degree-day and negative with the summation of rain between early veraison and ripening. Moreover, infection of all species was higher in the years when there was rain damage before harvest (Leong *et al.,* 2004).

2.8.2. TEMPORAL CHANGES OF FUNGAL POPULATIONS AND EFFECT OF ALTITUDE ON FUNGAL DIVERSITY, FOCUSING ON BLACK ASPERGILLI, AT POST HARVEST STUDIES

Overall, findings showed that, fungal richness decreased during the drying process in comparison to pre-harvest time. In additon, eveness of fungal species was also dramatically changed. With regards to black aspergilli, by the end of the drying process, *A. niger* group was commonly isolated as the dominant fungal species, regardless of the altitude. Incidences of *A. carbonarius* were also observed. Among black aspergilli, the relative frequency of isolation of *A. carbonarius*, which is the main ochratoxigenic fungus in sultanas, ranged between 20%-50%, depending on the year, at the medium levels, while at the other altitudes, this percentage was lower than 15%. These are in aggrement with previous field studies. Batillani e*t al*. (2006) demonstrated that the year significantly influenced the number of berries colonized by black aspergilli. Abarca *et al. (*2002) reported that *A. niger* aggregate was isolated from all positive samples of dried vine fruits and from 78.7% of the plated pieces, while *A. carbonarius* was found in 58% of the samples and only in 28.5% of the plated pieces. Previous studies showed that *A. niger* aggregate was the predominant fungus in dried vine fruits (Pitt *et al.,* 1997; El Halouat *et al.,* 1997; Giridhar *et al.,* 2001). In general, the literature body has confirmed the presence of black aspergilli (*A. niger* and *A. carbonarius*) in dried vine fruits originating from Australia (King *et*

al., 1981), Egypt (Abdel-Sater and Saber, 1999), Spain (Abarca *et al.,* 2003) and Greece (Tzamos *et al.,* 2004; Markaki *et al*., 2001; Dekanea, 2005).

Despite the fact that black aspergilli are post-harvest spoilage fungi, their populations in sultanas decreased shortly after harvest. This may be due to the fact that the grapes were dipped in solutions of 5-7% K_2CO_3 and 0.5% olive oil or 2.5% K_2CO_3 and 2% ethyl oleate solutions before drying. This pre-treatment accelerates the drying rate of the grapes resulting in a better quality final product. Also, this pre-treatment (a) resulted in an increase of pH level (more alkaloid environment) which negatively influence fungal growth (Mitchell *et al.*, 2004) and (b) washed out fungal spores from the surface of the grapes. Heenan *et al.* (1998) found that *A. niger* and *A. carbonarius* could be isolated from dried fruit wash water.

Overall, population dynamics of *A. niger* aggregate gradually increased during sun-drying, resulting in a high degree of contamination of the sultanas, just prior to storage. Currants have a high sugar content that becomes more concentrated as they get dried. Thus, they may provide a selective nutritional substrate, a conducive factor for the growth of xerotolerant spoilage fungi, such as black *Aspergilli*, and *A. niger* aggregate. Bucheli and Taniwaki (2002) found that coffee cherries contained enough water (25-50%) for supporting growth of black aspergilli, after five days of drying. These findings agree with our results which have pointed out a rapid and high loss of water, in the first five days of sun-drying. Thus, it may be a window during this period when both *Aspergillus* and OTA contamination is more likely to occur.

The current field studies also showed that, approximately 55% of *A. carbonarius* isolates were OTA producers. A characteristic positive fluorescence was observed when they were checked under UV light using either Coconut Cream Agar (CCA) or NH_4 solution. This agrees with a study in Corinthian raisins (Dekaena, 2005). Magnoli *et al*. (2004) reported that of 82.3% of *A. carbonarius* isolated, from dried vine fruits, were ochratoxigenic, while Abarca *et al*. (2003) reported a higher percentage (96.7%). The literature, overall, points that the percentage of *A. carbonarius* isolates able to produce OTA ranged from 25-100%, while this percentage is very low (1.7 - 30%) for *A. niger* aggregate (Abarca et al., 1994; Heenan *et al.,* 1998; Da Rocha Rosa *et al.,* 2002; Accensi *et al.,* 2004).

2.8.3. EFFECT OF A_W ON FUNGAL DIVERSITY

Our findings showed that lowering the levels of water availability (0.87 a_w), the fungal richness decreased to 3 fungal genera, at sea level, in comparison to higher a_w levels (7 genera). Fungal biodiversity decreased as the altitude increased. This is the first time that the impact of low a_w, combined with altitude, is investigated on fungal diversity of drying sultanas. At lower levels of a_w (< 0.82) no fungal species were isolated. Interestingly, no yeasts were found at lower a_w (0.87) levels. According to the literature, osmotolerant yeasts are able to grow in dry conditions (0.80-0.85 a_w) (El Halouat *et al.*, 1997) even under elevated (40%) CO_2 conditions (Smith *et al.*, 1983). Moreover, *A. flavus* and *Eurotium* spp isolates were also observed at < 0.90 a_w. These fungi are able to grow under several moisture conditions (0.81-0.95 a_w) (Gibson *et al.,* 1994).

Furthermore, recent studies have demonstrated that black aspergilli can grow in the drying fruits (at initial stages of drying) until water availability decreased at *ca* 0.85 a_w (Leong *et al.*, 2004). Valero *et al.* (2004) showed that environmental conditions, such as Mediterranean climates, where a_w levels drops gradually during sun-drying of grapes, are favourable mainly to *A. niger* aggregate, and then to *Penicillium* spp. and *Eurotium* spp. Our findings are in agreement with these.

2.8.4. OTA CONTAMINATION

The findings of the present study demonstrated that both parameters examined (altitude and time) and their interaction did not significantly influence OTA production, during drying. In general, ochratoxin A was detected in very low concentrations. Overall, black *aspergilli* are normally present in vineyards (Da Rocha *et al.,* 2002; Sage *et al.,* 2002) but OTA is not always detected, although the ochratoxigenic fungi might be present (Battilani *et al.,* 2003b). Similarly, OTA producer fungi are not always present when OTA is detected in the berries (Sage *et al.,* 2002). This is due to the fact that OTA production has different optimal conditions than *A. carbonarius* growth, at least under *in vitro* conditions (Mitchell *et al.,* 2004). Thus, there might be a correlation between the OTA contamination and the relative populations of *A. carbonarius* presence. However, more studies are required to evaluate this aspect.

Studies in synthetic grape juice medium have pointed out that OTA production by *A. niger* aggregate was optimized at 0.98-0.995 a_w, at the first 5 - 10 days of incubation (Belli *et al.*, 2004). Esteban *et al.*, (2006) showed that *A. niger* aggregate produced maximum OTA concentrations within the range of 0.94-0.98 a_w, after 5 - 10 days of

incubation while OTA accumulation was detected within 5 days. On the other hand, the optimal a_w level for *A. carbonarius* in synthetic grape-like medium, ranged between 0.95-0.99 a_w (Mitchell *et al.,* 2004; Belli *et al.*, 2005). The water availability levels were narrower for OTA production by *A. niger* aggregate (0.90-0.99 a_w, depending on the strain and the medium of the culture) than for fungal growth (Esteban *et al*., 2006). Moreover, in *vitro* experiments showed that damage of the grape skin might result in production of maximum amounts of ochratoxin A by *A. carbonarius* isolates, since entrance of fungal colonization could be facilitated (Belli *et al.,* 2004; Battilani *et al*., 2004; Leong *et al.,* 2007). Simultaneously, it is well known that several *A. niger* isolates, from grapes, could hydrolyze OTA into ochratoxin α (Abrunhosa *et al*., 2001) due to some of their enzymes. The literature reports up to 90% degradation of OTA, after one week incubation at 25^o C (Abrunhosa *et al.,* 2007).

Furthermore, studies in coffee (Bucheli *et al.,* 2002) pointed out that OTA formation could take place during drying, in particular under humid tropical conditions. Sufficient amounts of water for supporting fungal growth were observed, during the first 5 days of drying on the outer part of coffee cherries. This agrees with our results on sultanas, where the first 5 days of sun-drying contributed to a critical threshold for optimizing fungal growth and therefore OTA contamination.

Geographical regions seem to have a clear effect on fungal composition and OTA contamination (Otteneder *et al.,* 2000; Battilani *et al.,* 2001b; Battilani *et al.,* 2003; Stefanaki *et al.,* 2003). In addition, the type of soil may influence the soil contamination by *Aspergilli* spp. and microbial proportions. Recent findings pointed

out vineyard soil as primary reservoir of black *Aspergillus* (Kazi *et al*., 2004; Leong *et al*., 2006). More specific, *A. niger* aggregate were more frequently isolated from soil samples than *A. carbonarius* (Leong *et al*., 2007). Thus, different vineyards might have different fungal contamination and therefore OTA contamination (Sage *et al.,* 2002). The geographical distribution of fungi has been investigated by Battilani *et al.,* (2003*)*. Their survey was carried out in Italy. Uniseriates were more frequently isolated in the North of Italy, while a large number of biseriate strains were originated from the South. Relevant geographical differences were found in South America; incidence of *A. niger* was higher in Brazil than in Argentina (Da Rocha *et al.,* 2002).

2.8.5. CONCLUSIONS OF THE CURRENT SURVEYS

1. *Aspergillus niger* aggregate was the most predominant fungal species, followed by yeasts and *Penicillium* spp, at pre-harvest. No *P. verrucosum* was found. Very few populations of *A. carbonarius* were isolated.
2. Grapes touching the soil were more favourable to black aspergilli while grapes exposed to the direct sunshine, had the least population dynamics.
3. Populations of *A. niger* aggregate gradually decreased as the altitude was increased.
4. *A. carbonarius* was most frequently isolated from samples originated from intermediate altitudes (*ca* 200-500 m). Less *A. carbonarius* was isolated at sea level.
5. As *A. niger* aggregate populations increased, fungal dynamics of *A. carbonarius* was restricted.

6. OTA accumulation seems to be influenced by bunch position and not by the altitude. Bunches touching the soil had the less ochratoxin A contamination.

7. Loss of water volume from drying sultanas was very rapid and high, during the first five days of sun-drying.

8. On grapes, fungal richness was high (at least 7 genera were identified), but drying decreased richness (3 genera, on the 10^{th} day of drying).

9. Fungal populations of black aspergilli (*A. niger* aggregate and *A. carbonarius*) increased over time. *A. niger* aggregate was the dominant fungal species during drying.

10. *A. carbonarius* was more frequently isolated at higher altitudes. On the contrary, *A. niger* aggregate was more frequently isolated at sea level.

11. Lowering of a_w level, restricted the fungal diversity. Higher fungal richness was observed at sea level. Black aspergilli (*A.niger* group) were more frequently isolated at higher altitudes.

12. Ochratoxin A was detected in very low concentrations. It was not significantly affected by the altitude and time of drying.

Chapter Three

IMPACT OF INDUSTRIAL PROCESSING SYSTEMS

3.1. INTRODUCTION AND OBJECTIVES

One of the most profitable cultures of grapes in Greece is Sultanina (*Vitis vinifera* L.). This culture covers a great percentage of the total vine cultures of Crete. Sultanina raisins (known as "sultanas" or "sultana type" raisins) are produced from sun-dried Sultanina grapes (see Section 1.3).

After sun-drying, dried vine fruits undergo an industrial process in order to extend their shelf-life by improving their quality and hygiene properties (Staurakakis, 1998). During this process, sultanas are first thoroughly washed and sometimes sprayed with sulphur dioxide, resulting in a lighter colour. According to the Codex Standard 67 (1981), the maximum level of sulphur dioxide used in bleached raisins is up to 1.5 g Kg^{-1}. The Australian Capital Territory states that the permitted SO_2 concentration in dried fruits should not exceed 3 g Kg^{-1} (Department of Health ACT Government Health Information, 1997). Sultanas are then dried in a convective air drier at 85-95^{o} C to a water content of 14-15% (w/w). Eventually, sultanas are coated with a special vegetable oil (food grade) to prevent stickiness and facilitate pedicle removal. Maximum level of this oil is 5 g Kg^{-1} (Codex Standard 67, 1981).

There is still very little knowledge of the fluctuations in fungal populations during the industrial processing of sultanas. The objective of this study was to investigate the fungal population diversity (especially colonization by *A.niger* aggregate and *A. carbonarius*) and ochratoxin A (OTA) concentration in the final processed product. These data have been utilized to facilitate the identification of Critical Control Points (CCPs) during the specific stages of industrial processes to minimize risk from OTA contamination.

3.2. COMMON INDUSTRIAL PROCESSING SYSTEMS

3.2.1. BASIC PHASES OF INDUSTRIAL PROCEDURE WITH SO_2 TREATMENT

- ***Stage 1:*** Sultanas are classified into groups

Sultanas are categorized into several groups according to their sizes, colour and texture. As a result, there are nine groups in all of industrial processed sultanas. Table 3.1 shows these groups.

Table 3.1. Types of industrial processed sultanas

Sorts	Code of Type	Colour of the berry	Size (Ø) of the berry	Homomorphy of the colour (%)	% of crystallized berries and/or with different colour
Big size	No 00	Blond-golden	*ca* 10 mm	≥ 95%	≤ 0%
»	No 0	Blond-amber	*ca* 9 mm	≥ 85%	≤ 4%
»	No 1	Blond-amber	*ca* 8 mm	≥ 85%	≤ 5%
»	No 2	Blond-sorrel	*ca* 7.5 mm	≥ 80%	≤ 10%
»	No 4	Blond-sorrel	*ca* 7 mm	≥70%	≤ 17%
»	No 5	Dark brawn	> 6 mm	≥ 85%	≤ 40%
Small size	No 21	Blond-golden	6 mm	≥ 80%	≤ 10%
»	No 22	Blond	6 mm	≥ 80%	≤ 10%
»	No 24	Dark brown	6 mm	≥ 80%	≤ 20%

- ***Stage 2:*** Pre-washing.

After grouping the sultanas according to their physical characteristics, they are thoroughly washed to remove any foreign matter (i.e. granules of dust which may adhere to the surface of sultanas, or hollow berries). However, the most important part of this stage is to prepare them for absorption of SO_2 after wetting. SO_2 is more easily absorbed by wet berries. Pre-washing usually lasts 5 min and the water should be clean without any scent/odour (Staurakakis, 1998).

- ***Stage 3:*** SO_2 treatment

At this stage the sultanas are transferred into a special chamber with the aid of a screw belt conveyor system. In this chamber, the sultanas are treated with sulphur dioxide. It should be noted that the sultana industry uses exposure to SO_2 for colour lightening, not as a treatment to eliminate fungal populations. SO_2 absorption is facilitated by wetting the raisins at the previous phases (Staurakakis, 1998) accordin g to the following formula:

$$S + O_2 \leftrightarrow SO_2 + H_2O \leftrightarrow H_2SO_3 \leftrightarrow HSO_3^- + H^+ \leftrightarrow SO_3^{2-} + 2H^+$$

$$1 \xleftarrow{\quad pH \quad} 7$$

This treatment lasts 25 – 30 minutes, depending on the size of the raisins.

- ***Stage 4:*** Final washing

At this stage, the raisins are re-washed with fresh water, without any scent. The second washing is necessary for the raisins to be completely cleaned and to remove any traces of SO_2 that might have remained on the surface. This stage lasts for about 5 min.

- ***Stage 5:*** Heat treatment

The water content of the raisins should be no more than 18% (Staurakakis, 1998, Lydakis 2001). Thus, after the final washing, the sultanas are usually placed into five special ovens to reduce both their water content and to remove the superficial water. These five ovens incorporate five different thermal treatments. Initially, the temperature gradually rises up to 95^o C, before starting to decline to match room temperature. Raisins are sensitive to very high temperatures and therefore

temperature changes should be absolutely controlled during this critical stage. This procedure lasts 25 - 35 minutes dependent on the type of sultanas.

- ***Stage 6:*** Raisin selection and packaging

After air drying, the sultanas are coated slightly with a special vegetable oil to prevent stickiness and to facilitate pedicle removal. Air blowers and sieves are used to remove foreign matter (vegetative parts, hollow fruits, small stones etc) and magnets are used to trap metallic objects. The sultanas are then sorted either by hand or by using automatic systems to remove unwanted berries (dark coloured, diseased or infested ones). They are grouped according to their size and colour. Finally, the raisins are packed in small laminated bags (200 – 1,000 g) or in polyethylene bags (5 - 15 Kg) and placed in carton boxes. The content of each package must be uniform and must contain raisins of the same size, production year and quality. The total process lasts about one hour.

3.2.2. BASIC PHASES OF INDUSTRIAL PROCEDURE WITHOUT SO_2 TREATMENT

This industrial procedure consists of the same stages as previously described except for the application of SO_2, with the same order and the same application times (see Table 3.2).

Table 3.2. Differences between the two kinds of industrial process.

Type of industrial process	Stage 1 (Classification)	Stage 2 (Pre-wash)	Stage 3 (SO_2 treatment)	Stage 4 (Final Wash)	Stage5 (Heat treatment)	Stage 6 (Final product)
With SO_2	+	+	+	+	+	+
Without SO_2	+	+	-	-	+	+

(+): indicates that the current stage is taking place during the industrial process

(-): indicates that the current stage is not taking place during the industrial process.

3.3. MATERIALS AND METHODS

3.3.1. SAMPLING SYSTEMS AND EXPERIMENTAL DESIGNS

All samples originated from Cretan vineyards and especially from the region of Heraklion, a major production site for sultanas. Samples were selected in two consecutive years, 2005 and 2006, from the silo of the Co-Operative sultanas Associations (abrv.: KSOS, email: ksos@otenet.gr), a Cretan industry for sultanas, sited at the Industrial area of Heraklion

For the first experiment (2005), two types of sultanas (No2 and No22) of the 2004/05 vine crops were selected. Both kinds of sultanas received SO_2 treatment. Five samples (5 replicates) of each type were taken every ten minutes. By timing of the industrial stages (see section 3.2.1.), it was possible to take samples form each stage from the same batch. Thus, 60 samples (5 replicates *ca* 1 Kg each, x 6 stages) of each sultana type (x 2) were analysed. In general, sampling was carried out in accordance with the recommendations of the European Communities (Commission Directive, 2002/26/EC) for sampling methods for the official control of OTA content in foodstuffs. Wherever this was not possible to apply, the method was appropriately adapted.

In 2006 the experiment was updated following the same sampling pattern. In this case, the sultanas received two different industrial treatments: with and without SO_2 spraying. The fungal populations of sultanas and OTA accumulation were also determined in these studies.

3.3.2. MYCOLOGICAL ASSESSMENT DURING PROCESSING STAGES

Fungal identification of black *Aspergilli* was carried out to a species level only for *A. carbonarius*, while the other isolates were grouped in the *A. niger* aggregate. The aim was to determine the percentage of *A. carbonarius* in the *A. niger* aggregates as well as the total fungal mycoflora before and after industrial processing.

For this experiment, two identification media were used: Malt extract agar (MEA, Oxoid Ltd) with 0.985 a_w (MEA98) and Malt extract agar in which glycerol was added to obtain 0.954 a_w (MEA95) (see Table 2.1, in APPENDIX A). In both media a small amount of chloramphenicol was added prior to sterilization at 121^o C for 15 minutes, to inhibit bacterial growth.

10 grams of each sample were suspended in 90 mL diluent (sterile-distilled water + 0.5 g agar + 0.005% Tween 80) and homogenized in a Seward 200 Stomacher for ~ 5 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 (three replicates of each). Complementary to serial dilution, ten berries per sample were randomly selected and direct plated onto MEA98 and MEA95. The berries were aseptically cut in half before plating in Petri plates. All the Petri plates were incubated at 25º C. After 7 days the number of colonies in the serial dilution method were counted and reported as colony-forming units (CFUs) g^{-1} of raisins.

3.3.3. OCHRATOXIN A ANALYSIS

3.3.3.1. Sample preparation and IAC clean-up

The method suggested by Stefanaki *et al.* (2003) (based on the Visconti *et al*. method) for dried vine fruits was used in combination with a direct clean-up method using IAC as suggested by Rhone Diagnostics (Rhone Diagnostics, 1999). A 100 g portion of each sample was blended at high speed for 5 minutes with 80 mL of water solution containing 1% sodium bicardonate ($NaHCO_3$). This gave a homogeneous paste. 45 g of this paste (which contained 25 g sultanas and 20 g of the water solution) were mixed with 180 mL of the aforementioned water solution. The mixture, after homogenization for 5 min in a Seward 200 Stomacher, was filtered through Whatman filter paper. The next step was to add 10 mL PBS to 10 mL of the filtrate. The solution was then passed through an immunoaffinity column (Ochraprep, Rhone Diagnostics) which was attached on a vacuum manifold. The solution was either passed through under gravity or at a flow rate of 1 mL min^{-1}. After that, the column was washed with 10 mL of HPLC grade water at a flow rate of about 1-2 mL min^{-1} and dried with air. OTA was eluted by passing 1.5 mL of a mixture of methanol-acetic acid (98:2) through the IAC column by gravity, followed by 1.5 mL HPLC grade water. During the elution, back flushing (or reversing the direction of flow) of the desorption solution took place to ensure complete elution of the ochratoxin. When all the eluant was collected, air was pushed using the syringe to collect the last drops of eluant. The eluant (3 mL) was collected in graduated, dark coloured vials and stored at 4^{o} C until analysed.

3.3.3.2. HPLC analysis

OTA analysis was performed using a reverse-phase HPLC system as described in 2.1.5. OTA standards were prepared by using a stock solution of 50 μg g^{-1} of OTA (Sigma-Aldrich, Dorset, UK). Before each ochratoxin A analysis, fresh standards were prepared by diluting 5 μg g^{-1} of OTA of the stock solution with the appropriate volumes of methanol HPLC grade, giving the following concentrations of 0.3, 0.6, 0.9 and 1.2 μg g^{-1} OTA. (see APPENDIX A: Fig. 2.1).

3.4. STATISTICAL ANALYSIS

Univariate Analysis of Variance (ANOVA) was used. Significance was determined at the 95%, 99% and 99.9% confidence level depending on the data analysed. Statistical handling of the data (i.e. data input, data manipulation) was performed by Statistical Programe for Society Sciences (SPSS), version 12.0. The graph plotting was carried out using Microsoft Excel 2003 and Sigma Plot version 10.0. The data associated with the serial dilution isolate method, were transformed prior to analysis in order to homogenise variance (Log_{10} CFUs g^{-1}).

3.5. RESULTS

3.5.1. FUNGAL DIVERSITY

3.5.1.1. Effect of type of sultanas with SO_2 treatment on fungal populations

In 2005, the fungal population dynamics and the potential effect of sultana type on the fungal diversity were investigated. Thus, two types of sultanas (No2 and No22) treated with SO_2, were examined. Table 3.3 shows the individual properties of these specific categories of sultanas.

Table 3.3. Properties of sultanas No2 and No22.

Sorts	Code of Type	Colour of the berry	Size (Ø) of the berry	Homomorphy of the colour (%)	Percentage of crystallized berries and/or with different colour
Big size	No 2	Blond-sorrel	*ca* 7.5 mm	≥ 80%	≤ 10%
Small size	No 22	Blond	6 mm	≥ 80%	≤ 10%

Figure 3.1 shows fungal population dynamics of the most dominant genera and species isolated from two types of sultanas (No2 and No22) treated with SO_2, from the 2004/05 vine crop. This is based on isolation on MEA98 (0.985 a_w). Black aspergilli, and more specific *A. carbonarius* and *A. niger* aggregate, were the most dominant fungi during the industrial process followed by yeasts spp. and *Penicillium* spp. Both types of sultanas had similar fungal population loads during the initial stages (stage 1-4) while after heat-treatment (stage 5) the total fungal populations were dramatically reduced, especially for the No2 sultanas. At the final sampling the No2 sultanas had practically no fungal populations remaining. Similar patterns were found on the drier medium MEA95 (0.954 a_w) used (see Fig. 3.1, APENDIX A.)

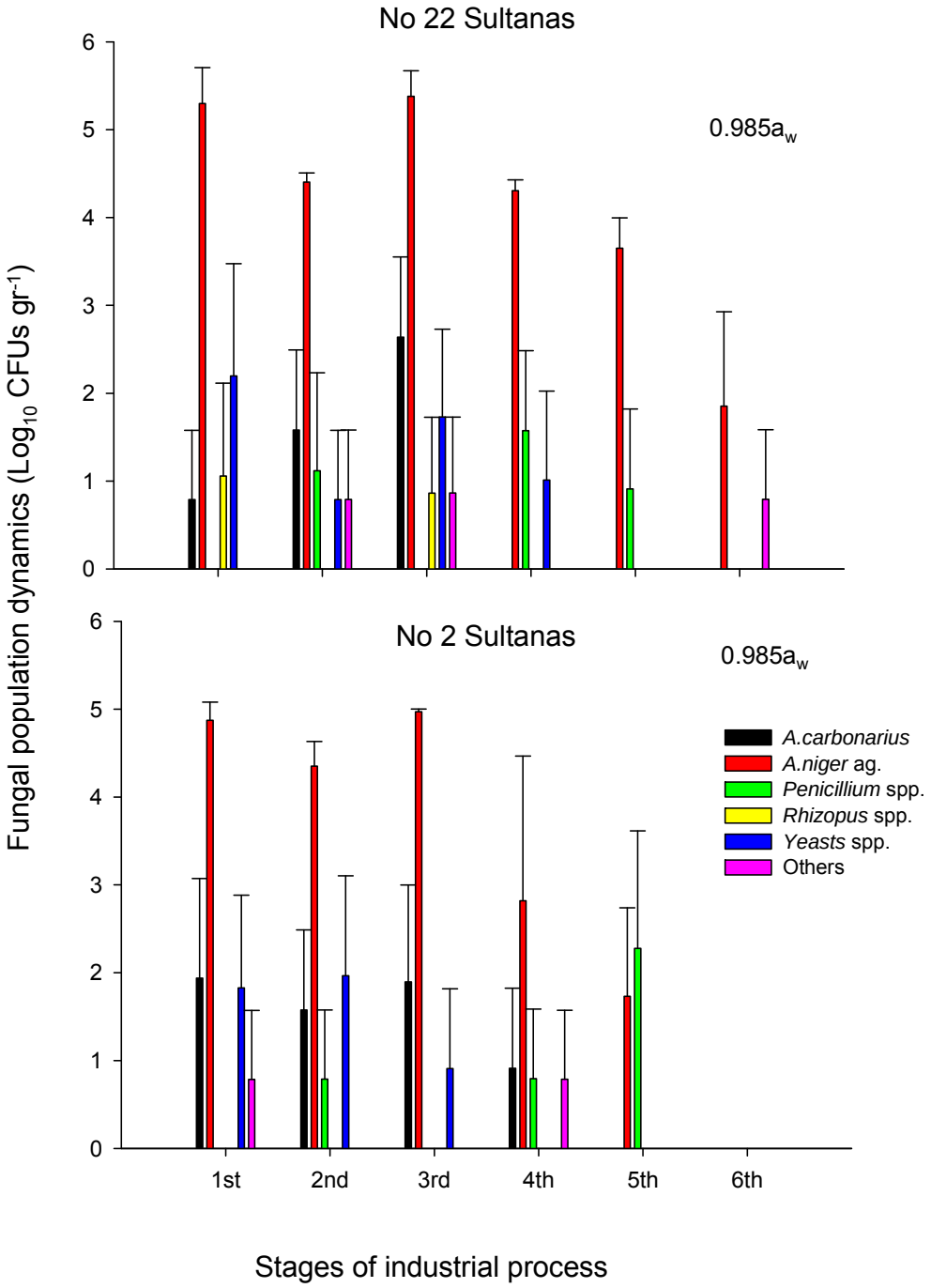


Figure 3.1. Population dynamics of the most dominant fungi, on two types of sultanas (No22 and No2 from 2004/05 vine crop) treated with SO_2, on MEA98 (0.985 a_w) isolation medium, during industrial processing. The values are the means of four replicates. Error bars indicate standard error of mean. Keys-words: 1st=classification stage, 2nd=pre-washing, 3rd=SO_2 treatment, 4th=final washing, 5th=heat-treatment, 6th=final product.

Based on direct plating of sultanas a similar pattern was obtained with respect to the relative frequency of isolation on MEA98 medium (Figure 3.2.). More specifically, black aspergilli were the dominant fungal genera; they were isolated from about 60% of sultanas during the initial stages (stage 1 - 4) of the process, regardless the type of sultanas, following by *Penicillium* and yeasts spp. After heat-treatment (stage 5) the fungal diversity was markedly reduced, with the black aspergilli the most frequently isolated (90% on No2 sultanas). In the final product, black aspergilli remained the most frequently isolated fungi for No22 sultanas (*ca* 80%) while on No2 sultanas they were isolated from about 40%. In contrast, *A. niger* aggregate were the only species frequently isolated on MEA95 (0.954 a_w) medium in the final product of No2 sultanas (see Fig. 3.3, APENDIX A.)

Figures 3.3 and 3.4 show both population dynamics and relative frequency of isolation of black aspergilli, and more specifically of *A. carbonarius* and *A. niger* aggregate. *A. carbonarius* was initially isolated in about Log_{10} 2 CFUs gr^{-1} which was almost eliminated in the finals stages, regardless of the type of sultanas. In contrast, *A. niger* aggregate were isolated at about $Logs_{10}$ 5 CFUs gr^{-1} in the initial stage of the process in both types of sultanas. In the final product, after all treatments, population dynamics of these fungi were reduced by 50% on No22 sultanas, while on No2 sultanas, the industrial method resulted in the elimination of black aspergilli. The same patterns were observed on MEA95 (0.954 a_w) medium (see Fig. 3.3 in APENDIX A) Figure 3.4 shows that the relative frequency of isolation of *A.carbonarius* ranges from *ca* 20-30% before processing begins, depending on type of sultanas. It was significantly reduced after heat-treatment. Thus, in the final product, the relative frequency of isolation was 5% and 0% for No22 and No2 sultanas,

respectively. The opposite pattern was found for isolation of *A. niger* aggregate. While the initial isolation rate was *ca* 80% and 60% on No2 and No22 sultanas respectively, in the final product it was almost 100%, in both types of raisins. *A. niger* aggregate was more frequently isolated than *A. carbonarius*. Similar patterns are shown on Figure 3.4. in APPENDIX A, on the second medium MEA95 (0.954 a_w) used. In this case, A. *carbonarius* was less frequently isolated in comparison to other black aspergilli.

Statistical analyses

- *A. carbonarius*

Tables 3.4 and 3.5 show the results of statistical analyses (Univariate Analysis of Variance) for *A. carbonarius* isolated by the serial dilution method. According to these Tables, "stages" significantly effected on fungal populations. After final-washing (stage 4), the population size was reduced, while, after heat-treatment it was almost eliminated. SO_2 treatment appeared not to have a statistically significant effect on population dynamics of fungus during processing. According to Tables 3.6-3.8, the relative frequency of isolation of *A. carbonarius* was affected by both factors examined ("stage" and "type"). The final stages of industrial processing (stages 5-6) are usually grouped together clarifying that heat-treatment is the key-factor for elimination of the fungal populations, during industrial processing.

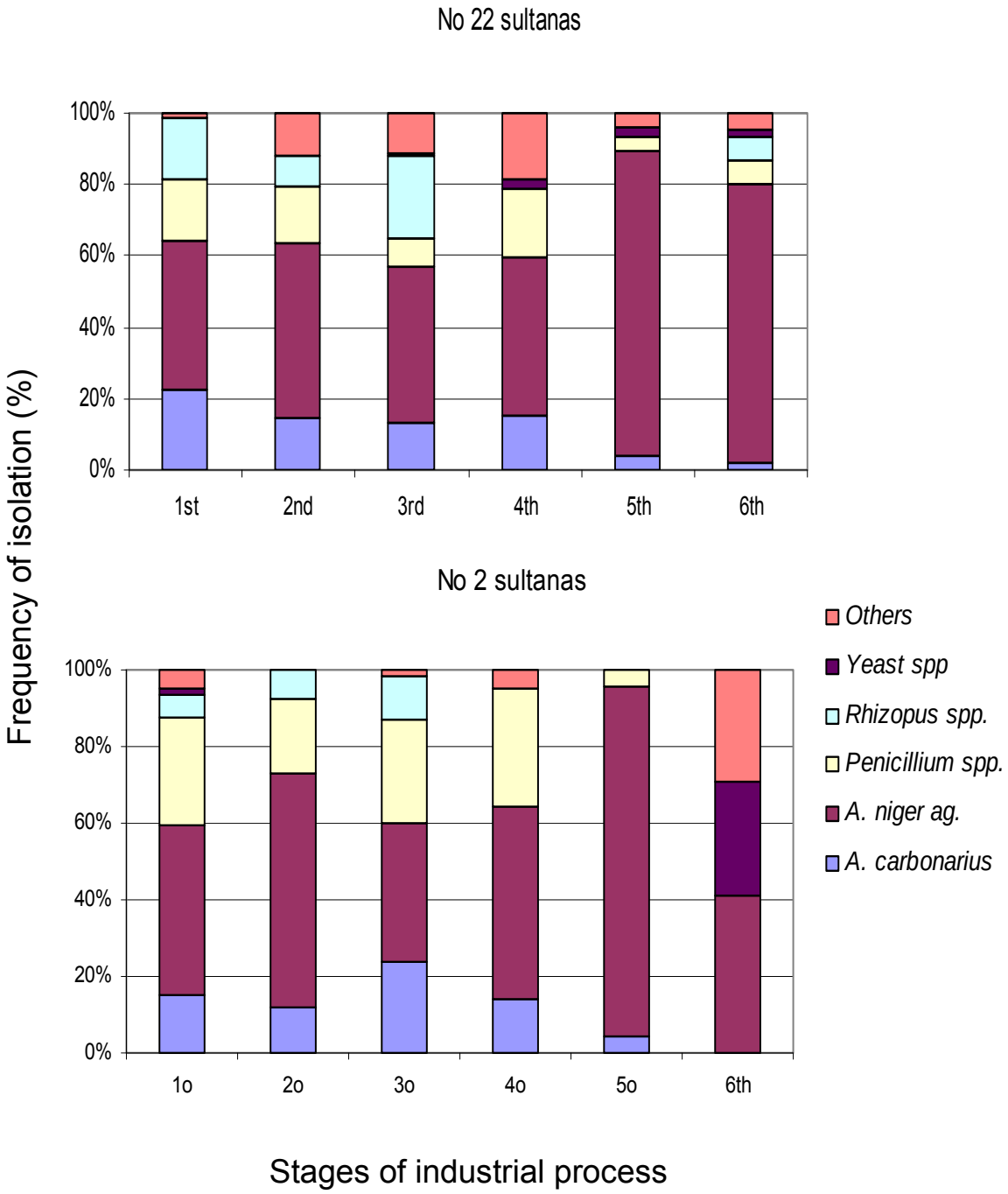


Figure 3.2. Percentage (%) of frequency of isolation of the most dominant fungal genera and species on both types of sultanas (No2 and No22 from the crop of 2004/05) treated with SO_2, on MEA98 (0.985 a_w) isolation medium, during industrial processing. The values are the means of four replicates. Keys-words: 1st=classification stage, 2nd=pre-washing, 3rd=SO_2 treatment, 4th=final washing, 5th=heat-treatment, 6th=final product.

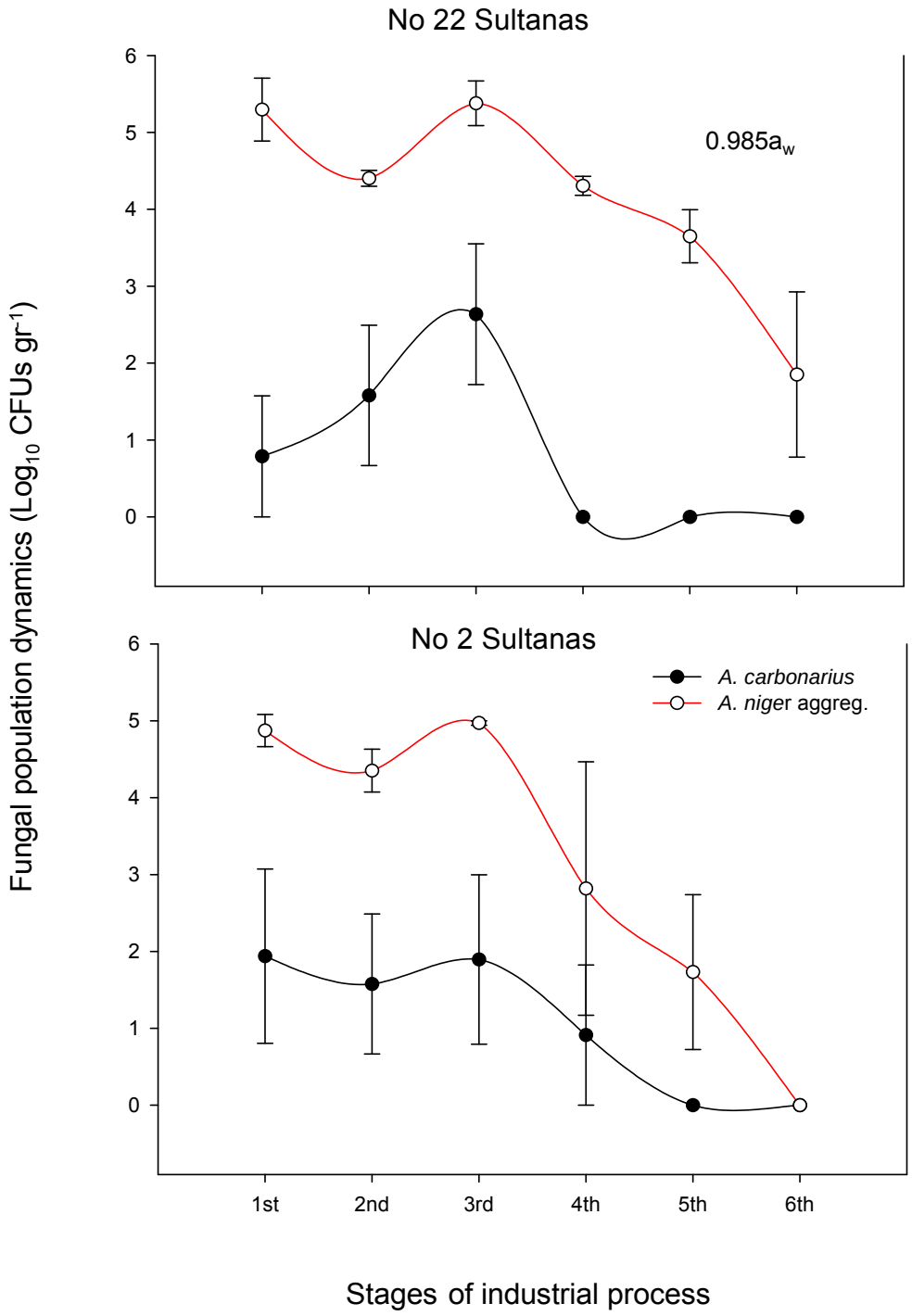


Figure 3.3. Population dynamics of *A. carbonarius* and *A. niger* aggregate, on MEA98 (0.985 a_w) medium, during industrial processing of sultanas treated with SO_2, from 2004/05 vine crop. The values are the means of four replicates. Error bars indicate standard error of mean. Keys-words: 1st=classification stage, 2nd=pre-washing, 3rd=SO_2 treatment, 4th=final washing, 5th=heat-treatment, 6th=final product.

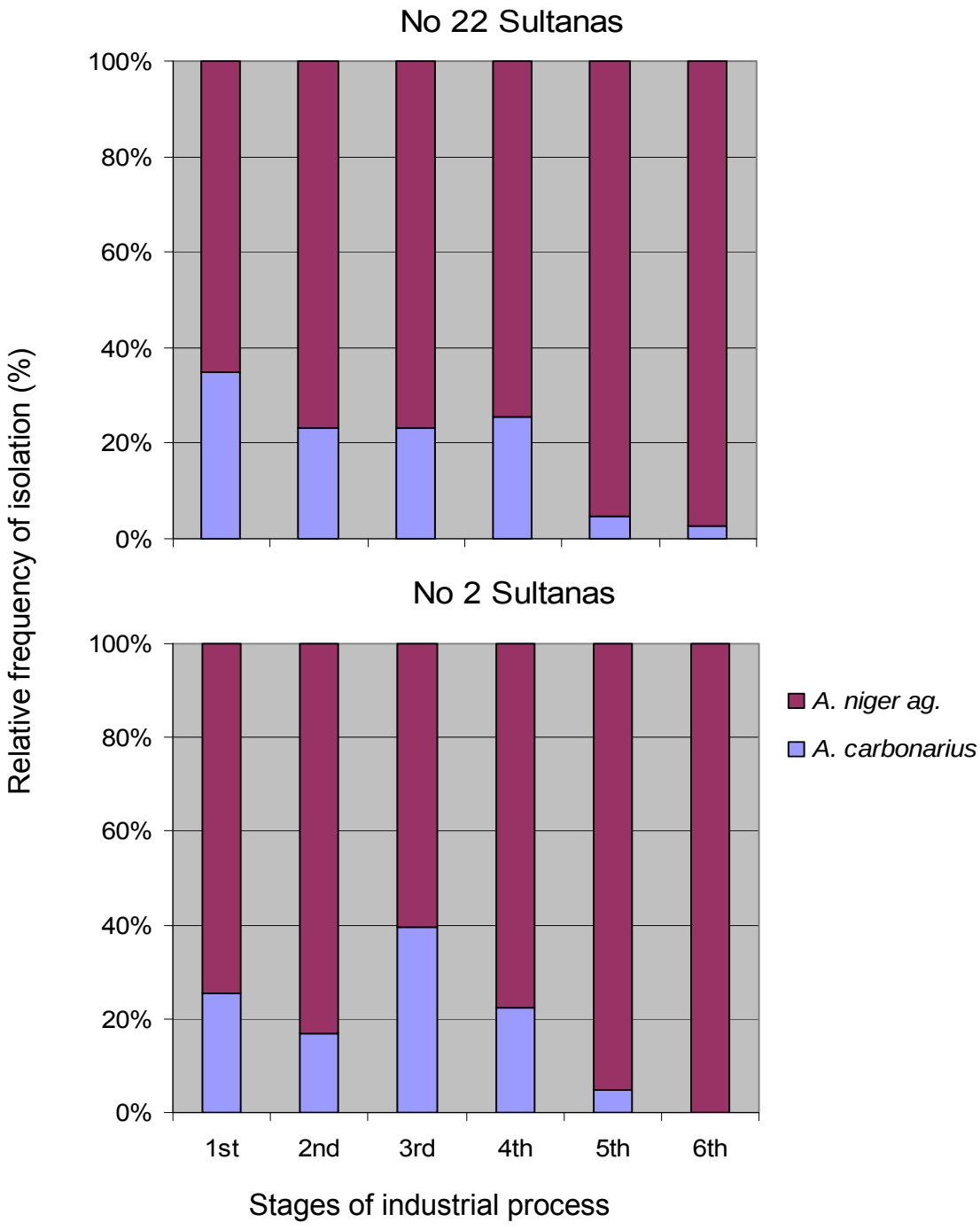


Figure 3.4. Relative frequency of isolation of *A. carbonarius* and *A. niger* aggregate, on two types of sultanas (No2 and No22) from the crop of 2004/05, during industrial processing with SO_2. The isolation medium used is MEA98 (0.985 a_w). The values are the means of four replicates. Keys-words: 1st=classification stage, 2nd=pre-washing, 3rd=SO_2 treatment, 4th=final washing, 5th=heat-treatment, 6th=final product.

Table 3.4. Summary statistical table of significance (Univariate Analysis of Variance) of the effects of two abiotic parameters examined (stage, type) and their interaction on *A. carbonarius* population dynamics. The serial dilution method was used on MEA98 isolation medium.

Source	df	F	Sig.
Stages	5	3.235	0.016*
Type	1	0.269	0.607
Stages * type	5	0.449	0.811

(*) significant at P<0.05

Table 3.5. Homogenous subsets of the effects of stage on *A. carbonarius* populations isolated using the serial dilution method, on MEA98 isolation medium.

	Stages	N	Subset	
			1	2
Duncan[(a,b)]	5 a	8	0.0000	
	6 a	8	0.0000	
	4 a	8	0.4560	
	1 ab	8	1.3636	1.3636
	2 ab	8	1.5784	1.5784
	3 b	8		2.2664

Significance level = 0.05

Table 3.6(a,b). Summary statistical table of significance (Univariate Analysis of Variance) of the effects of two abiotic parameters examined (stage, type) and their interaction on *A. carbonarius,* isolated by direct plating isolation method on (a) MEA95 and (b) MEA98 media.

(a)				(b)			
Source	df	F	Sig.	Source	df	F	Sig.
Stages	5	8.837	0.000***	Stages	5	13.489	0.000**
Type	1	4.058	0.045**	Type	1	6.316	0.012**
Stages * type	5	2.705	0.020***	Stages * type	5	1.298	0.264

(***) significant at P<0.001 (**) significant at P<0.01

Table 3.7(a,b). Effect of types of sultanas on *A. carbonarius* isolated by direct plating isolation method on (a) MEA95 and (b) MEA98 isolation media.

(a)			(b)		
Type	Mean	Std. Error	Type	Mean	Std. Error
No22 sultanas	,033	0.009	No22 sultanas	0.254	0.025
No2 sultanas	,008	0.009	No2 sultanas	0.167	0.025

Table 3.8(a,b). Homogenous subsets of the effects of stage on *A. carbonarius* dynamics isolated by direct plating isolation method on (a) MEA95 and (b) MEA98 media.

(a)

	Stages	N	Subset	
			1	2
Duncan[(a,b)]	3 a	80	0.00	
	4 a	80	0.00	
	5 a	80	0.00	
	6 a	80	0.00	
	2 a	80	0.01	
	1 b	80		0.11

Significance level = 0.001

(b)

	Stages	N	Subset		
			1	2	3
Duncan[(a,b)]	6 a	80	0.01		
	5 a	80	0.04		
	4 b	80		0.24	
	2 bc	80		0.25	0.25
	3 bc	80		0.31	0.31
	1 c	80			0.41

Significance level = 0.01

- *A. niger* aggregate

Tables 3.9–3.14 show the statistical analyses for *A. niger* aggregate. Both population loads and relative frequency of isolation of *A. niger* aggregate were statistically influenced by the "stage" and "type", regardless of a_w levels. During the final stages of industrial processing (stages 5-6) they were grouped together showing that heat-

treatment was the key-factor for elimination of the fungal populations. Fungi were more frequently isolated from No22 sultanas (see Table 3.10). Moreover, in some cases, fungal loads were higher in the third stage of the production line with SO_2 treatment.

Table 3.9(a,b). Summary statistical table of significance (Univariate Analysis of Variance) of the effects of two abiotic parameters examined (stage, type) and their interaction on *A. niger* aggregate isolated by serial dilution isolation method on (a) MEA95 and (b) MEA98 isolation media.

(a)				(b)			
Source	df	F	Sig.	Source	df	F	Sig.
Stages	5	8.282	0.000*	Stages	5	11.787	0.000*
Type	1	10.204	0.003*	Type	1	6.996	0.012*
Stages * type	5	0.868	0.512	Stages * type	5	0.752	0.590

(*) significant at P<0.05

Table 3.10(a,b). Effect of types of sultanas on *A. niger* aggregate populations, isolated by serial dilution isolation method on (a) MEA95 and (b) MEA98 isolation media.

(a)			(b)		
Type	Mean	Std. Error	Type	Mean	Std. Error
No22 sultanas	4.153	0.310	No22 sultanas	4.149	0.274
No2 sultanas	2.752	0.310	No2 sultanas	3.124	0.274

Table 3.11(a,b). Homogenous subsets of the effects of stage on *A. niger* aggregate, isolated by serial dilution isolation method on (a) MEA95 and (b) MEA98 media.

(a)				
	Stages	N	Subset	
			1	2
Duncan[(a,b)]	6 a	8	1.2618	
	5 a	8	1.8690	
	4 b	8		3.6088
	2 b	8		4.3926
	3 b	8		4.5293
	1 b	8		5.0547

(b)

	Stages	N	Subset			
			1	2	3	4
Duncan[a,b]	6 a	8	0.9267			
	5 b	8		2.6912		
	4 b	8		3.5626	3.5626	
	2 bc	8			4.3781	4.3781
	1 c	8				5.0852
	3 c	8				5.1756

Significance level = 0.01

Table 3.12. Summary statistical table of significance (Univariate Analysis of Variance) of effects of stage, type and their interaction on *A. niger* aggregate isolated by direct plating isolation method on MEA98 isolation medium (same pattern is presented on MEA95 isolation medium, see Appendix B).

Source	df	F	Sig.
Stages	5	21.819	0.000***
Type	1	163.018	0.000***
Stages * type	5	14.048	0.000***

(***) significant at P<0.001

Table 3.13. Effect of types of sultanas on *A. niger* aggregate isolated by direct plating isolation method on MEA98 isolation medium (pattern is presented on MEA95 isolation medium, see Appendix B).

Type	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
No22 sultanas	0.967	0.021	0.899	1.035
No2 sultanas	0.596	0.021	0.528	0.664

Table 3.14. Homogenous subsets of the effects of stage on *A. niger* aggregate isolated by direct plating isolation method on MEA98 isolation medium, (same pattern is shown on MEA95 isolation medium, see Appendix B).

	Stages	N	Subset		
			1	2	3
Duncan[a,b]	6 a	80	0.53		
	4 b	80		0.74	
	3 b	80		0.75	
	5 b	80		0.75	
	1 c	80			0.93
	2 c	80			1.00

Significance level = 0.001

3.5.1.2. Effects of SO_2 treatment combined with heat-treatment on fungal population dynamics of industrial processed sultanas

In 2006, the effect of SO_2 treatment combined with heat-treatment on fungal population dynamics was specifically investigated. Hence, there were samples (vine crop of 2005/06) exposed to SO_2 treatment (No2 sultanas) and some others which did not receive SO_2 treatment (No22 sultanas). All samples undertook heat treatment. The sampling pattern was similar to that of the previous year.

Figure 3.5 shows the fungal diversity of industrial processed sultanas from the 2005/06 vine crop based on two industrial methods, with and without SO_2 treatment. More specifically, it shows population dynamics (Log_{10} CFUs gr^{-1} sultanas) of the most dominant fungal genera/species on MEA98 medium, at four basic stages of two industrial processes studied. Although at classification stage the fungal population diversity was not the same in both kinds of treatements, black aspergilli, and *A. niger* aggregate in specific, were the most dominant fungi, during both industrial processes. They were followed by yeasts and *Penicillium* spp. Fungal population loads were

almost eliminated, after their exposure to SO_2, except of of *A. niger* group ones. The overall contamination levels were decreased from 5 and 6 Log_{10} CFUs gr^{-1} to 2.5 and 4.0 Log_{10} CFUs gr^{-1} after SO_2 application combined with heat-treatment and heat-treatment alone, respectively. The same patterns were observed on MEA95 (0.954 a_w) isolation medium (see Fig.3.5, APENDIX A) where efficacy of the first method ($+SO_2$) is more clearly observed.

Figure 3.6 focus to the population dynamics of black aspergilli, on MEA98 medium. The dominance of *A. niger* aggregate group over *A. carbonarius* is clearly observed. In the untreated sultanas, *A. carbonarius* was isolated in about of 0-2 Log_{10} CFUs gr^{-1} while *A. niger* aggregate species were isolated in 5.5 - 6.3 Log_{10} CFUs gr^{-1}. However, in the final products, *A. carbonarius* was not isolated at all, while *A. niger* aggregate were isolated in 2.5-3.0 Log_{10} CFUs gr^{-1}. It is notable that *A. niger* isolates were present in 4.5 Log_{10} CFUs gr^{-1} in the final product, after the application with heat-treatment alone (see Figure 3.6, APPENDIX A).

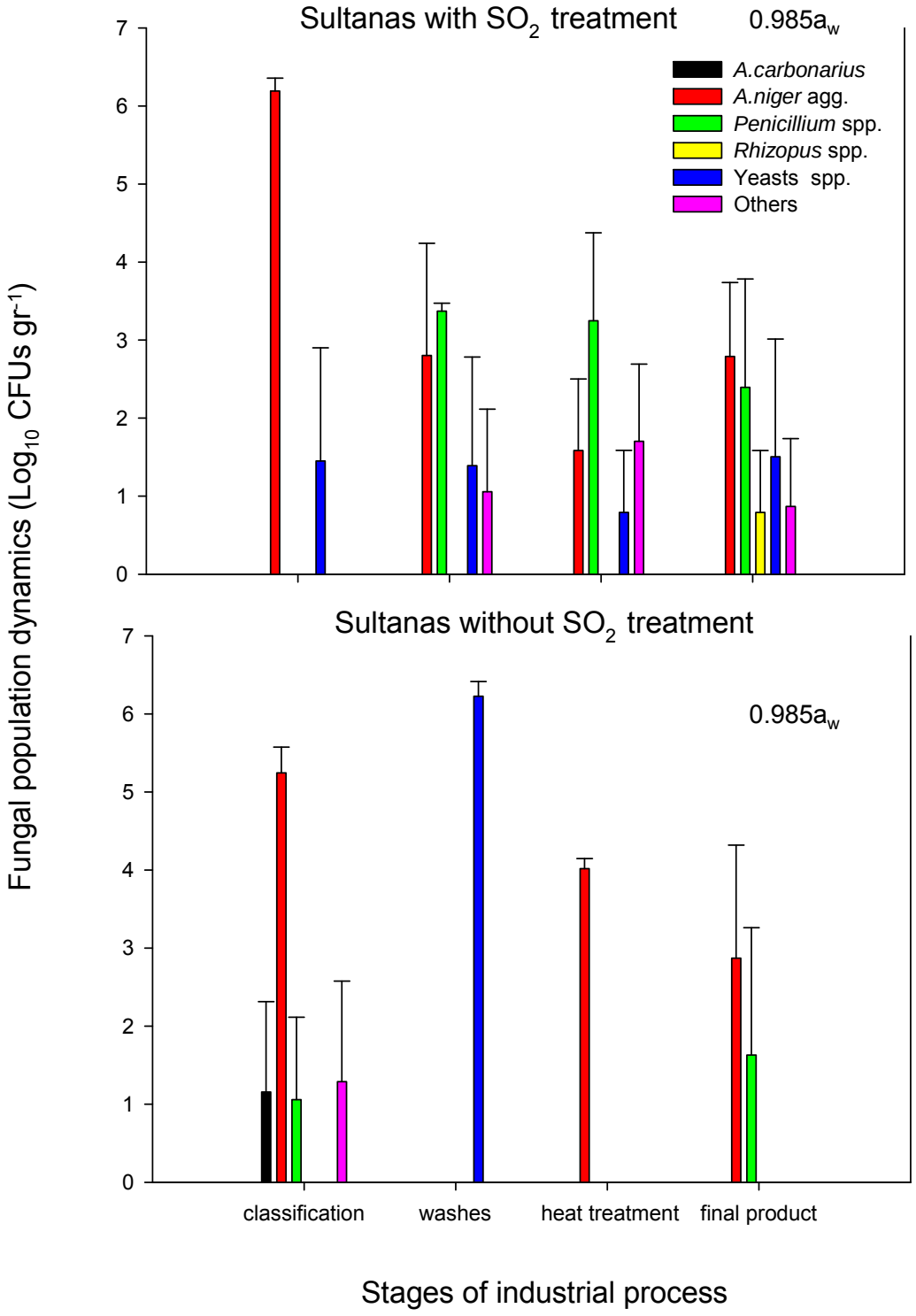


Figure 3.5. Population dynamics of the most dominant fungal species/genera on sultanas from 2005/06 crop, treated (No 2 sultanas) or untreated (No 22 sultanas) with SO_2, on MEA98 (0.985 a_w) medium, during industrial processing. The values are the means of three replicates. Bars indicate standard error of the mean.

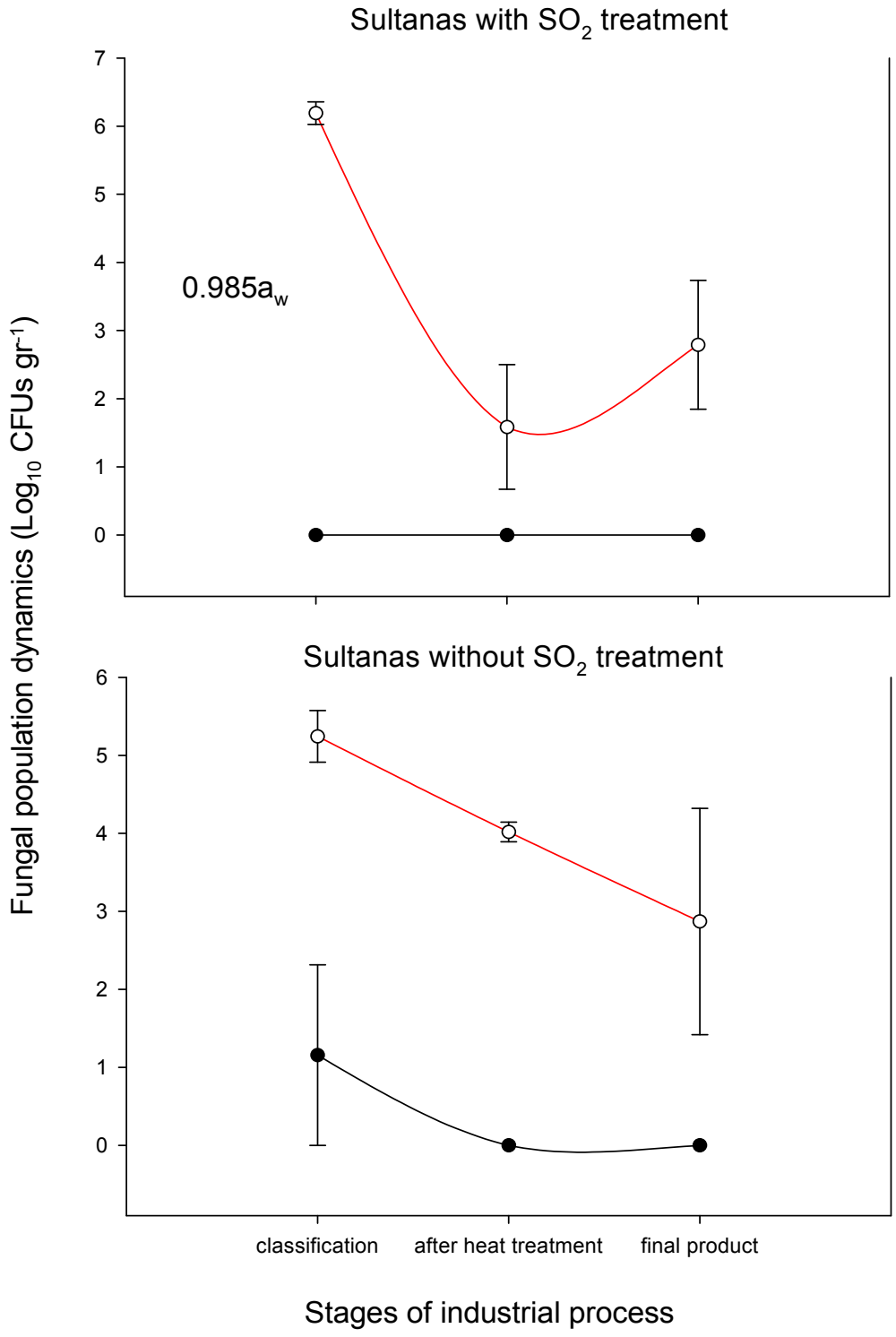


Figure 3.6. Population dynamics of *A. carbonarius* and *A. niger* aggregate, on sultanas treated (No 2 sultanas) or untreated (No 22 sultanas) with SO_2, on MEA98 (0.985 a_w) isolation medium, during industrial processing. The values are the means of three replicates. Bars indicate standard error of the mean.

Figure 3.7 shows the population dynamics of *A. niger* aggregate isolated on MEA95 medium. It shows three basic stages of industrial processes and compares the two industrial treatments examined; (i) SO_2 application combined with heat-treatment and (ii) heat- treatment alone. Fungal populations were more restricted after both SO_2 and heat-treatment applications than heat-treatment alone (see stage '*after heat-treatment*'); *A. niger* aggregate species were isolated in 1.6 and 4.0 Log_{10} CFUs gr^{-1}, respectively. However, the population dynamics of black asrpergilli seem not to be effectively eliminated, in the final product. A possible cross-contamination or bad hygienic practices may have been taken place.

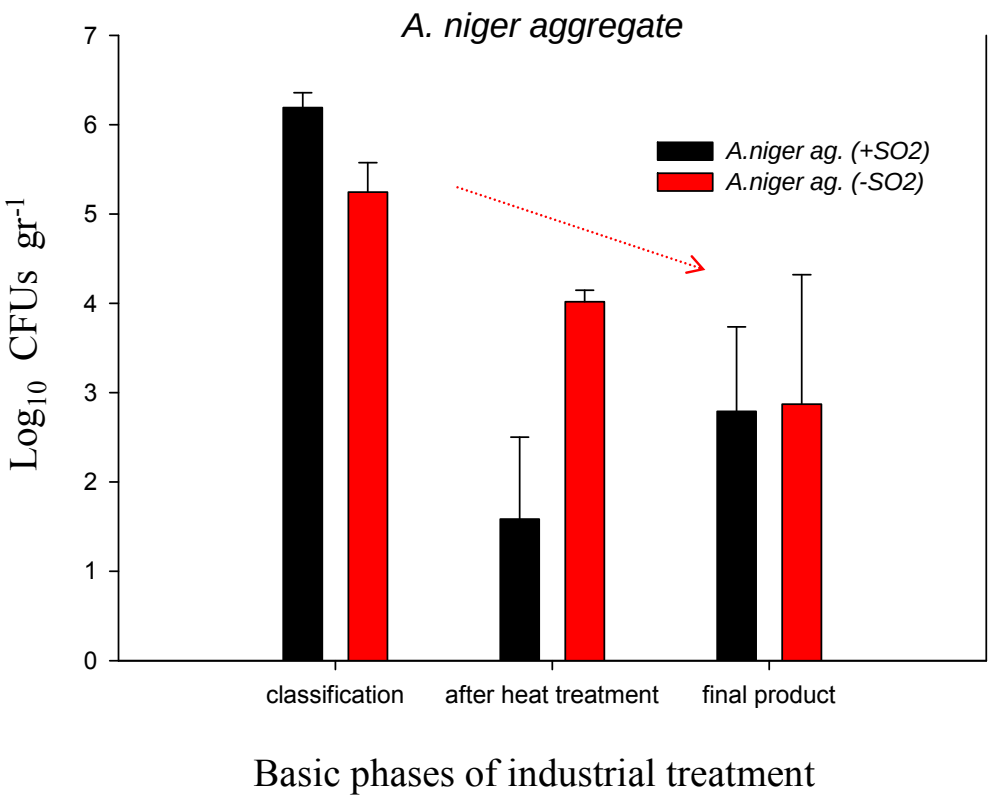


Figure 3.7. Effect of two industrial methods studied on *A. niger* aggregate populations, isolated from sultanas treated with and without SO_2 (2005/06 vine crop) on MEA95 (0.954 a_w) isolation medium. The values are the means of three replicates. Bars indicate standard error of the mean.

Statistical analyses

- *A. carbonarius*

Tables 3.15 and 3.16 show the results of statistical analysis for *A. carbonarius,* isolated by the serial dilution method. According to these, stages of industrial process significantly affected the fungal population dynamics.

Table 3.15. Summary statistical table of significance (Univariate Analysis of Variance) of effects of stage, type and their interaction on *A. carbonarius,* isolated by the serial dilution method

Source	df	F	Sig.
Type	1	0.218	0.644
Stages	2	3.369	0.048*
type * Stages	2	0.218	0.806

(*) significant at P<0.05

Table 3.16. Homogenous subsets of the effect of stages on *A. carbonarius,* isolated by serial dilution isolation method.

	Stages	N	Subset	
			1	2
Duncan(a,b)	washes	12	0.0000	
	final product	12	0.0000	
	Untreated product	12		0.8825

Significance level = 0.05

- *A. niger* aggregate

Tables 3.17-3.19 show the results of statistical analysis for *A. niger* aggregate species. Fungi were influenced by the type of industrial method used. It seems that heat-treatment combined with SO_2 application might be more efficient than heat-treatment alone.

Table 3.17(a,b). Summary statistical table of significance (Univariate Analysis of Variance) of effects of stage, type and their interaction on *A. niger* aggregate isolated on MEA98 (a) and MEA95 (b) by using serial dilution isolation method.

(a)				(b)			
Source	df	F	Sig.	Source	df	F	Sig.
type	3	7.798	0.623	type	1	1.948	0.182
Stages	1	0.251	0.002 **	Stages	3	14.468	0.000 **
type * Stages	3	2.398	0.106	type * Stages	3	3.116	0.056

(**) significant at P<0.01

Table 3.18(a,b). Homogenous subsets of *A. niger* aggregate isolated on MEA98 (a) and MEA95 (b) by using serial dilution isolation method.

(a)

	Stages	N	Subset	
			1	2
Duncan(a,b)	Washes	6	1.4013	
	final product	6	2.6010	
	Oven	6	3.0665	3.0665
	Classification	6		5.7179

(b)

	Stages	N	Subset		
			1	2	3
Duncan(a,b)	Washes	6	0.6285		
	Oven	6	2.5560	2.5560	
	final product	6		3.4406	3.4406
	Classification	6			5.6051

Significance level = 0.01

3.5.2. OCHRATOXIN CONTAMINATION DURING INDUSTRIAL PROCESS

Industrial process did not statistically (P<0.05) influence OTA contamination. The mean level of OTA accumulation in all samples tested were 12 µg Kg^{-1} at the untreated berries up to 3.2 µg $K.g^{-1}$ at the final products. Types of sultanas (No2 and No22 sultanas) as well as type of industrial processing system (sulphur dioxide added or not added to sultanas) had no significant effect on OTA content in the final product.

3.6. DISCUSSION

3.6.1. EFFECT OF SULTANAS TYPE ON CONTAMINATION WITH BLACK ASPERGILLI

This study examined the potential impact of sultanas type (No22 and No2) on fungal populations during industrial process with both SO_2 application and heat-treatment, focusing on black aspergilli. Findings pointed out black aspergilli as the most dominant fungal species in sultanas, which compares well with findings from field studies (see chapter 2). Moreover, although fungal population dynamics were slightly decreased at the washing phases (stages 2 and 4) an increase of fungal diversity was observed. This can be explained by the fact that rinsing water, used in the washing steps, was half-recycled and therefore, cross-contamination could easily have occured.

Furthermore, a non significant increase in fungal population load was observed, after SO_2 treatment. A recent survey showed that exposure to medium concentrations (100-250 mg L^{-1}) of sodium metabisulphite (NaMBS) stimulates fungal growth of *A. carbonarius* (Pateraki *et al*., 2007). It is notable that fungal populations decreased after heat-treatment (stage 5). This indicates that heat-treatment is the key-procedure to eliminate fungal populations. It is worth noting that heat-treatment appeared to be more efficient when applied to No2 sultanas. This could be explained by the fact that No2 sultanas undertook heat-treatment for a longer time than No22 type. A longer duration of this treatment (even only for a few minutes) made it more efficient. In addition, No22 sultanas have a bigger total surface per Kg in comparison to No2 sultanas, due to their smaller size. Thus, for a successful minimizing or eliminating of fungal contamination, they might need a longer exposure period to high temperatures.

Similar patterns were observed when relative frequency of isolation was considered. Fungal diversity, interestingly, was high during the final stages of processing. In the final products, black aspergilli remained the most frequently isolated fungi. Among the black aspergilli, *A. niger* aggregate was the dominant fungal group against *A. carbonarius*, regardless of a_w levels. Overall, as the statistical analysis showed, both black aspergilli groups were affected by the stage and the type of sultanas, regardless of water activity level. It seemed that black aspergilli were more frequently isolated in sultanas No22. This indicates that the method used for fungal elimination, was more efficient on No2 sultanas. The washing steps reduced fungal populations by thorough rinsing of the external surfaces of sultanas.

High temperature treatment (up to 90^o C) apperared to be the most effective way to eliminate the fungal population load. A decrease of *A. niger* aggregate dynamics was observed in about 45% and 65%, in No22 and No2 sultanas respectively, in comparison to the initial counted loads. In summary, the results suggest that the process currently followed by the KSOS industry is not able to fully eliminate fungal populations of *A. niger* group from the final product. The above may be the cause of quality degradation and shorter shelf-life of the treated sultanas.

3.6.2. COMPARISON BETWEEN SULTANAS PROCESSING METHODS REGARDING FUNGAL ELIMINATION.

In the current survey, two industrial methods were examined in order to estimate their efficacy (a) heat-treatment combined with sulphur dioxide application (SO_2) and (b) heat-treatment alone.

All patterns were similar in both years studied. Among the black aspergilli, *A. carbonarius* was less frequently isolated (1 Log_{10} CFUs g^{-1}), while *A. niger* aggregate species were isolated in the range of 5.5-6.5 Log_{10} CFUs g^{-1}, in untreated sultanas. In the final products, populations of *A. carbonarius* were practically eliminated in comparison to other black aspergilli, which were just reduced. As statistical findings indicate, *A. carbonarius* was not influenced by the industrial treatment method. However, care is needed because of the very low populations isolated. Populations of *A. niger* aggregate seemed to become more restricted by the combination of SO_2 and heat treatment. For this kind of industrial treatment only No2 sultanas were used. Hence, heat-treatment is more efficient with these samples based on previous surveys although that the most fungal spores are destroyed during thermal inactivation (Earle and Putt, 1984; Legan, 1993)

Overall, based on statistical analyses of the data, SO_2 application, combined with heat treatment, did not statistically significantly affect fungal dynamics, and more specifically black aspergilli populations. However, earlier studies indicated that different concentrations of applied SO_2 may have different effects on microbial populations (King *et al.*, 1981). Low amounts (1.5 ppm) of undissociated H_2SO_3 concentration were found to have a similar effect to that of free SO_2. Studies on filamentous fungi, including *Aspergillus* and *Penicillium* spp., indicated that exposure to 2.66 ppm of SO_2 for 10 mins stimulated fungal activity, while after 60 mins, fungal growth was inhibited (Singh *et al*., 1990). Moreover, previous studies pointed out that moist spores are more sensitive to SO_2 application than dry ones (Magan, 1992). Moreover, a survey on *Alternaria* spp. showed that germination of wet spores was decreased by about 60% by treatment with 50 ppm SO_2, for 24 mins, while >100 ppm

of SO_2 was needed to restrict dry spores germination, at high level Relative Humidity (RH=98%). More recent studies (Pateraki *et al*., 2007) on *A. carbonarius* isolates demonstrate that intermediate concentrations of NaMBS (100 - 250 mg L^{-1}) could facilitate spore germination, fungal growth and OTA production. In contrast, higher concentrations of NaMBS (>500 mg L^{-1}) resulted in complete inhibition. Overall, several parameters (i.e. formulation of SO_2, duration, pH of the sulphate solution, temperature, water availability) all have to be taken into account when considering SO_2 treatments. Interactions with these abiotic factors could make SO_2 application more or less efficient.

Finally, the literature suggests that the threshold SO_2 concentration may significantly vary depending on the tolerance of fungi. Previous surveys showed that yeasts are more sensitive to SO_2 exposure while some isolates of *Penicillium* spp were found to be more tolerant. The tolerance of fungi to high concentrations of SO_2 might be due to their ability to transport SO_2 into their mycelia (Tweedie and Segel, 1970). These considerations may explain the dominance of *Penicillium* species over yeasts and, occasionally, of black aspergilli after SO_2 treatments (see Fig.3.6, APPENDIX A).

3.6.3. OTA CONTAMINATION IN THE UNTREATED/FINAL PRODUCTS

Toxin was detected in very low concentrations in sultanas with SO_2 treatment while OTA contamination was higher in sultanas without SO_2 fumigation. More specific, OTA was detected in about 3.5 μg Kg^{-1} and 205 μg Kg^{-1} in untreated and final products of sultanas without SO_2 fumigation, respectively. However, statistical analyses pointed out that levels of ochratoxin A contamination were not statistically effected by industrial process, regardless of type of raisins (No22 and No2 sultanas)

and the method used (with and without SO_2). However, in some samples, OTA concentration was detected in excess in comparison to untreated samples. These cases are mainly associated with SO_2 treatments. This is in agreement with earlier studies conducted on filamentous fungi (Pateraki *et al.*, 2007). For example, both *Neosartoya fischeri* and *Byssochlamys nivea* were found to produce mycotoxins in the presence of 100 ppm and 50 - 75 ppm SO_2, respectively (Magan *et al*., 1991).

On the other hand, heat treatment may enhance OTA decomposition or adsorption. Recent studies demonstrated that dead cells of *Phuffia rhodzyma* isolates were able to adsorb significant amounts of the toxin, up to 250 ng mL^{-1} (Peteri *et al.*, 2007). Moreover, studies on wheat pointed out that OTA decomposition is absolutely dependant on temperature and duration of heat-treatment. Previous studies also demonstrated that processing of coffee beans appears to reduce but not eliminate OTA (Tsubouchi *et al.,* 1987; Micco *et al.,* 1989; Heilmann *et al.,* 1999).

Moisture conditions are also a crucial parameter. OTA could be greatly decomposed (> 60%) by heating in wet conditions. More specific, at 100^{o} C and 150^{o} C, the presence of water (50%) appeared to increase this function (Boudra *et al*., 1995). However, the literature also suggests that OTA can be detoxified by several microorganisms and their enzymes, such as bacteria (Hwang and Draughon, 1994), filamentous fungi (Varga *et al.,* 2005) and numerous yeasts (Molnal *et al.*, 2004). Interestingly, yeasts (Bejaoui *et al.*, 2004) and conidia of black aspergilli (Bejaoui *et al.*, 2005) could also adsorb OTA.

3.6.4. POTENTIAL CRITICAL CONTROL POINTS (CCPs) OF OTA CONTAMINATION RISK ON INDUSTRIAL PROCESSED SULTANAS

The current survey suggests that there are some potential critical points during the industrial process which need to be monitored. Firstly, storage conditions should be in accordance with recommendations suggested by the European Communities (Commission Directive, 2002/26/EC) or other official committee. By improving the hygienic conditions, microbial contamination risk can be successfully controlled.

Some other critical parameters that should be taken into account are formulation of SO_2, duration of the SO_2 treatment, pH and water availability of the product. As mentioned above, these abiotic factors could influence efficiency of SO_2 by enhancing or inhibiting its toxicity. Furthermore, SO_2 may act as a stimulator to fungal populations and toxin production, under specific conditions. Concerning the heat-treatment, which is the key-procedure for fungal elimination, the appropriate temperature and duration of the treatment could determine the efficiency of the industrial procedure. Another critical factor, which should be taken into consideration, is the purity of the rinsing water, as the presence of contaminants can lead to cross-contamination. Heenan *et al.* (1998) found that *A. niger* and *A. carbonarius* could be isolated from dried fruit wash water. Bucheli *et al*. (2002) suggested that OTA contamination could effectively be controlled by good agricultural practices, as well as appropriate post-havesting handling (i.e. techniques for drying, transportation and storage).

3.6.5. CONCLUSIONS OF THE CURRENT SURVEYS

1. The fungal populations of untreated sultanas were mainly composed of black aspergilli (dominant fungi), *Penicillium* spp, yeasts and *Rhizopus* spp.

2. *A. carbonarius* was isolated in low levels (1.5-2 Log_{10} CFUs gr^{-1}) and frequently isolated in about 20% of untreated sultanas, regardless of type of sultanas. After heat treatment, populations of *A. carbonarius* were almost eliminated.

3. *A. niger* aggregate were the predominant fungal species among black aspergilli, regardless of type of sultanas. They were isolated at about of 5.8 Log_{10} CFUs gr^{-1} and frequently isolated from up to 45% in untreated sultanas. Overall, these fungi were detected in about 1.8 - 4.0 Log_{10} CFUs gr^{-1} and frequently isolated up to 85%, after heat treatment. They were not eliminated.

4. Types of sultanas (No22 and No2) significantly influenced the population dynamics of black aspergilli. In general, higher contamination levels were found in No2 sultanas.

5. Heat treatment (up to 90^{o} C) is the key-procedure for the elimination of fungal populations. Several parameters, such as duration of the treatment, might improve the efficiency of the method.

6. SO_2 treatment using the specific considerations (spraying with sulphur dioxide) does not seem to statistically alter the fungal population loads. However, interactions with several abiotic factors (i.e. formulation of SO_2, duration, pH of the sulphate solution, temperature, water availability) should

be taken into consideration to optimize the conditions of the aforementioned process.

7. The levels of ochratoxin A contamination are not statistically different in untreated and in final industrially processed sultanas, regardless of he type of the raisins (No22 and No2 sultanas) and the method used (with and without SO_2) in the two seasons studied.

8. It is important to ensure purity of the rinsing water to prevent cross-contamination.

Chapter Four

ECOLOGICAL STUDIES *IN VITRO* AND POTENTIAL CONTROL OF *A. CARBONARIUS*

4.1. INTRODUCTION AND OBJECTIVES

In nature, there is a range of abiotic and biotic factors which influence fungal activity (see section 1.7). Water availability (a_w), several preservatives (e.g. SO_2), modified atmospheres, fungal interactions, nutrition sources and temperature are some parameters which should be taken into account when the ecological behaviour of a fungus is studied.

In the current set of *in vitro* experiments, some of the above factors were examined in accordance with the fungal activity of black aspergilli, and in particular of *A. niger* aggregate and *A. carbonarius*. Work has concentrated on the latter due to its ochratoxigenic ability; it is the key-fungus for OTA contamination in grapes and their derivatives, especially in Mediterranean climates (Markaki *et al.,* 2001, Battilani *et al.,* 2003a).

There is a significant body of literature on the effect of the water availability (a_w) on the ecological behaviour of black aspergilli (*A. carbonarius* and *A. niger* aggregate) but much less on the effects of combination of a_w x SO_2 or a_w x CO_2 (modified atmospheres), are known, especially in relation to vine fruit production. Simultaneously, antagonism is an ubiquitous phenomenon in microbial systems. It deals with the fact that several species could occupy similar general niches. Therefore, competition between microbial organisms or potential overlap, due to their ecologically similar behaviour in a community, could take place. Antagonism of *A carbonarius* against other fungi has not been studied in detail, especially under conditions relevant to drying vine fruits.

Overall, this study attempted to investigate the influence of some major abiotic and biotic factors that influence both fungal activity and OTA production by these ochratoxigenic fungi.

The objectives were the followings:

- To examine the effects of SO_2 and a_w on lag phase prior to germination, on germination, mycelial growth and OTA production on both agar (grape–juice-like media) and on sultanas.

- To examine the effects of modified gas atmospheres and a_w on lag phase prior to germination, germination, mycelial growth and OTA production on both agar (grape–juice-like media) and on sultanas.

- To investigate the effect of fungal competition between *A. carbonarius* and other species using the Index of Dominance approach and on OTA production on both agar (grape–juice-like media) and in sultanas.

4.2. MATERIALS AND METHODS

4.2.1. EFFECT OF A_W AND SODIUM METABISULPHITE (NaMBS) ON GROWTH AND OTA PRODUCTION BY *ASPERGILLUS CARBONARIUS*

4.2.1.1. Fungal isolates

Three strains of *A. carbonarius* (GRE105, GRE119 and GRE117) were isolated from sultanas. They originated from Cretan ecosystems (crop year 2004/05) for which ochratoxigenic ability was previously assessed using both Coconut Cream Agar (Heenan *et al.,* 1998) and by exposure to ammonia before checking them under UV light (see §2.3). For higher reliability, HPLC analysis was also used (see §2.4). An Italian isolate (isolate IMI388653) from red wine grapes with known OTA producing ability was also used for comparison (Mitchell *et al.,* 2004; CABI BioSciences, Egham, Surrey).

4.2.1.2. Preparation of the media

White Grape Juice Agar medium (WGJM) was used as the basal medium throughout the *in vitro* studies. The medium was prepared by mixing 25% (v/v) supermarket long life white grape juice (Sainsbury's, pure white grape juice, UK, see Table 4.1. in APPENDIX A) and 2.5% agar (Oxoid, UK technical agar No.3) in distilled water, autoclaved at 121^{o} C for 15 mins cooled to approximately 50^{o} C and poured into sterile 90 mm diameter plastic Petri plates.

4.2.1.3. Modification of the medium water activity and pH

Adjustment of a_w to the required levels was done by adding D(+) glucose or glycerol (Fisher chemicals, F.w. 92.10) to the basal medium as detailed in Table 2.1, APPENDIX A. 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 were used: 0.965 and 0.93. They were obtained by adding 18.73 and 50.35g D(+) glucose 100 g^{-1} medium, respectively. The pH of all treatments was modified to 4.2 ± 0.2, (similar to those of sultanas), using a buffer solution (McIlvaine, 1921, see Table 4.2, APPENDIX A). A 100 mL solution of this buffer was prepared 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).

4.2.1.4. Adjustment of concentration of sodium metabisulphite (NaMBS)

Sodium metabisulphite ($Na_2S_2O_5$; NaMBS; BDH Chemicals Ltd, Poole, England) was used as source for the SO_2. Seven concentrations of Sodium metabisulphite were used: 0, 50, 100, 250, 500, 750 and 1,000 mg L^{-1}. In order to obtain these concentrations, a stock solution (30,000 mg L^{-1}) was prepared by adding 30g of NaMBS in 1 L distilled water. It should be highlighted, that 100 g of NaMBS releases 67.4 g of SO_2. No stock solution was added to the control treatment. In summary, in each flask an amount of white grape juice and buffer solution were added in a proportion of 1:4, respectively. Then the appropriate amounts of glucose or glycerol, agar (2.5%) and NaMBS were added. In total 18 different treatments were evaluated.

4.2.1.5. Inoculation and incubation

Inocula were prepared by growing the strains on Malt Extract Agar (Oxoid) at 25° C for 7 days, to obtain heavily sporulating cultures. Spore suspensions of each isolate were prepared by harvesting spores from these cultures and suspending them in sterile distilled water containing 0.005% of a wetting agent (Tween 80) to assist dispersion of the spores. The final concentration of the spore suspension was measured by using a haemocytometer slide, and was adjusted to approx. 10^6 spores mL^{-1}. The Petri plates of each treatment were centrally inoculated with a small loop of spore suspension (Pitt and Hocking, 1997) for mycelial growth studies. 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 with the same a_w were enclosed in polyethylene bags to ensure no fluctuation in a_w. All experiments were carried out with four replicates per treatment, incubated at 25° C and carried out twice.

4.2.1.6. Measurement of spore germination, germ tubes extension and mycelial growth

The effects of a_w x NaMBS on spore germination, germ tubes extension and mycelial growth were evaluated. Three agar plugs from each replicate plate were cut out with a sterile cork borer (1.5 cm) and placed on a slide. Plugs were removed after 4, 6 or 8, 12, 24, 36 and 48 hours of incubation. The disks were stained with lactophenol blue solution (Fluka) and examined microscopically. A total of 50 spores per plug (150 per agar plate) were counted. Spores were considered to have germinated when the germ-tube length was equal to or greater than the diameter of the spore. Germ-tube extension was measured for 3 x 30 spores for each treatment, at random, using an eyepiece micrometer. Moreover, mycelial growth rates were determined by daily

measurement of the colony diameter in two directions at right angles to each other. Measurements were carried out for 2-10 days. Linear regression of the colony radius against time (days) was used to obtain the growth rates (cm day^{-1}) under each set of growth conditions.

4.2.1.7. Ochratoxin A extraction and HPLC analysis

Ochratoxin A analysis was performed by cutting three agar plugs one from the inner and two from the middle areas of each colony using a cork borer (1.5 cm) (see Figure 4.1). Each sample was introduced into a preweighed Universal bottle. Five mL of HPLC grade methanol (Fisher, UK) were added and the samples were shaken and incubated at room temperature for 60 minutes. The extracts were then filtered (Millex HV 13MM, Millipore) directly into amber HPLC vials (Jaytee Biosciences LTD, UK) using a filter agent (celite 545, Aldrich®) and stored at 4° C until HPLC analysis was performed as detailed in §2.4.3.

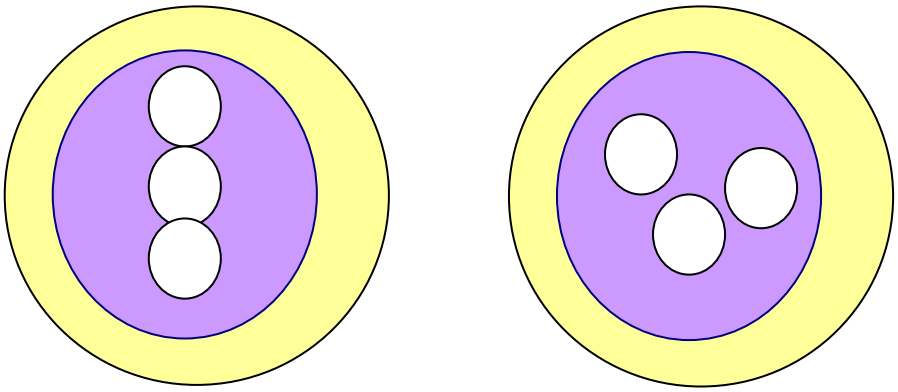


Figure 4.1. Two examples of agar plugs removed randomly from agar plates.

4.2.2. EFFECT OF GAS COMPOSITION AND A_W ON SPORE GERMINATION, MYCELIAL GROWTH AND OCHRATOXIN A PRODUCTION IN WHITE GRAPE JUICE MEDIUM

4.2.2.1. Fungal isolates

Two isolates of *A. carbonarius* (strains GRE105, GRE117) with high ochratoxigenic properties, isolated from Cretan sultanas of the crop year 2004/05, were used in this experiment. Their ochratoxigenic ability was previously assessed using CCA medium (Heenan *et al.,* 1998) and by HPLC analysis (see § 2.3 and 2.4). For comparison, an Italian *A. carbonarius* strain (isolate IMI388653) originated from red wine grapes with known OTA producing ability, was also used (Mitchell *et al.,* 2004; CABI BioSciences, Egham, Surrey).

4.2.2.2. Preparation of the media

White Grape Juice Agar Medium (WGJM) was used as the basal medium throughout the controlled atmospheres trials (see §4.2.1.2). The pH of all treatments was modified to 4.2 ± 0.2, (similar to those of sultanas), using a buffer solution (see §4.2.1.3). The media were also prepared as described in §4.2.1.3.

4.2.2.3. Medium water activity (a_w) adjustment

White Grape Juice Agar Medium (WGJM) was modified to two water availability levels (0.965 and 0.93 a_w) by adding 18.73 and 50.35g D(+) glucose (Fisher chemicals,) 100 g^{-1} medium, respectively (see §4.2.1.3). The a_w of this basic medium was 0.985, determined with a Humidat Sprint IC II thermoconstanter (Novasina, Zurich, Switzerland). A calibration curve of water activity (Fig. 4.1, APPENDIX A) for white grape juice was previously obtained (Dallyn and Fox, 1980).

4.2.2.4. Inoculation and incubation

Inoculation and incubation steps were performed as described previously, in §4.2.1.5.

4.2.2.5. Gas mixtures adjustment

Immediately after inoculation, the plates with the same treatment were placed in appropriate plastic chambers (36 L volume). Each chamber had inlet and outlet tubes to allow gas mixtures to be passed through them. The inlet tube was connected inside the chamber to a spurger which was placed in a flask containing 500 mL of water or glycerol solutions as appropriate to maintain the equilibrium relative humidity of the gas mixtures and the atmosphere in each chamber at the target a_w level for agar treatments (0.985, 0.965 and 0.930 a_w). A computerized gas blender (Signal Series 850 Gas blender, Camberely, UK) was used to provide the three treatments: (a) air (21% O_2, 0.03% CO_2 and 79% N_2); (b) 25% CO_2 (1% O_2, 25% CO_2 and 74% N_2); (c) 50% CO_2 (1% O_2, 50% CO_2 and 49% N_2). Gas compositions were also periodically checked with a Gas Chromatographer (GC; Carlo Erba model GC-8340, Carlo Erba Instruments, Hemel Hempstead, Herts., UK). The chambers were flushed continuously and the Petri plates were measured and replaced immediately. Experiments were carried out at 25^{o} C.

4.2.2.6. Measurement of spore germination, germ tubes extension and mycelial growth

Spore germination, germ tube extension and mycelial growth were evaluated as described in §4.2.1.6. However, in the controlled atmospheres trial, the germ tube extension was evaluated after 24 and 48 hrs incubation while the mycelium diameter was measured after the 5th and 10th day of incubation, at 25^{o} C.

4.2.1.7. Ochratoxin A extraction and HPLC analysis

The OTA extraction method and the analysis technique are described in detail in §4.2.1.7.

4.2.3. EFFECT OF CONTROLLED ATMOSPHERES AND WATER AVAILABILITY (A_W) ON *A. CARBONARIUS* GROWTH AND OTA PRODUCTION IN SULTANAS

4.2.3.1. Fungal isolates

In this experiment, one isolate of *A. carbonarius* (strain GRE105) with abundant ochratoxigenic ability, isolated from Cretan sultanas of the crop year 2004/05, was used. Its ochratoxigenic ability was previously assessed using CCA medium (Heenan *et al.,* 1998) and by examination under UV light after exposure to ammonia (see §2.3). For higher reliability, HPLC analysis is also used (see §2.4).

4.2.3.2. Substrates and water activity modification

For this experiment, Cretan sultanas of the vine crop of 2005/06 were used as substrates. All treatments were modified to two a_w levels (0.884 and 0.928 a_w) which reflect those of drying vine fruits, at the first days of sun-drying (see Figure 2.9). In order to reach the target a_w levels, appropriate volumes of water were added to three types of sultanas (*ca* 0.65-0.70 a_w initially), according to the water absorption curve of sultanas (see Fig. 4.2, in APPENDIX A). Sultanas samples came from three different processing stages: (i) "A type" (sultanas after 10 days sun-drying period and before industrial process), (ii) "B type" (sultanas immediately after SO_2 treatment of industrial process) and (iii) "C type" (sultanas at the final stage of the industrial process with SO_2 treatment). After water addition, sultanas were left at 4^o C overnight

to assist water equilibration. A Humidat Sprint IC II thermoconstanter (Novasina, Zurich, Switzerland) was used to determine the final a_w of the sultanas.

4.2.3.3. Inoculation and incubation

Inocula were prepared by growing the strain GRE105 of *A. carbonarius* on Malt Extract Agar medium (Oxoid) at 25° C for 7 days, to obtain heavily sporulating cultures. Spore suspensions were prepared by harvesting spores as detailed in section 4.2.1.5. The final concentration of the spore suspension was assessed by using a haemocytometer slide, and was adjusted to approx. 2.5 x 10^2 spores mL^{-1}. 5 mL of the aforementioned inocula were added to each sultanas treatment with the same water activity treatments (250 g of raisins), in order to ensure uniform contamination of sultanas by *A. carbonarius*.

Treatments of the same a_w were enclosed in airtight chambers containing flasks with water or water-glycerol solutions in order to ensure no fluctuation in a_w. The experiment was carried out twice, at 25° C, with two replicates per treatment. Fungal assessment was performed after both 7 and 14 days of incubation period, while OTA analysis performed only after 14 days of incubation, at 25° C.

4.2.3.4. Adjustment of gas mixtures

A computerized gas blender (Signal Series 850 Gas blender, Camberely, UK) was used to provide three gas treatments: (a) air (21% O_2, 0.03% CO_2 and 79% N_2); (b) 25% CO_2 (1% O_2, 25% CO_2 and 74% N_2); (c) 50% CO_2 (1% O_2, 50% CO_2 and 49% N_2) (Plate 4.1.) . Adjustment of gas mixtures was performed as previously described in §4.2.2.5.

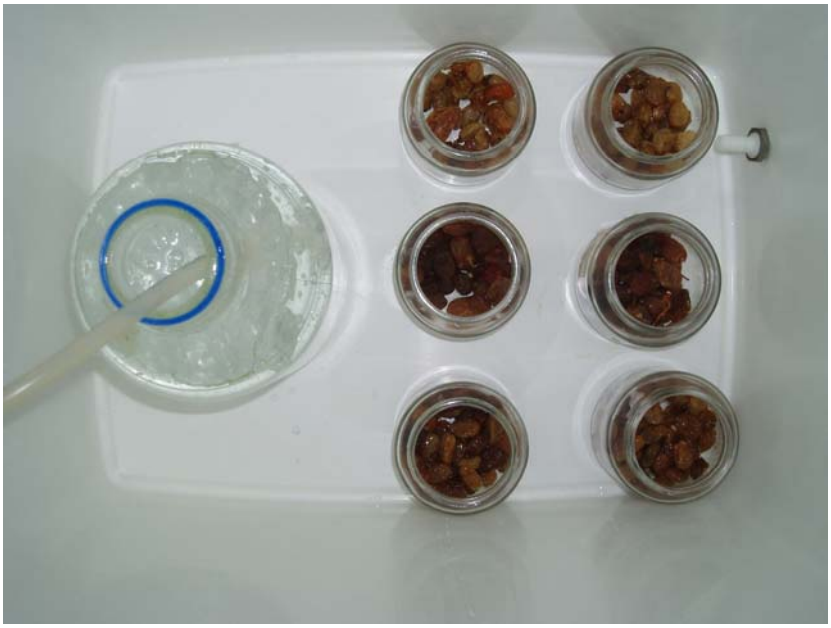


Plate 4.1. A computerized gas blender was used to provide gas treatments into a chamber containing a flask with water-glycerol solutions in order to ensure no fluctuation in a_w. Incubation time: 12 days.

4.2.3.5. Fungal assessment

Fungal identification of black aspergilli was carried out to species level only for *A. carbonarius*. The remaining isolates of black aspergilli were grouped in the *A. niger* aggregate while the other isolated fungi were identified to genera level only. To determine the mycoflora on sultanas, Malt extract agar (Oxoid Ltd), modified to 0.971 a_w (MEA97), was prepared by adding 14.72 g of glycerol (Sigma) 100 mL^{-1} distilled water, according to Table 2.1 in APPENDIX A. The a_w of this medium was determined with a Humidat Sprint IC II thermoconstanter (Novasina, Zurich, Switzerland). Two complementary isolation techniques were used: (a) serial dilution and (b) direct plating. For the first method, 10 g of each replicate (2 replicates) of each treatment were suspended in 90 mL diluent (see §2.2.2.1) prior to

homogenisation and serial dilution. The second one, direct plating method, is described in detail in section 2.2.2.1.

4.2.3.6. Sample preparation and ochratoxin A analysis

Sample preparation, immunoaffinity clean-up and HPLC analysis methods were detailed in 2.4.2 and 2.4.3 sections.

4.2.4. INTERACTIONS BETWEEN FUNGAL GENERA AND SPECIES ON GRAPE-LIKE JUICE AGAR MEDIUM

4.2.4.1. Fungal isolates

One isolate of *A. carbonarius* (strain GRE105) isolated from Cretan sultanas of the crop year 2004/05, was used in this experiment. Its ochratoxigenic ability was previously assessed using CCA (Heenan *et al.,* 1998) and by examination under UV light after exposure to ammonia (see §2.3). For better reliability, HPLC analysis was also used (see §2.4). Table 4.1 shows fungal genera, isolated from Cretan ecosystem (Sultanina and sultanas), used as competitors against to *A. carbonarius* isolate (GRE105).

4.2.4.2. Preparation of the media and modification of a_w levels

White Grape juice agar medium (WGJM) was used as the basal medium throughout all fungal interaction studies. Preparation of the medium (MEA) is described in §4.2.1.2. Two more a_w treatments were also used: 0.954 (MEA95) and 0.932 a_w (MEA93). The media were modified with glycerol to achieve the required a_w levels (Table 2.1, APPENDIX A). All a_w levels were confirmed by a Humidat Sprint IC II thermoconstanter (Novasina, Zurich, Switzerland).

Table 4.1. Competitor fungal genera/species used against to *A. carbonarius.*

Fungal genera/ species	Fungal spore concentration (spores mL^{-1})
Aspergillus carbonarius (GRE105)	2.1×10^6
Aspergillus niger aggregate (GRE0119)	2.4×10^6
Penicillium sp (GRE0657)	2.3×10^6
Penicillium sp (GRE0664)	2.3×10^6
Penicillium sp (GRE0636)	2.7×10^6
white yeast sp. (GRE0686)	2.1×10^6
red yeast sp. (GRE0689)	2.3×10^6
Botrytis cinerea (GRE0624)	1.1×10^6
Eurotium sp. (GRE0659)	2.4×10^6
Cladosporium sp. (GRE0611)	1.8×10^6
Rhizopus sp. (GRE0623)	2.3×10^6
Aspergillus flavus (GRE0680)	1.5×10^6

4.2.4.3. Inoculation and incubation

Inocula were prepared by growing the strains on Malt Extract Agar (Oxoid) at 25° C for 7 days, to obtain heavily sporulating cultures. Spore suspensions of fungal isolates were prepared as described in section 4.2.1.5. The final concentrations of spore suspensions were assessed by using a haemocytometer slide, and were adjusted to approx. 2.3×10^6 spores mL^{-1} (Table 4.1).

All Petri plates of White Grape Juice Agar Media were inoculated with a small loop (0.2 μL) of spore suspensions of *A.carbonarius.* For mycelial growth studies, a small loop (0.2 μL) of competitor fungal species was also inoculated in the same plates. The distance between the two inoculation points, in the same plate, depended on a_w level. Thus, for 0.985 a_w the distance was approximately 4cm while for 0.965 and 0.93 a_w the distance was decreased to 3cm and 2cm, respectively. Controls were inoculated centrally with the same loop (0.2 μL) of spore suspension. Plates with the

same a_w were enclosed in polyethylene bags to ensure no fluctuation in a_w. All experiments were carried out with three replicates per treatment and incubated at 25^o C for 10 days.

4.2.4.4. Measurement of fungal growth and interaction score

Fungal growth was determined by measuring colony diameter in two directions to assist in obtaining the area covered by each interacting species. This data was also used for determining relative growth rates by linear regression. The colonies were checked regularly for interactions by macroscopic analysis. The interactions were then scored using the Score Index of Dominance (I_D) indicating fungal competitiveness and overlap, as suggested by Magan and Lacey (1984) (see Table 1.9). In the case of the dominance, the higher score was always awarded to the more competitive fungus (Magan & Lacey, 1984b).

4.2.4.5. Ochratoxin A extraction and HPLC analysis

Ochratoxin A analysis was performed by cutting 8 agar plugs of each plate using a cork borer (1.5 cm) as described in Figure 4.2. Each sample was introduced into a preweighed Universal bottle. 20 mL 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) using a filter agent (celite 545, Aldrich®) and stored at 4^o C until HPLC analysis was performed, which is detailed in §2.4.3.

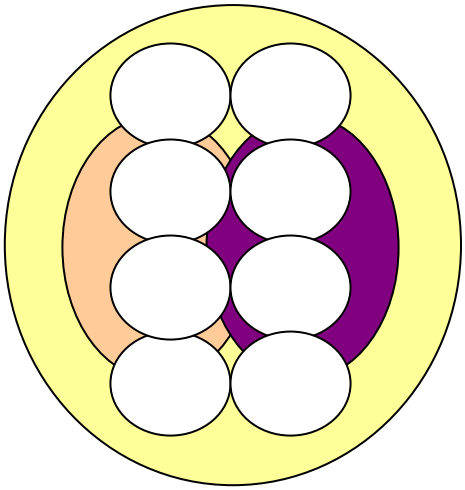


Figure 4.2. An example of agar plugs removed randomly from agar plates, under fungal antagonistic conditions.

4.2.5. INTERACTIONS BETWEEN THE MOST COMMON FUNGAL GENERA/SPECIES ON SULTANAS

4.2.5.1. Fungal isolates

Four fungal species were used in the current study. One isolate of *A. carbonarius* (GRE0105), one isolate of *A.niger* aggregate, (GRE0119 strain), one isolate of *Penicillium* sp (GRE0657) and finally one isolate of a red yeast species (GRE0689).

The same fungal species as in the previous study (see §4.2.4.1.) were used for comparison.

4.2.5.2. Substrate and modification of water activity (a_w)

The sultanas used had already undergone industrial process and originated from Cretan vineyards (2005/06 crop). Water availability was modified at 0.94 a_w level for all treatments by reference to the water adsorption curve for sultanas (see Fig. 4.2 in APPENDIX A). Sultanas were kept at 4^o C overnight to assist water equilibration. A Humidat Sprint IC II thermoconstanter (Novasina, Zurich, Switzerland) was used to determine a_w levels.

4.2.5.3. Inoculation and incubation

Inocula were prepared by growing the strains on Malt Extract Agar (Oxoid) at 25° C for 7 days, to obtain heavily sporulating cultures (GRE105, GRE0119 and GRE0657 strains). Spore suspensions of fungal isolates were prepared by harvesting spores from these cultures and suspending them in sterile distilled water containing 0.005% of a wetting agent (Tween 80) to assist dispersion of the spores.

Three different final concentrations of the spore suspensions were assessed by using a haemocytometer slide. More specific, both *A. niger* aggregate and *Penicillium* spp isolates were used in approximately of 1 x 10^2, 1 x 10^4 and 1 x 10^6 spores mL^{-1} while red yeast sp. isolate were used in spore concentrations of 1 x 10^3, 1 x 10^5 and 1 x 10^7 spores mL^{-1}. These fungal spore concentrations reflect spore populations that have been determined in the field experiments. The incubation temperature was 25° C for one week. The experiment was carried out in 3 replicates with 2 sub-samples of each.

4.2.5.4. Samples preparation, clean-up method and ochratoxin A analysis

Sultana samples were prepared and directly cleaned-up using the methodology suggested by Stefanaki *et al.* (2003) with some modifications and Rhone Diagnostics (1999), respectively. A portion of approximately 40 g of each sample was blended with 120 mL of a water solution containing 1% sodium bicarbonate ($NaHCO_3$), for 5 minutes, to give a homogeneous paste. After a further ~2 min. homogenisation 100g of this paste (which contained 25g of sultanas and 75g of water solution) were mixed with another 125 mL of the aforementioned water solution. Then the mixture, after being homogenized for a second time during 5 min in the same Stomacher, was filtered through Whatman folded filter paper. Both clean up method and HPLC analysis are detailed in §2.4.1 and §2.4.3, respectively.

4.3. RESULTS

4.3.1. THE INFLUENCE OF A_W AND SODIUM METABISULPHITE CONDITIONS ON FUNGAL SPORE GERMINATION, FUNGAL GROWTH AND OTA PRODUCTION BY STRAINS OF *A. CARBONARIUS*

4.3.1.1. Effects of sodium metabisulphite (NaMBS) x a_w on fungal spore germination

Figures 4.3 and 4.4 show two examples of the effect of NaMBS concentration (0 - 750 mg L^{-1}) at three a_w levels (0.985, 0.965 and 0.93 a_w) on spore germination after 6 or 8, 12, 24 and 36 hours of incubation at 25^o C, using White Grape Juice Medium (WGJM) as substrate. Spore germination was not significantly affected by 0 - 500 mg L^{-1} of NaMBS at 0.985 a_w, regardless of incubation time. At intermediate a_w level (0.965), a decrease in the percentage of germinated spores was observed at 8 hrs incubation. This inhibition of germination spores was in a range 30 - 55% at 250 mg L^{-1} and 500 mg L^{-1} NaMBS. After 12 hrs incubation, the pattern of spore germination was similar, but a lower percentage of non-germinated spores were observed at 250 mg L^{-1} (20%) and 500 mg L^{-1} (30%) NaMBS. At 100 mg L^{-1} NaMBS, almost all spores germinated, regardless of the incubation time. After 24 and 36 hrs, almost all spores had germinated.

In drier conditions (0.93 a_w), the percentage of spore germination was about 7%, after 8 hrs incubation, with 100-200 mg L^{-1} NaMBS. At higher concentrations of NaMBS (≥500 mg L^{-1}) no germination occurred. After 12 hours incubation, 60% of the spores had germinated at 100 - 200 mg L^{-1} of NaMBS, while at 500 mg L^{-1} this was restricted to 40%. However, these results were higher when compared to the control

treatment (10% germination). A stimulation of spore germination had occurred, at intermediate NaMBS concentrations, when combined with low a_w (0.93). In the range of 100 - 500 mg L^{-1} NaMBS, a significant (P=0.05) stimulation was observed between 100 - 250 mg L^{-1}. In contrast, after 24 and/or 36 hrs incubation the opposite phenomenon was observed at the higher NaBMS concentrations (>500 mg L^{-1}).

Figure 4.4 shows spore germination of wine-grape strain (IMI388653) of *A. carbonarius*. This clearly shows that in the first 6 hours of incubation a more intense effect of NaMBS was observed. Germination rate gradually decreased as the concentration of NaMBS increased, regardless of a_w levels. Patterns of all other treatments are similar with those previously described.

Overall, in all treatments and all examined strains, germination was inhibited at the highest examined concentrations of NaMBS (≥750 mg L^{-1}), regardless of a_w and incubation time, (even after 48 hours of incubation, data not shown).

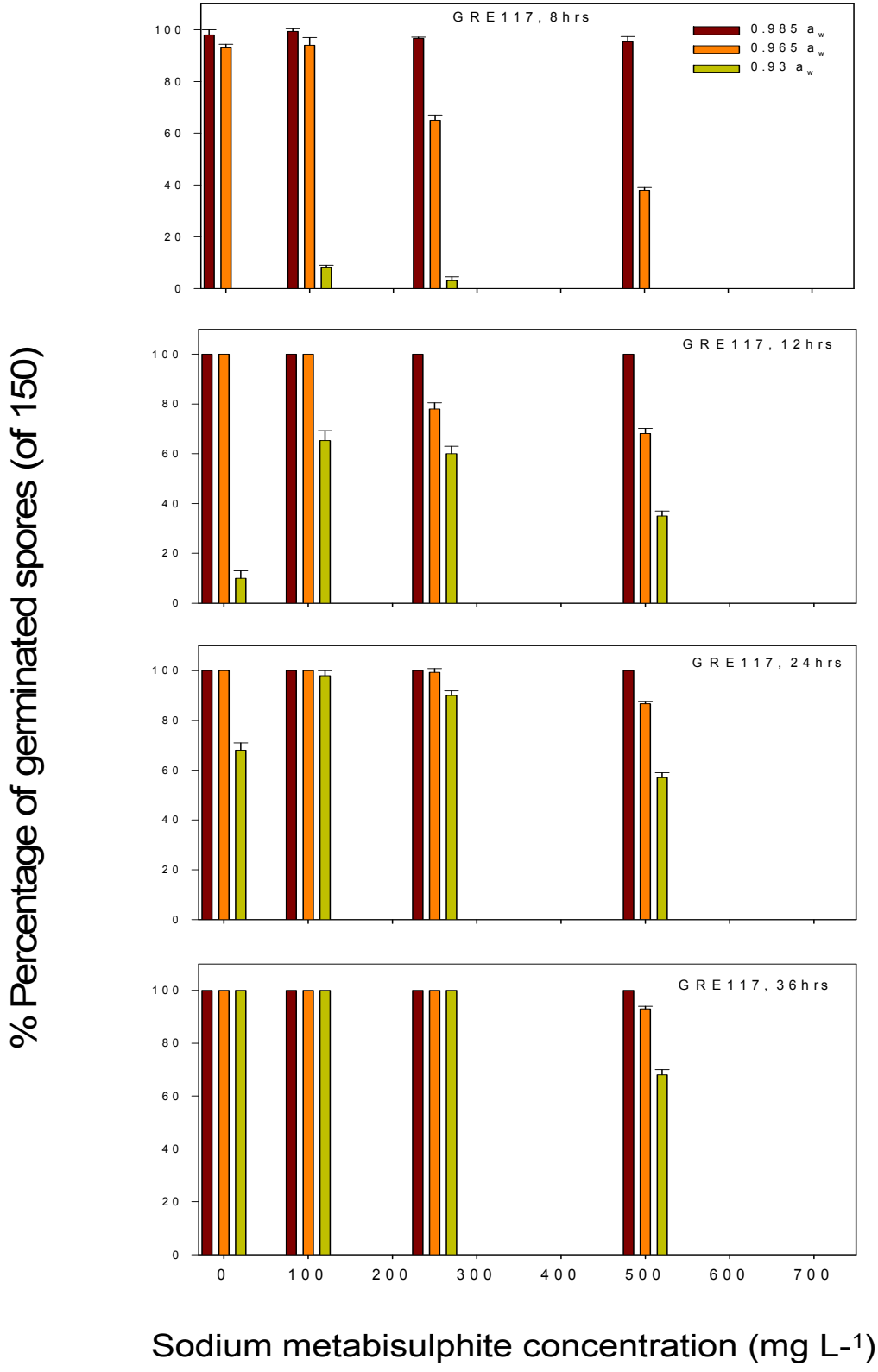


Figure 4.3. Effects of sodium metabisulphite concentration on number of germinated spores (out of 150) of an isolate (GRE117) of *A. carbonarius* on WGJM, modified to three water activity levels, at 25° C. Bars indicate standard error of the mean.

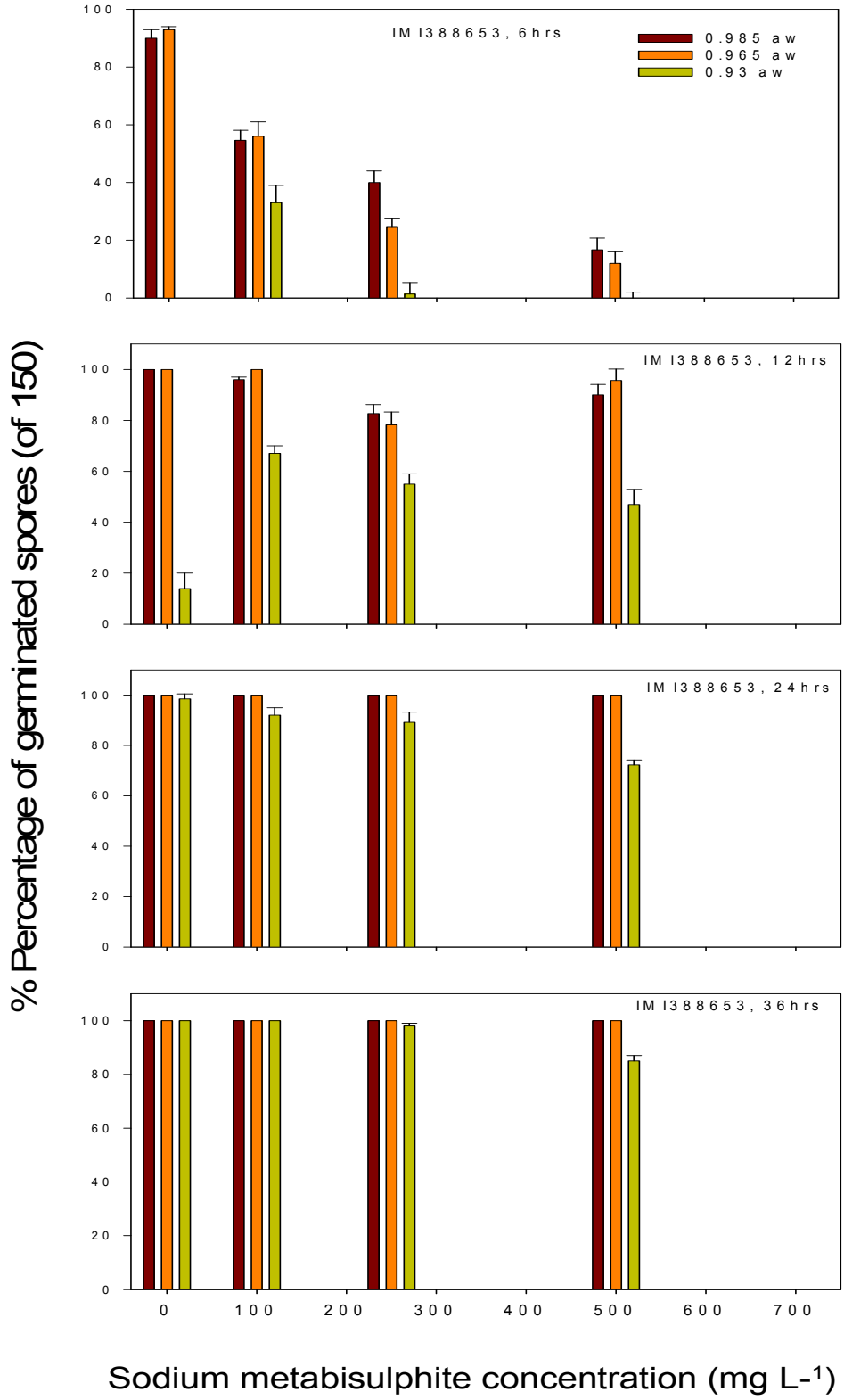


Figure 4.4. Effects of sodium metabisulphite concentration on number of germinated spores (out of 150) of an isolate (IMI388653) of *A. carbonarius* on WGJM, modified to three water activity levels, at 25° C. Bars indicate standard error of the mean.

♦ *Statistical analysis*

The concentrations of NaMBS examined, as well as a_w levels, and their interaction (a_w x NaMBS) significantly affected spore germination (see Tables 4.2(a,b) and 4.3(a,b))

Table 4.2. Homogenous subsets of the effects of a_w levels on spore germination of *A. carbonarius* strains, after 6-8 hours incubation, on WGJM at 25° C. Same patterns are observed at longer incubation time (see APPENDIX B).

	a_w	N	Subset	
			1	2
Duncan(a,b)	0.93	14	0.03238	
	0.965	14		0.46151
	0.985	14		0.54190

Table 4.3(a,b). Homogenous subsets of the effect of NaMBS concentrarions on spore germination of *A. carbonarius* strains, after (a) 6-8 hrs and (b) 12 hrs of incubation, on WGJM at 25° C. Same patterns are observed at longer incubation time (see APPENDIX B).

(a)

	NaMBS	N	Subset		
			1	2	3
Duncan(a,b)	750	6	0.00000		
	1000	6	0.00000		
	500	6		0.27000	
	250	6		0.38407	0.38407
	50	6			0.56444
	100	6			0.57500
	0	6			0.62333

(b)

	NaMBS	N	Subset		
			1	2	3
Duncan(a,b)	750	6	0.0000		
	1000	6	0.0000		
	250	6		0.6732	
	0	6		0.7117	
	500	6		0.7263	
	50	6		0.8114	0.8114
	100	6			0.8805

Significance level = 0.05

4.3.1.2. Effects of sodium metabisulphite x a_w on germ tube extension

In this experiment, the impact of NaMBS x a_w on conidial germ tube extensions (μm) of *A. carbonarius* was investigated. Several concentrations of NaMBS were used; 0, 50, 100, 250, 500, 750 and 1,000 mg L^{-1}. Measurements were taken after 4, 8, 12 and 24 hours of incubation at 25° C. Table 4.4 shows germ tube extension of an isolate of *A. carbonarius*, (GRE117). The same pattern was observed for the others isolates examined.

Results showed that after 4 hrs of incubation, only at 0.965 a_w, control conidia showed germ tube extension (20 μm). After doubling incubation time to 8 hours, germ tube extension was measurable in almost all treatments, except those at the lower level of a_w (0.93). After 12 and/or 24 hours, at higher levels of water availability (0.985 a_w), the hyphal growth phase had occurred, making measurements impossible even with 0-100 mg L^{-1} of NaBMS. At the same incubation time and at slightly drier conditions (0.965 a_w), germ tube extensions were up to *ca* 392 μm (100 mg L^{-1}) while at drier conditions (0.93 a_w), they had extended only 30 μm.

It was noticeable that when the water availability was decreased (0.965 and 0.93 a_w), an intermediate concentration of NaMBS of 100 μg L^{-1} resulted in stimulation after 8-12 hrs of incubation at 25° C. In the wettest a_w treatment (0.985), an increase in NaMBS concentrations resulted in a significant decrease in germ tube length.

Table 4.4. Effects of sodium metabisulphite (NaMBS) x water activity (a_w) on germ tube extension (µm) of *A. carbonarius* (GRE117 strain), on WGJM, after incubation for 4, 8, 12 and 24 hours, at 25° C.

		Incubation time			
Germ tube extension (µm)	NaMBS (µg L^{-1})	4 hours	8 hours	12 hours	24 hours
0.985 a_w	0	ng	71.43	na	na
	50	ng	70.71	na	na
	100	ng	47.00	na	na
	250	ng	65.71	443.33	na
	500	ng	55.75	270.48	na
	750	ng	ng	ng	ng
	1000	ng	ng	ng	ng
0.965 a_w	0	20.00	28.75	220.95	na
	50	ng	26.90	106.88	na
	100	ng	35.71	392.50	na
	250	ng	22.98	156.7	254.29
	500	ng	11.81	104.05	762.86
	750	ng	ng	ng	ng
	1,000	ng	ng	ng	ng
0.93 a_w	0	ng	ng	30.76	490.00
	50	ng	ng	26.07	354.50
	100	ng	ng	32.60	352.22
	250	ng	ng	ng	ng
	500	ng	ng	ng	ng
	750	ng	ng	ng	ng
	1,000	ng	ng	ng	ng

(ng): no growth, (na): germ tube extension > 1,000 µm, non accountable

4.3.1.3. Effects of sodium metabisulphite x a_w on mycelial growth

Mycelial growth rates were determined by daily measurement of the colony diameter in two directions at right angles to each other. Measurements were carried out for 2-10 days. Linear regression of the colony radius against time (days) was used to obtain the growth rates (cm day^{-1}) under each set of growth conditions.

The use of sodium metabisulphite significantly affected the growth rate of all isolates, although high concentrations of NaMBS were required for an effective control (Fig. 4.5). For >50% inhibition, concentrations of at least 500 mg L^{-1} NaMBS are required, depending on the water availability (see Table 4.5). For complete inhibition, >700 up to 1,000 mg L^{-1} were required for all examined strains, over the experimental period. For all *A. carbonarius* strains, growth was stimulated by medium concentrations of NaMBS (100 - 500 mg L^{-1}). The optimal a_w for growth was 0.985 in the presence of 100 mg L^{-1} 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 conditions tested. Only isolate GRE117 was completely inhibited by 750 mg L^{-1}, at both 0.985 and 0.965 a_w levels.

Table 4.5. The range of effective doses (ED_{50} and ED_{90}) concentrations of NaMBS required for inhibiting radial growth of the isolates of *A. carbonarius* examined, grown on WGJM, at 25° C.

	NaMBS (mgL^{-1})	
Water activity	ED_{50}	ED_{90}
0.985	750-880	950-980
0.965	600-700	800-950
0.93	430-580	700-730

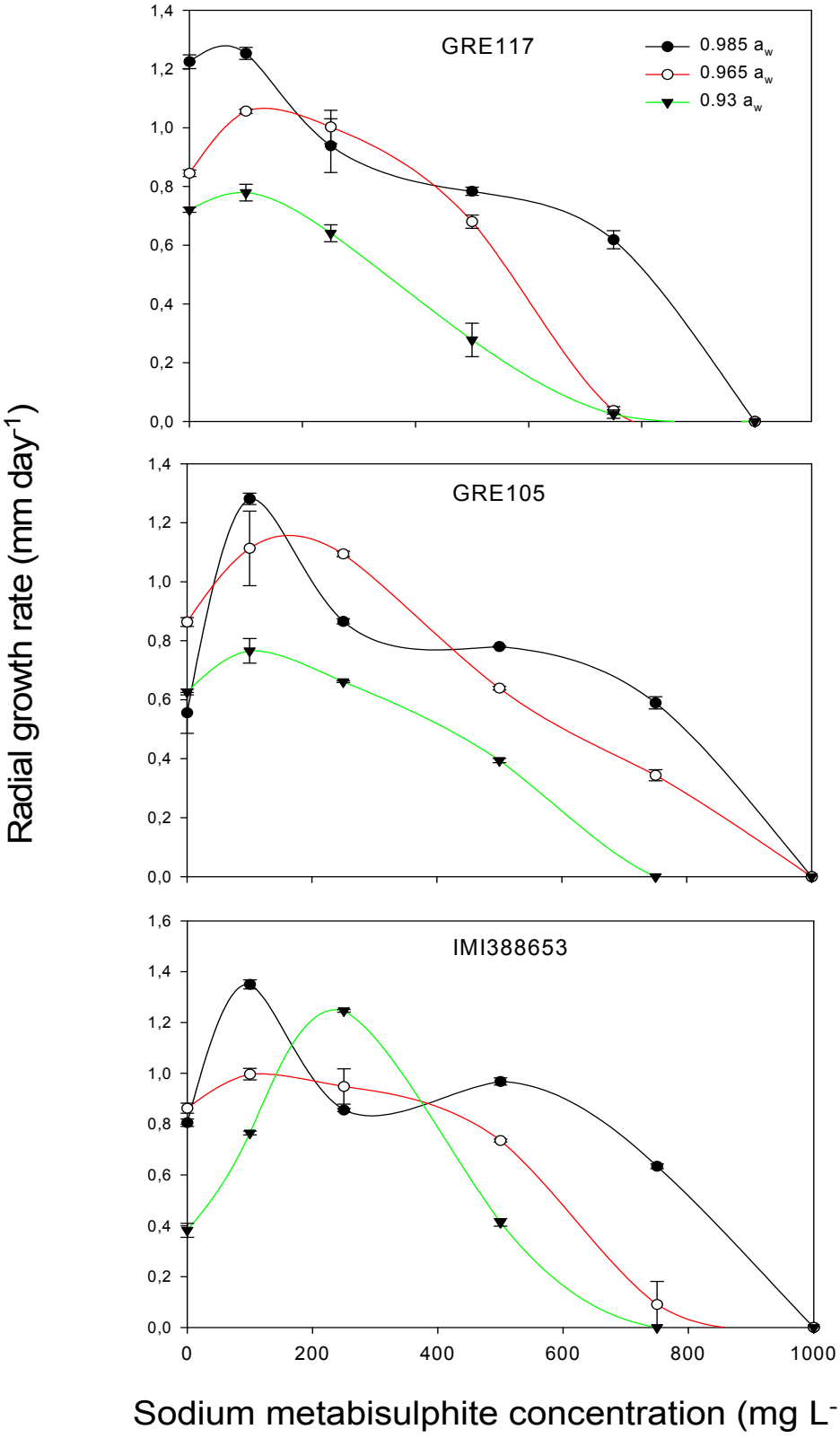


Figure 4.5. Effects of sodium metabisulphite concentration on radial growth rate (mm day^{-1}) by three isolates of *A. carbonarius* on WGJM, modified to three water activity levels, at 25^{o} C. Bars indicate standard error of the means.

♦ *Statistical analysis*

The examined strains of *A. carbonarius* favoured higher levels of a_w (0.985 a_w), under the presence of sodium metabisulphite (see Table 4.6). Moreover, 100 mg L^{-1} of NaBMS potentially stimulated growth rate (see Table 4.7)

Table 4.6. Homogenous subsets of the impact of a_w (x NaMBS) on radial growth (cm).

	a_w	N	Subset		
			1	2	3
Duncan(a,b,c)	0.93	84	0.4667		
	0.965	83		1.6998	
	0.985	84			2.5373

Table 4.7. Homogenous subsets of the impact of NaMBS (x a_w) on radial growth (cm).

	NaMBS	N	Subset					
	(mg L^{-1})		1	2	3	4	5	6
Duncan(a,b,c)	1,000	36	0.0000					
	750	36		0.2586				
	500	36			0.6336			
	250	36				0.8479		
	0	36				0.8612		
	50	35					0.9424	
	100	36						1.0402

Significance level = 0.05

4.3.1.4. Control of OTA production using sodium metabisulphite

Figure 4.6 shows the interaction between a_w x NaBMS on OTA accumulation after 10 days growth, on WGJM, at 25° C. According to this, the best treatment for OTA inhibition was at lower a_w (0.93), regardless of the NaMBS concentration. Maximum OTA (1.5 µg g^{-1}) content was obtained in the 0.985 a_w/0 mg L^{-1} NaBMS treatment by GRE105 strain.

Overall, at marginal water activity (0.985 and 0.965 a_w) levels there was a fluctuation in the amount of OTA production under different NaMBS concentrations. In general, medium levels of sodium metabisulphite (100-500 mg L^{-1}), in combination with higher levels of a_w (0.985), can potentially faciliate ochratoxin A production by *A. carbonarius*. However, the concentration of NaMBS when stimulation of OTA occurs differs, depending on the fungal strain. Strain GRE117 had a high capacity for OTA production at 250 m gL^{-1}, at the highest a_w tested, while strains GRE105 and IMI358853 produced higher OTA concentrations at 500 m gL^{-1} x 0.985 a_w. Table 4.8 shows the ED_{50} and ED_{90} values for inhibition of OTA production.

Table 4.8. The range of effective doses (ED_{50} and ED_{90}) concentrations of NaMBS required for inhibiting OTA production by the isolates of *A. carbonarius* examined, grown on WGJM at 25° C.

	NaMBS (m gL^{-1})	
Water activity	ED_{50}	ED_{90}
0.985	50-100 and 600-680	900-990
0.965	200-550	850-950
0.93	400-450	750-880

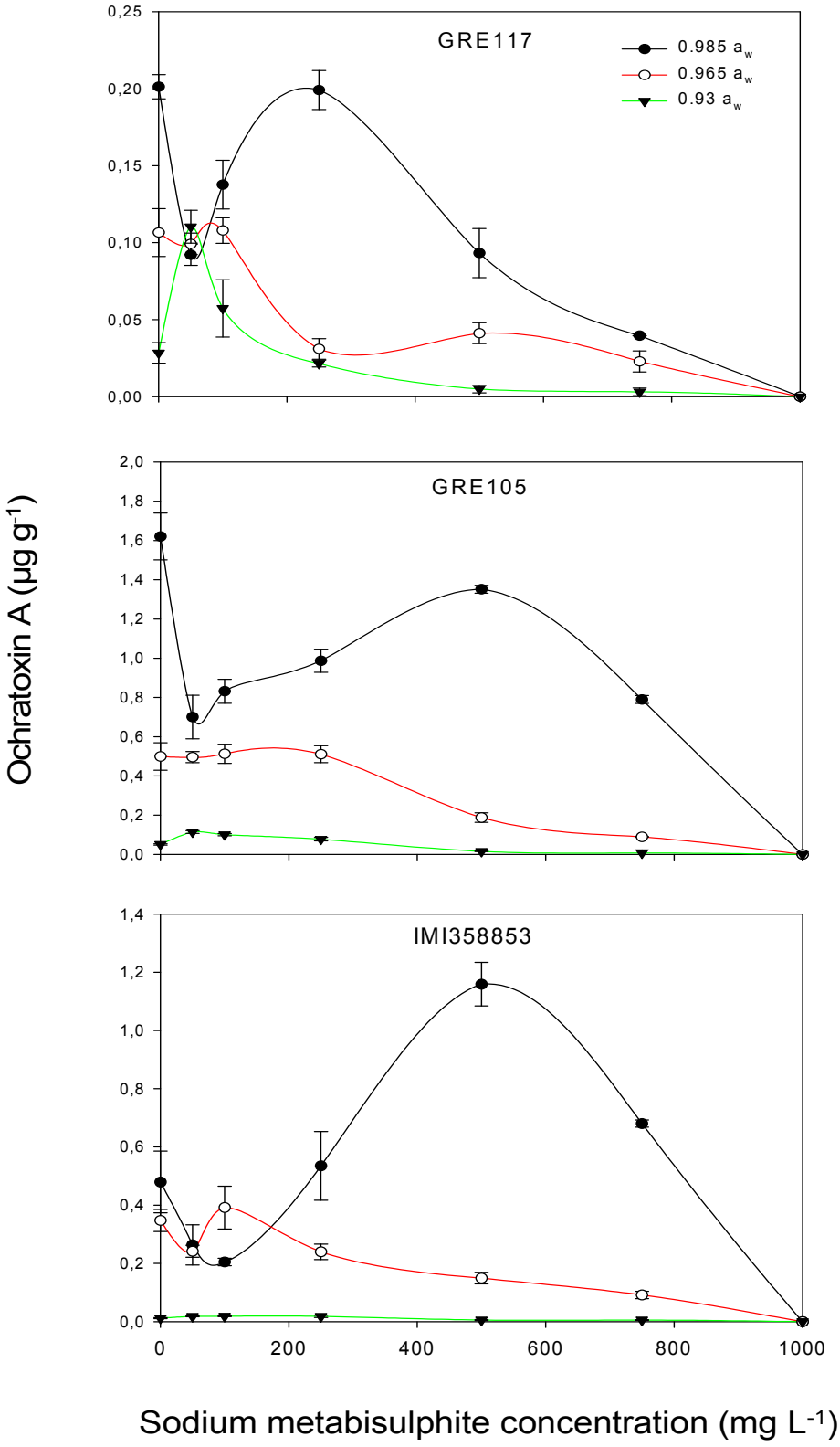


Figure 4.6. Effects of sodium metabisulphite concentration on OTA production by three isolates of *A. carbonarius*, on WGJM modified to three water activity levels, at 25° C. Bars indicate standard error of the means.

♦ *Statistical analysis*

The three levels of water activity studied significantly affected OTA biosynthesis, under the specific experimental conditions (see Table 4.9). Moreover, under drier conditions, intermediate concentrations of NaMBS significantly stimulated toxin production (see Table 4.10(a,b)).

Table 4.9. Homogenous subsets of the impact of a_w (x NaMBS) levels on OTA production.

	aw	N	Subset		
			1	2	3
Tukey HSD(a,b,c)	0.93	60	0.05032108		
	0.965	59		0.26157954	
	0.985	57			0.57543718

Significance level = 0.05

Table 4.10 (a,b). Homogenous subsets of the impact of NaMBS (x a_w) concentration on OTA production, according to drier (≤0.965) a_w levels examined.

(a)

0.965 a_w	NaMBS (mg L^{-1})	N	Subset	
			1	2
Tukey HSD(a,b,c)	500	12	0.12617312	
	50	11	0.26126542	0.26126542
	250	12	0.26519247	0.26519247
	0	12	0.31776443	0.31776443
	100	12		0.33747609

(b

0.93a_w	NaMBS (mg L^{-1})	N	Subset	
			1	2
Tukey HSD(a,b)	500	12	0.00849275	
	0	12	0.02898279	0.02898279
	250	12	0.03919743	0.03919743
	50	12	0.07970763	0.07970763
	100	12		0.09522482

Significance level = 0.05

4.3.2. THE EFFECT OF CONTROLLED ATMOSPHERES x A_W TO CONTROL SPORE GERMINATION, FUNGAL GROWTH OF *A. CARBONAIRUS* AND OTA ACCUMULATION IN GRAPE-LIKE MEDIUM

4.3.2.1. Inhibition of spore germination by modified atmospheres

Figure 4.7 details the effect of a_w and controlled atmospheres on spore germination of two strains of *A. carbonarius* originated from the Cretan ecosystem (GRE117 and GRE105) and another isolated from Italian wine grapes (IMI388653). The number of germinated spores (average of 150 spores) after 24/48 hrs was compared.

In the control treatment (air: 0.03% CO_2), a high percentage of germinated conidia was achieved within 24 hours; 60-100% of spores of GRE105 and IMI388653 germinated, depending on a_w level. Isolate GRE117 was more sensitive to CO_2 and it was completely inhibited at high CO_2 (50%) concentrations, at 24 hours.

Under conditions of 25% CO_2 x a_w, conidia germinated in a range of 60 - 100%, after 24 hours, depending also on a_w level. In general, as the level of a_w decreased, a stronger inhibition was observed. However, this specific pattern was not so representative for all strains. Thus, under 25% x 0.965 a_w conditions, a stimulation of spore germination of strain GRE105 was observed, in comparison to higher level of a_w (0.985 a_w).

At 50% CO_2 exposure, similar patterns were observed. However, the percentage of germinated spores, after 24 hrs incubation, decreased considerably (0-60%),

depending on a_w. More specifically, spores originating from the wine fungal isolate (IMI388653) had higher percentage of germination (60%), at intermediate a_w (0.965), indicating that CO_2 may act as stimulation factor.

Doubling the incubation time (48 hours), at combinations of high levels of a_w (≥0.965 a_w) with low concentrations of CO_2 (≤25%), all spores germinated regardless of strains. At 0.985a_w x 50% CO_2 conditions, spores germinated in a range of 90-100%, regardless of strains. Similar results obtained for 0.965a_w x 50% CO_2 conditions. At the driest water availability (0.928 a_w) combined with the higher concentration of CO_2 (50%), a total inhibition of spore germination occurred, regardless of strains. No spore germination occurred after 24 or 48 hours of incubation time.

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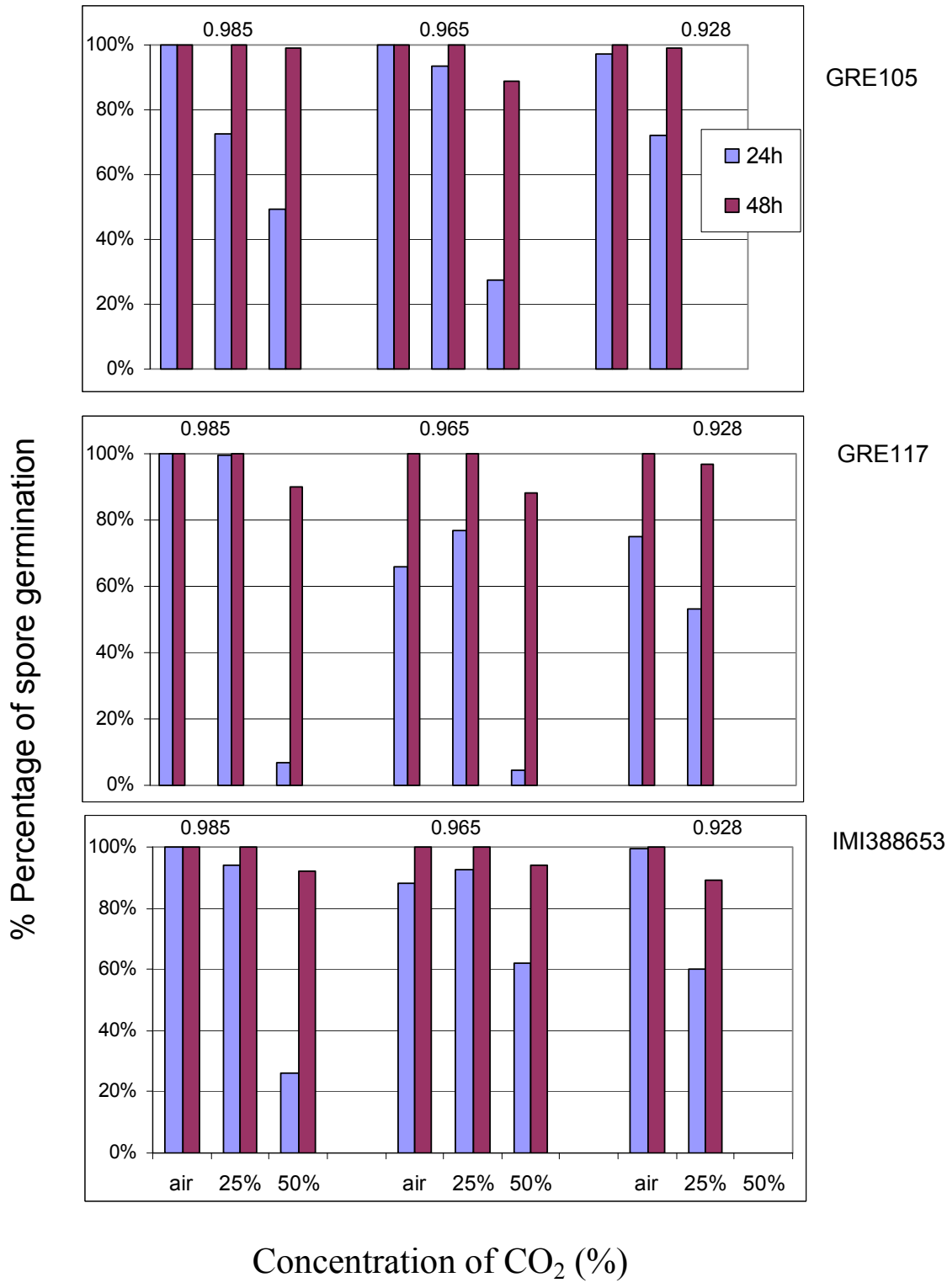


Figure 4.7. Effect of CO_2 concentration on spore germination. The experiment used three strains of *A. carbonarius* on (GRE105, GRE117 and IMI388653), grown on a White Grape Juice Medium (WGJM), modified at three a_w levels (0.985, 0.965 and 0.928 a_w), at 25° C.

Statistical analysis

Overall, both two abiotic factors studied (a_w, CO_2), as well as their interaction (a_w x CO_2), significantly affected spore germination, after 48 hours of incubation, at 25° C (see Table 4.11(a,b)). Table 4.12 shows the homogenous subsets of the impact of the two abiotic parameters (a_w and CO_2 concentration), after 48 hours incubation.

Table 4.11(a,b). Summary statistical tables of significance (Univariate Analysis of Variance) of the impact of a_w, controlled conditions (CO_2) and their interaction on spore germination of *A. carbonarius*, after (a) 24 hours and (b) 48 hours of incubation time.

(a)				(b)			
24 hours incubation	df	F	Sig.	48 hours icubation	df	F	Sig.
CO_2	2	56.244	0.000**	CO2	2	644.064	0.000**
a_w	2	4.793	0.051	a_w	2	471.654	0.000**
CO_2 * a_w	4	1.278	0.315	CO2 * a_w	4	401.164	0.000**

(**) significant at P<0.01

Table 4.12(a,b). Homogenous subsets of the impact of (a) the a_w (b) CO_2 on spore germination of *A. carbonarius*, after 48 hours at 25° C. Same regime for CO_2 was shown after 24 hours (see APPENDIX B).

(a)

	a_w	N	Subset	
			1	2
Duncan(a,b)	0.928	9	0.649630	
	0.965	9		0.967778
	0.985	9		0.978889

(b)

	CO_2	N	Subset	
			1	2
Duncan(a,b)	50%	9	0.613333	
	25%	9		0.982963
	air	9		1.000000

(**) significant at P<0.01

4.3.2.2. Effects of modified atmospheres on germ tube extension

Figure 4.8 shows the impact of elevated CO_2 at three a_w levels (0.985, 0.965 and 0.928a_w) on germ tube extension of three strains (GRE105, GRE117 and IMI388653) of *A. carbonarius* examined after 24 and 48 hours of incubation at 25^oC.

Control treatment (0.03% CO_2), combined with higher a_w (0.985 a_w), resulted in conidial germ tubes extensions of about 1,000 - 1,200 μm., within 24 hrs of incubation, in all strains studied. As the level of a_w was decreased, germ tube extensions also decreased. Thus, at 0.965 a_w, germ tubes extended from 400 - 740 μm, depending on the strain, while at 0.928 a_w, a significant reduction (100 μm) of germ tube extension was observed in all strains. Overall, the same pattern occurred after 48 hrs incubation. At higher a_w (0.985), germ tubes lenght did not permit any measuring (they were not accountable). At intermediate a_w levels, germ tubes extended in a range of 800 - 1,200 μm, depending on the strain. At the lowest a_w (0.928), significant inhibition of germ tube extension occurred.

At 25% CO_2, significant inhibition (30 - 210 μm) of germ tubes extension was observed, after 24 hrs, regardless of strain or level of a_w. After 48 hours, the range of tube extension slightly increased (300-800 μm), depending on the strain. No stimulation was observed.

To sum up, at 50% CO_2 conditions, germ tube extension was almost eliminated. A very strong inhibition was observed, regardless of a_w levels, incubation time or strain of *A. carbonarius*.

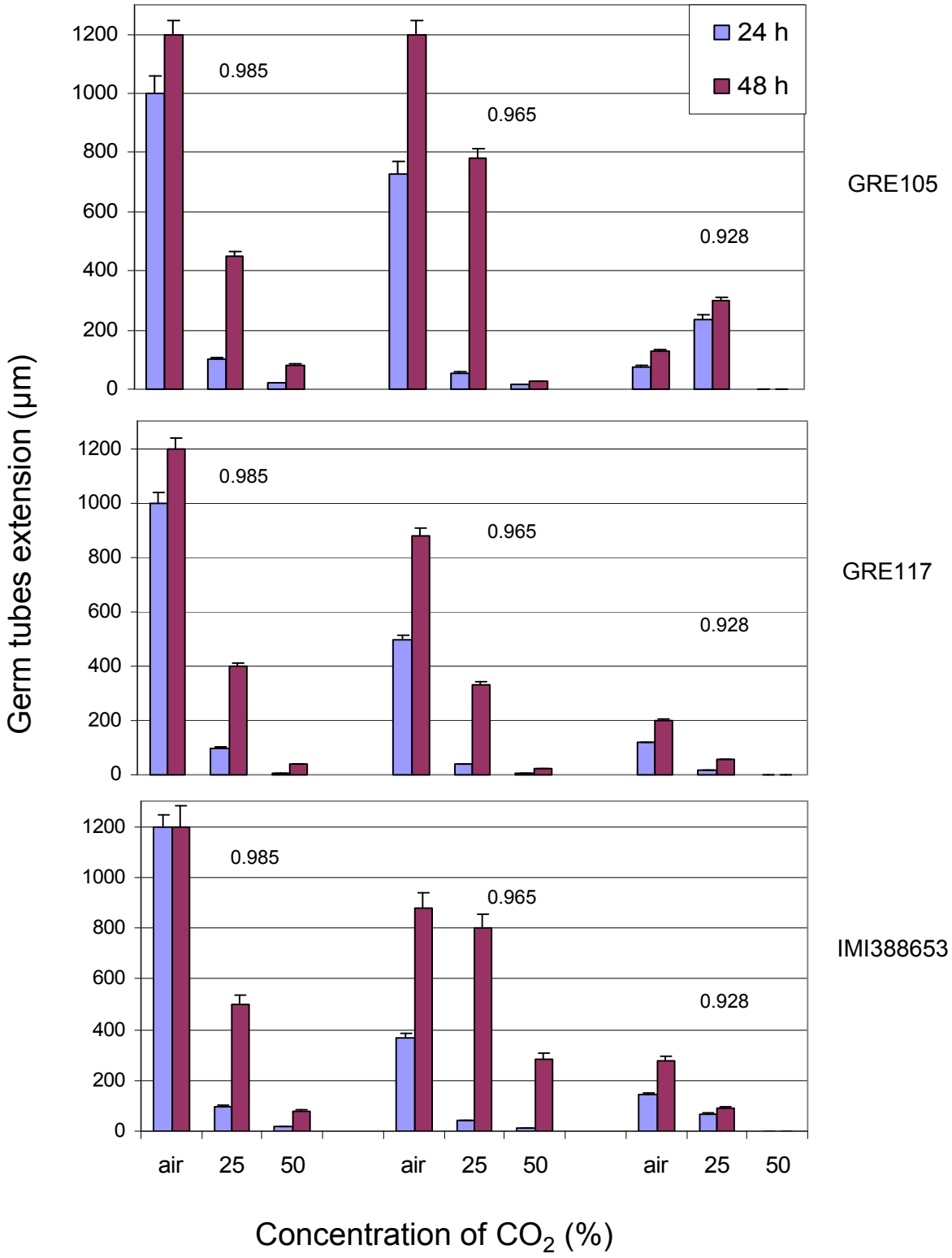


Figure 4.8. Effect of CO_2 concentration on germ tubes extension. The experiment used three strains of *A. carbonarius* (GRE105, GRE117 and IMI388653), grown on a White Grape Juice Medium (WGJM), modified at three a_w levels (0.985 a_w, 0.965 a_w and 0.928 a_w), at 25° C. Bars indicate the standard error of the mean.

♦ *Statistical analysis*

Table 4.13 shows the effect of elevated CO_2 at three a_w levels (0.985, 0.965 and 0.928 a_w) on germ tube extension of three strains (GRE105, GRE117 and IMI388653) of *A. carbonarius* examined, after 24 hours of incubation, at 25° C. The levels of a_w, CO_2 concentration and their interaction had a significant effect on germ tube extension. Table 4.14 shows the homogenous subsets of the impact of the two abiotic parameters (a_w and CO_2 concentration) examined, after 24 hours incubation.

Table 4.13. Summary statistical tables of significance (Anova) of the impact of a_w, CO_2 and their interaction on germ tube extension of *A. carbonarius,* after 24 hours. The same pattern is observed after 48 hours of incubation (see APPENDIX B)**.**

Source	df	F	Sig.
a_w	2	35.022	0.000*
CO_2	2	124.152	0.000*
a_w * CO_2	4	33.950	0.000*

(*) significant at P<0.05

Table 4.14(a,b). Homogenous subsets of the effect of (a) the a_w (b) CO_2 on germ tube extension of *A. carbonarius,* after 24 hours incubation at 25° C.

(a)

	a_w	N	Subset		
			1	2	3
Duncan(a,b)	0.928	9	0.073381		
	0.965	9		0.195240	
	0.985	9			0.393565

(b)

	a_w	N	Subset	
			1	2
Duncan(a,b)	50%	9	0.008758	
	25%	9	0.085056	
	air	9		0.569373

Significance level = 0.05

4.3.2.3. Effects of modified atmospheres on mycelial extension

Figure 4.9 shows mycelial colony diameter for *A. carbonarius* (GRE117), under conditions of different concentrations of CO_2 (0.03%, 25% and 50%). Measurements were taken after 5 and 10 days of incubation on WGJM, at 25° C.

Exposure to 25% CO_2 did not favour mycelial extension. A 60-70% inhibition in colony diameter took place, after five days of incubation, at 25° C. Colony diameter was slightly extended (1.9 cm) at 0.965 a_w, when compared with 0.985 a_w (1.1 cm.) and 0.928 a_w (0.8cm) levels. A non statistically significant inhibition was observed at 0.965 a_w x 25% CO_2. At 50% CO_2 conditions, fungal growth was significantly delayed, at all water availability levels examined.

After 10 days of incubation, mycelial growth was slightly restricted (10%-20%), in comparison to the control treatments, at 0.985 a_w level. At the driest conditions (0.928 a_w), mycelial growth was inhibited by 50% and 70%, when exposed continuously to 25% and 50% CO_2, respectively. This also shows that 0.965 a_w is a more favourable environment for mycelial growth, when compared to 0.985 a_w and 0.928 a_w. It is worth noting that at 0.965 x 25% CO_2, mycelial growth was similar to the control treatment. For all strains studied, effects of CO_2 x a_w on mycelial extension were similar (see Fig. 4.3, 4.4. in APPENDIX A).

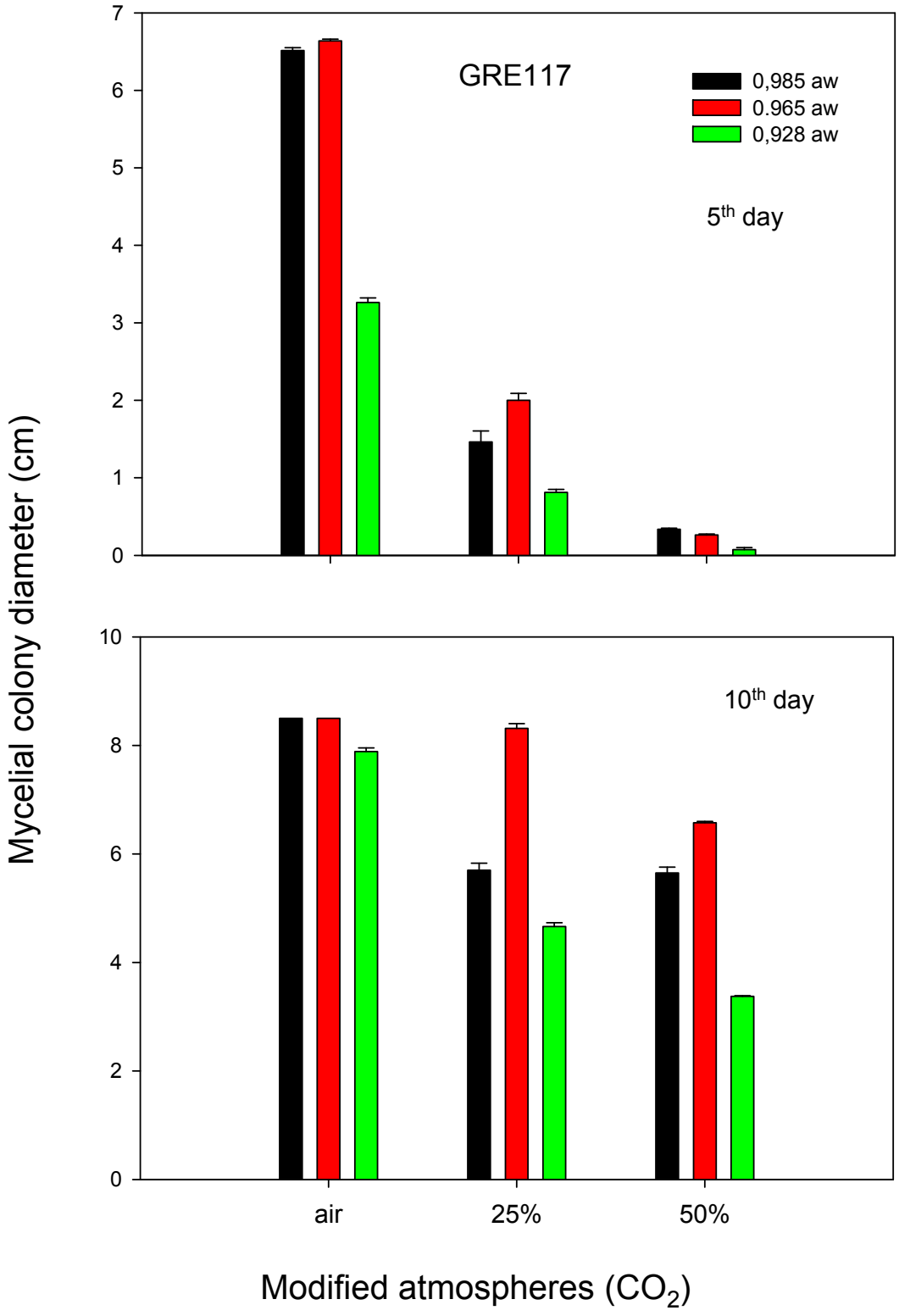


Figure 4.9. Effect of controlled atmospheres on mycelial colony diameter (cms) of *A. carbonarius* (GRE117), at three different levels of a_w. Measurements were taken on the 5th and 10th day of incubation, at 25° C. Bars indicate standard error of the mean.

♦ *Statistical analysis*

Levels of water availability (a_w), modified conditions (different concentrations of CO_2) and their interactions significantly affected mycelial colony diameter of *A. carbonarius* (Tables 4.15-4.17). Radial growth was similar at 0.985 a_w and 0.965 a_w, at the 5th day of incubation. At 10th day of incubation, growth of *A. carbonarius* was better at 0.965 a_w (Table 4.16a,b).

Table 4.15. Summary statistical tables of significance (Anova) of the impact of a_w, x CO_2 and their interaction on mycelial colony diameter of *A. carbonarius,* after 5 days at 25^o C. The same pattern is observed after 10 days incubation (see APPENDIX B).

Source	df	F	Sig.
a_w	2	300.,276	0.000**
CO_2	2	2872.241	0.000**
a_w * CO_2	4	119.466	0.000**

(**) significant at P<0.01

Table 4.16 (a,b). Homogenous subsets of the impact of a_w after (a) 5 days and (b) 10 days of incubation, at 25^o C.

			(a)		(b)		
	a_w	N	Subset (5th day)		Subset (10th day)		
			1	2	1	2	3
Duncan(a,b)	0.928	9	1.3135		5.1181		
	0.985	9		2.7016		6.8139	
	0.965	9		2.8619			7.7597

Significance level = 0.01

Table 4.17. Homogenous subsets of the impact of CO_2 after 5 days of incubation, at 25^o C. The same regime is observed after 10 days incubation (see APPENDIX B).

	CO_2	N	Subset		
			1	2	3
Duncan(a,b)	50%	9	0.2125		
	25%	9		1.4111	
	air	9			5.2534

Significance level = 0.01

4.3.2.4. Effect of controlled atmospheres on ochratoxin A production

Figure 4.10. shows the impact of elevated CO_2 concentrations on OTA production. In the control treatment, OTA production decreased as water activity levels also decreased. However, higher OTA concentrations (1.6 µg g^{-1}) were observed at higher a_w when 25% CO_2 was present, in comparison to the control treatment (1.4 µg g^{-1}). In this case, CO_2 seemed to act more as a stimulating factor. In general, the presence of CO_2 did not significantly influence OTA production. It was clear that the effect of a_w on OTA production was stronger than the impact of controlled atmospheres.

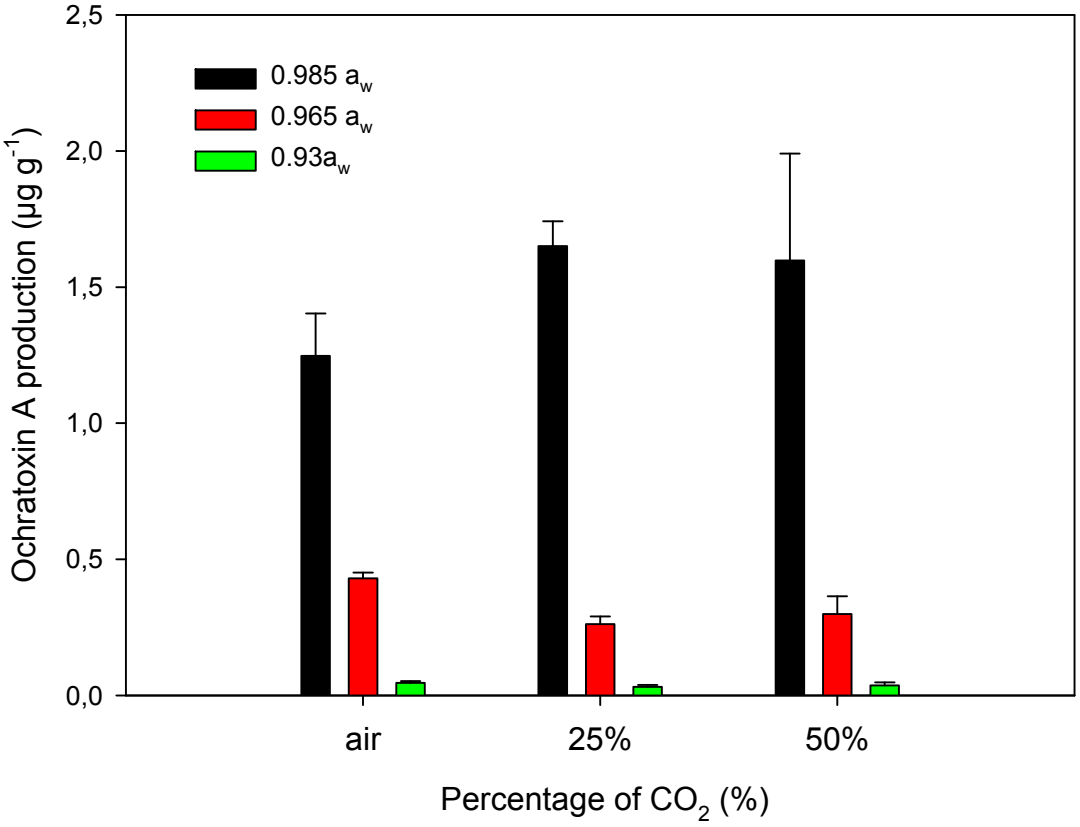


Figure 4.10. Effect of modified atmospheres x a_w on OTA production by three strains of *A. carbonarius* (GRE105, GRE117, IMI388653) examined, on WGJM, modified to three a_w levels. Bars indicate standard error of the mean.

♦ *Statistical analysis*

The level of water availability (a_w) statistically influenced OTA production by *A. carbonarius* isolates, under the specific experimental conditions (see Tables 4.18 and 4.19).

Table 4.18. Summary statistical tables of significance (Univariate Analysis of Variance) of the impact of a_w, controlled conditions (CO_2) and their interaction on OTA production by *A. carbonarius*, after 10 days of its exposure to the controlled conditions, at 25° C.

Source	df	F	Sig.
a_w	2	537.491	0.000***
CO_2	2	1.141	0.324
a_w * CO_2	4	2.413	0.054

(***) significant at P<0.001

Table 4.19. Homogenous subsets of the effects of a_w (x CO_2) on OTA biosynthesis, after 10 days of incubation, on White Grape Juice Medium, at 25° C.

	a_w	N	Subset (OTA Log_{10})		
			1	2	3
Duncan(a,b)	0.928	36	-1.5239		
	0.965	36		-0.3875	
	0.985	36			0.3266

(***) significant at P <0.001

4.3.3. EFFECT OF CONTROLLED ATMOSPHERES X A_W ON FUNGAL POPULATION OF BLACK ASPERGILLI AND OTA PRODUCTION BY *A. CARBONARIUS* IN SULTANAS

4.3.3.1. Effects on fungal growth

In this experiment, the effects of controlled atmospheres x a_w on fungal populations, and more specifically on black aspergilli (*A. carbonarius* and *A. niger* aggregate), grown on three different kinds of sultanas (A type, B type and C type), were studied. Two isolation methods were used for estimating the effects on fungal population loads: (i) serial dilution and (ii) direct plating. Simultaneously, the impact of controlled atmospheres on OTA accumulation was assayed.

Figure 4.11 shows the influence of a_w x CO_2 on black aspergilli populations, on A type of sultanas (sultanas before undertaking industrial process), modified to 0.928 a_w. It shows the temporal progress of fungal population dynamics under modified atmospheres (at 7^{th} and 14^{th} day of incubation, at 25^{o} C). A decrease of 10% and 50% was observed in *A. niger* populations, under conditions of 25% and 50%, respectively, after 7 days of incubation. Same pattern was observed for *A. carbonarius*. At 50% CO_2, *A. carbonarius* growth was totally inhibited.

After 14 days of incubation, the population loads of *A. carbonarius* were increased (3 times) at control treatment, while the loads of *A. niger* aggregate slightly decreased (from 6.1 to 5.1 Log_{10} CFUs g^{-1}). This may be due to the antagonism between these fungal species. Under 25% CO_2 conditions, a constant decrease of *A. niger* populations was observed, while *A. carbonariu*s populations were significantly influenced (from 5 to 1 Log_{10} CFUs g^{-1}). A total inhibition of *A. carbonarius* at 50% CO_2 treatment was seen (see Plate 4.2).

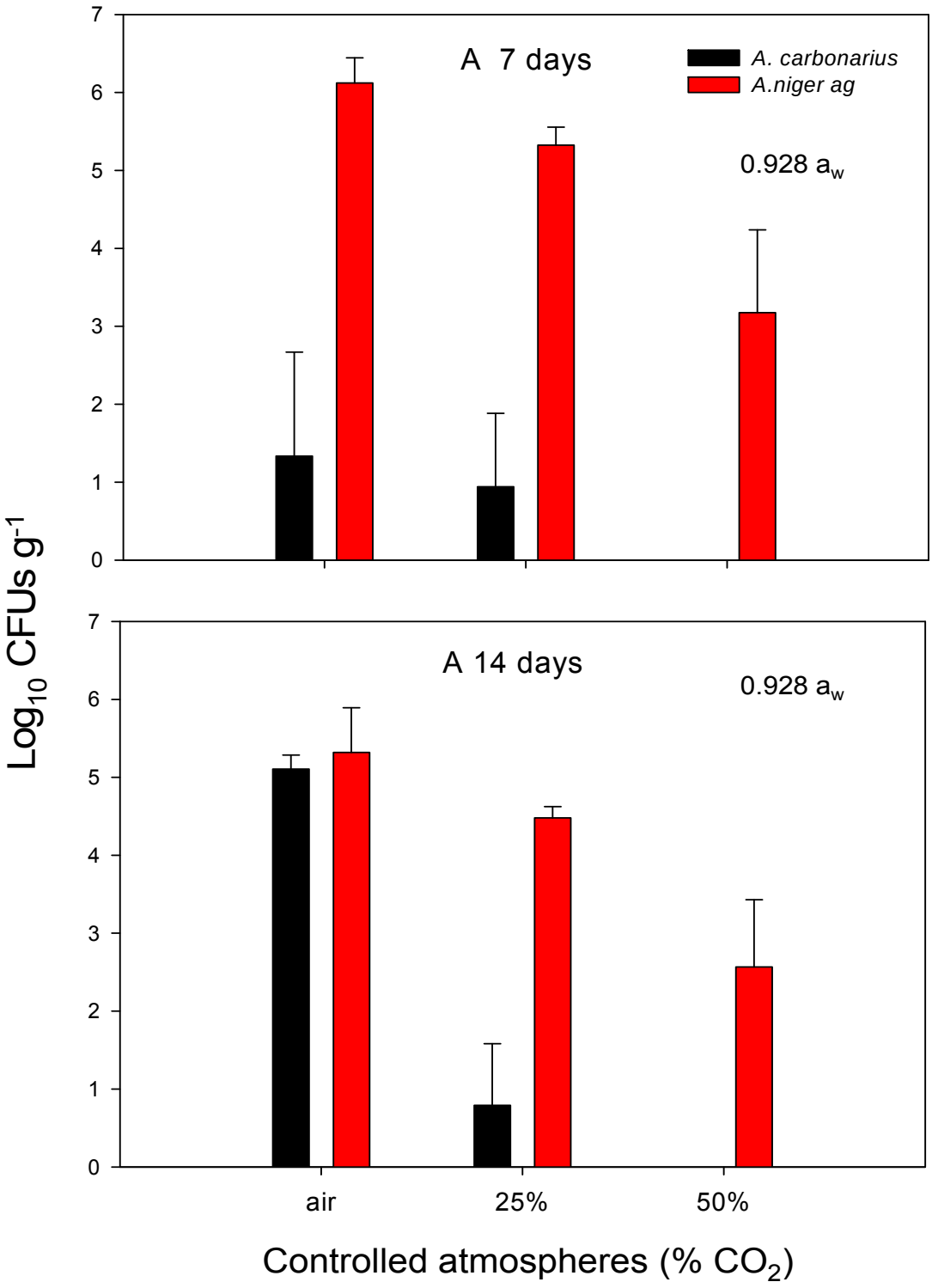


Figure 4.11. Population dynamics of black aspergilli on A type (pre-industrial) sultanas, modified to 0.928 a_w, under controlled atmospheres (air, 25% CO_2 and 50% CO_2). Incubation time: 7 and 14 days. Isolation medium: MEA97. Bars indicate standard error of mean.

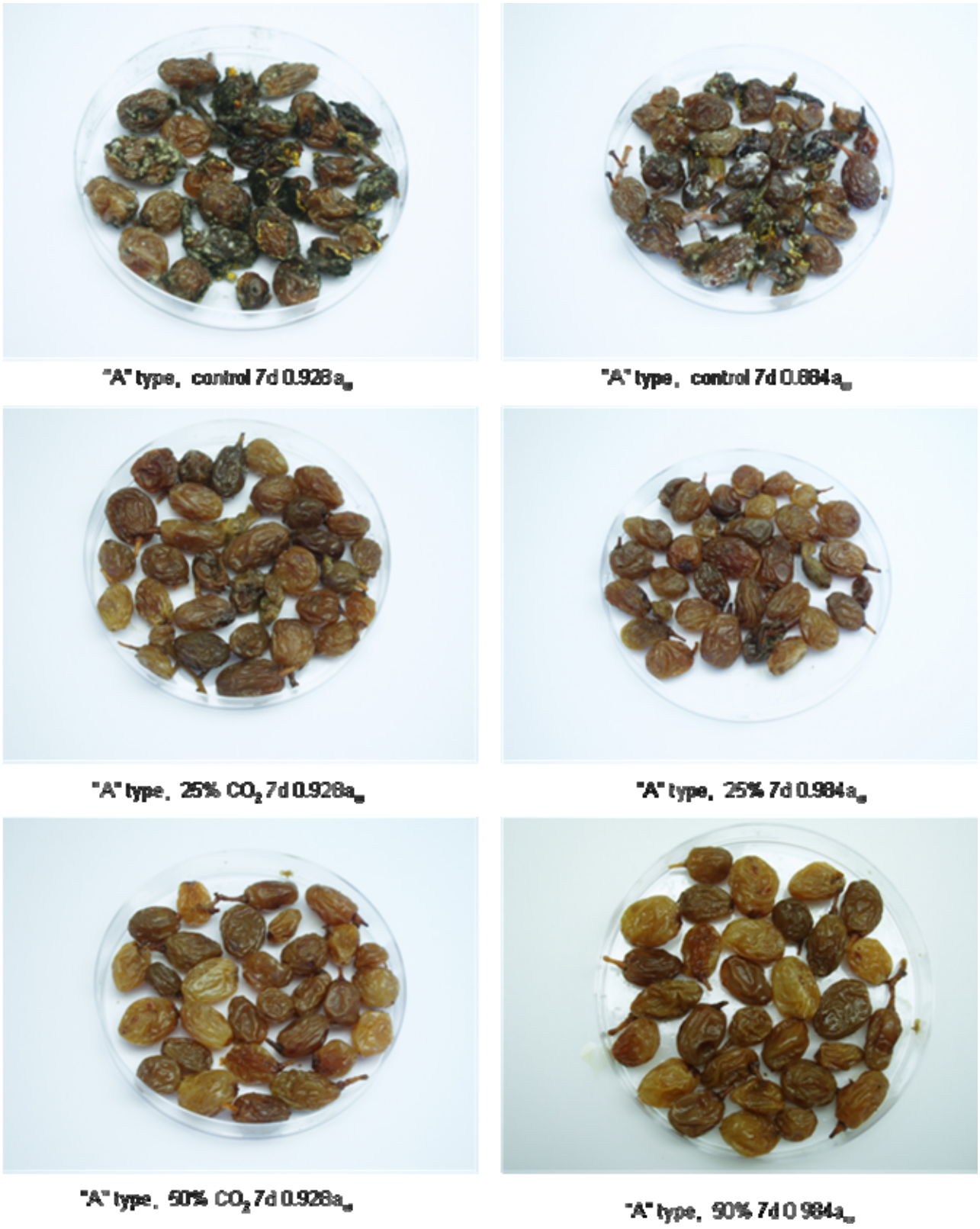


Plate 4.2. "A" type of sultanas, exposed to various concentrations of CO_2, after 7 days of incubation, at 25° C.

Figure 4.12. shows the influence of a_w x CO_2 on populations of black aspergilli. Ssultanas "Type B" (sultanas after SO_2 treatment, during industrial process) were used as a substrate, modified to 0.982 a_w. After 7 days of incubation there was no evidence of *A. carbonarius* growth. In contrast, growth of *A. niger* aggregate species were stimulated by the presence of 25% CO_2 (*ca* 3 Log_{10} CFUs g^{-1}), in comparison to control treatment (*ca* 1 Log_{10} CFUs g^{-1}). Under conditions of 50% CO_2, no populations of black aspergilli were isolated.

After 14 days of incubation the populations of *A. niger* aggregate had increased, at control conditions (from *ca* 1 to 3.8 Log_{10} CFUs g^{-1}). Exposure to 25% CO_2 did not significantly influenced fungal population dynamics over time. It is interesting that under higher concentrations of CO_2 (50%), combined with SO_2 presence and low water availability (0.928 a_w), black aspergilli were stimulated, after 2 weeks exposure to these conditions.

Figure 4.13 shows the effect of modified conditions on black aspergilli, on 'C type' sultanas (final industrial product). After 7 days of incubation, at 25% CO_2, dynamics of *A. niger* aggregate were decreased by half, while at 50% CO_2 a stimulation of populations was evident. After 14 days of incubation, populations of *A.niger* group remained constant, at both elevated CO_2 treatments (25% and 50% CO_2). However, in the control samples, no *A. niger* ag. were isolated. *A. carbonarius* was totally inhibited, regardless of the incubation time. Overall, no specific patterns in B and C types of sultanas could be found.

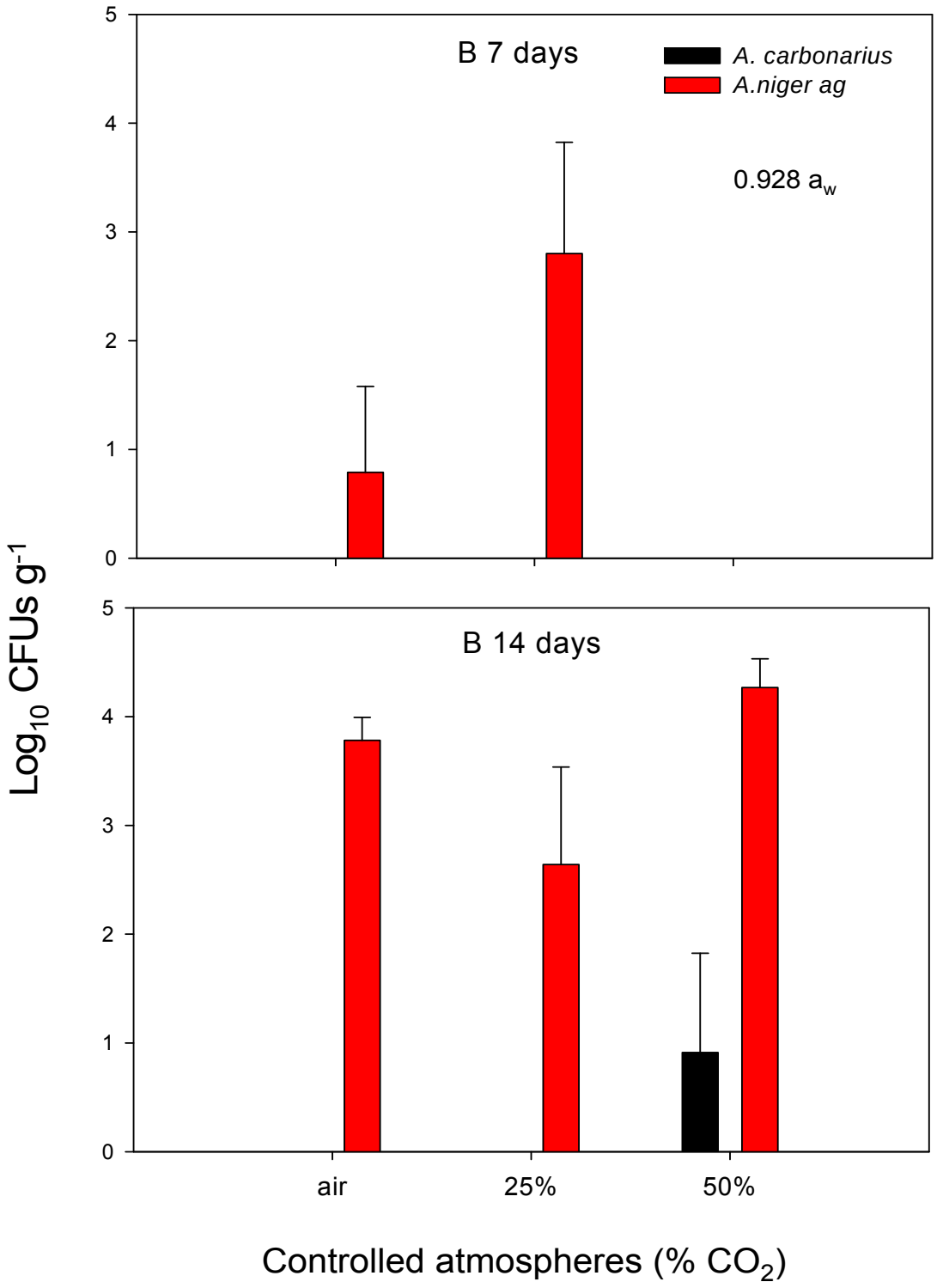


Figure 4.12. Population dynamics of black aspergilli on B type (after SO_2 treatment at industrial process) sultanas, modified to 0.928 a_w, under controlled atmospheres (air, 25% CO_2 and 50% CO_2). Incubation time: 7 and 14 days. Isolation medium: MEA97. Bars indicate standard error of mean.

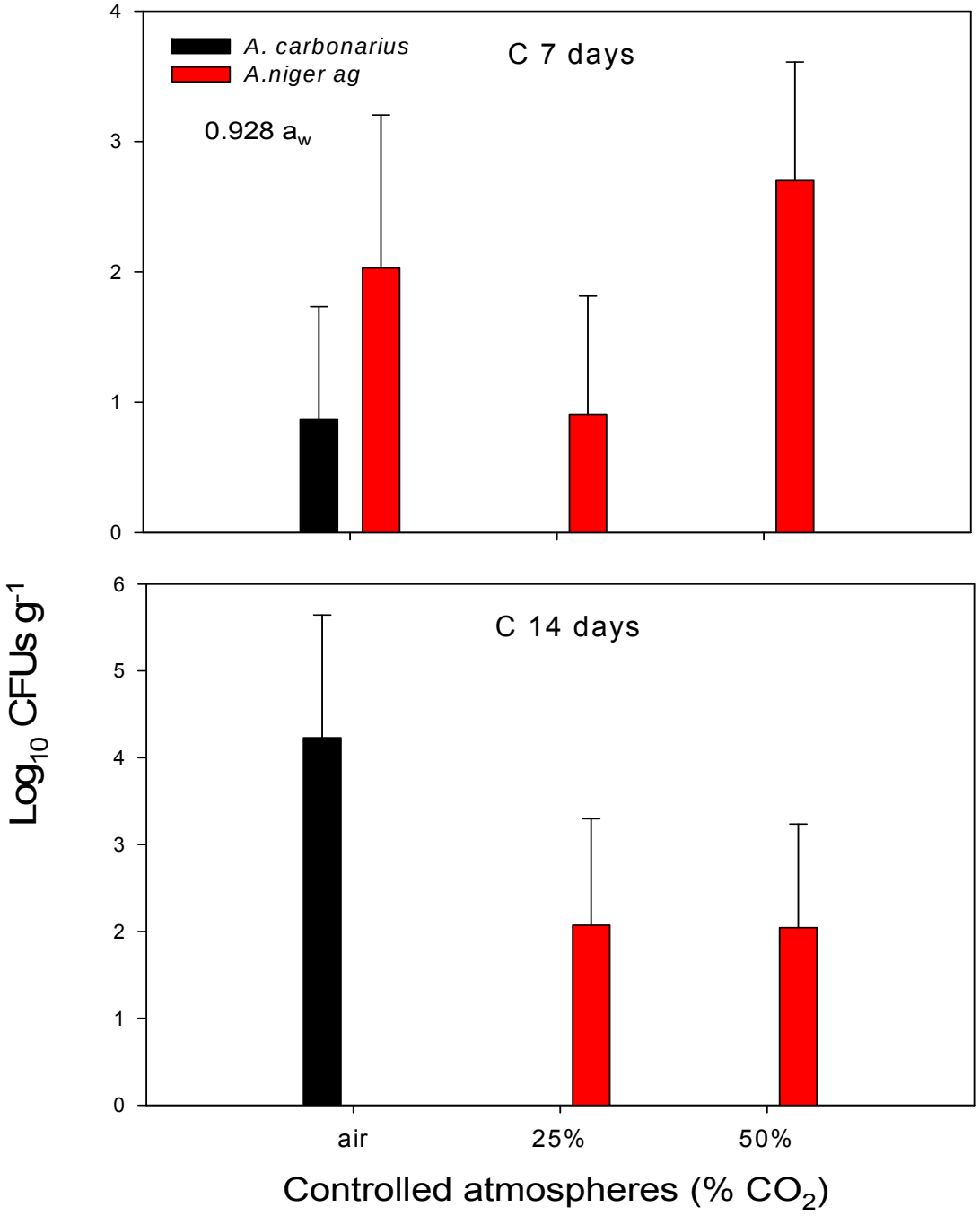


Figure 4.13. Population dynamics of black aspergilli on C type (final industrial product with SO_2) sultanas, modified to 0.928 a_w, under controlled atmospheres (air, 25% CO_2 and 50% CO_2). Incubation time: 7 and 14 days. Isolation medium: MEA97. Bars indicate standard error of mean.

Figure 4.14 shows the population dynamics of black aspergilli (*A. carbonarius* and *A. niger* aggregate) grown on the three examined "types" of sultanas (A, B, C types), modified to 0.882 a_w (see Plate 4.3). Three different concentrations of CO_2 were also used (0.03%, 25% and 50%) in order to modify growth conditions. Exposure to these conditions lasted for 14 days at 25^o C.

At drier conditions (0.884 a_w) the patterns are clearer after 14 days of incubation. The effects of CO_2 on black aspergilli populations on A "type" of sultanas are similar to those at 0.928 a_w. A similar, slight inhibition was observed on population loads of *A. niger* aggregate (*ca* 30% inhibition), at both elevated concentrations of CO_2 (25% and 50%). No *A. carbonarius* was isolated, under the above conditions, regardless of the "type" of sultanas, indicating a total inhibition.

Patterns for B and C "types" of sultanas are similar to those of A "type". In B type, the populations of *A. niger* aggregate were slightly decreased (*ca* 8%), under elevated CO_2 conditions. Overall, the total population dynamics of *A. niger* aggregate was higher than the representative ones, at 0.928 a_w. This is due to the fact that *A. carbonarius* cannot grow well in so drier conditions (Thom and Raper, 1945; El Halouat *et al.,* 1997) therefore, *A. niger* aggregate, which are more tolerant to these conditions, have taken an advance. *A. carbonarius* is completely eliminated under elevated CO_2 conditions.

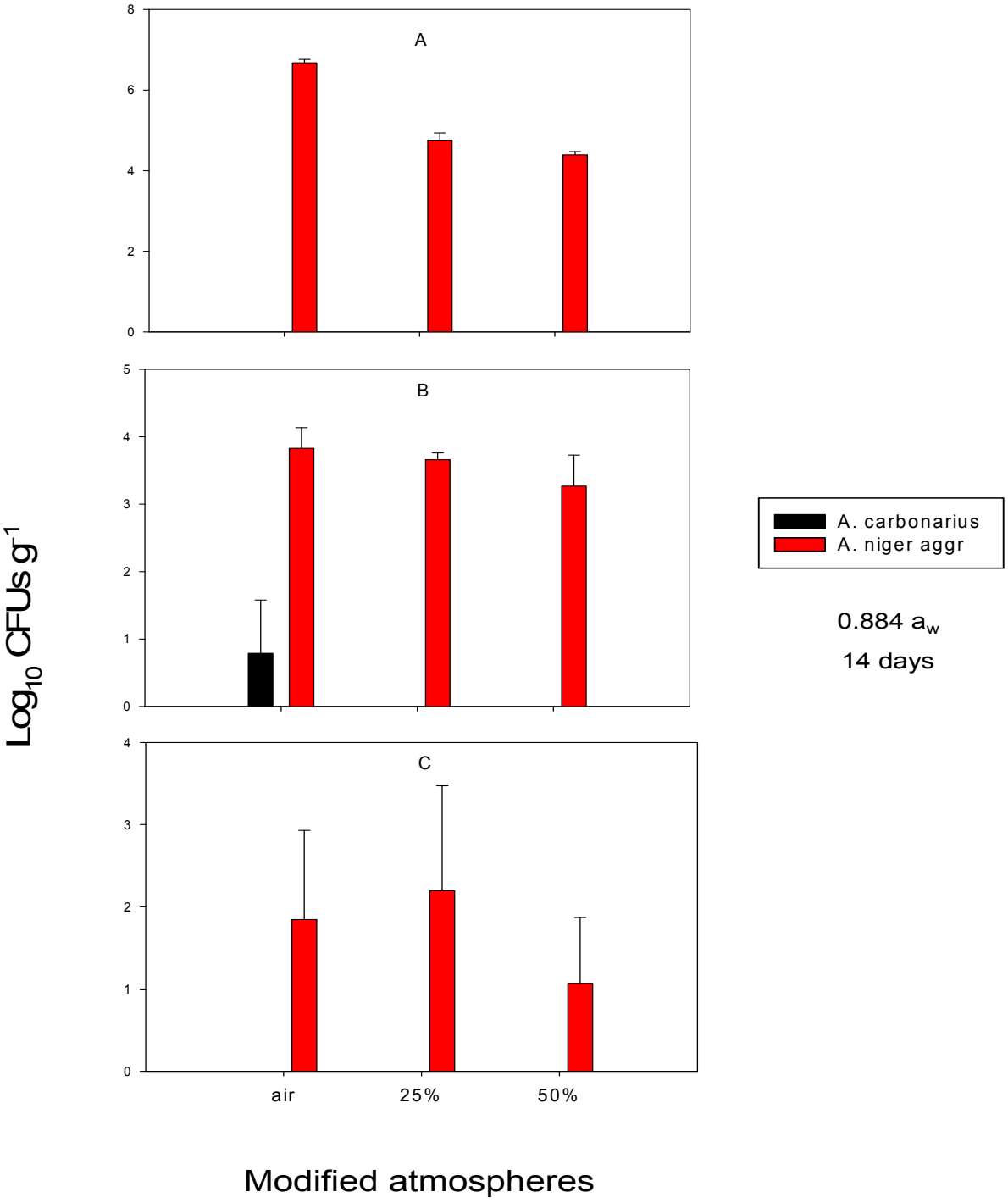
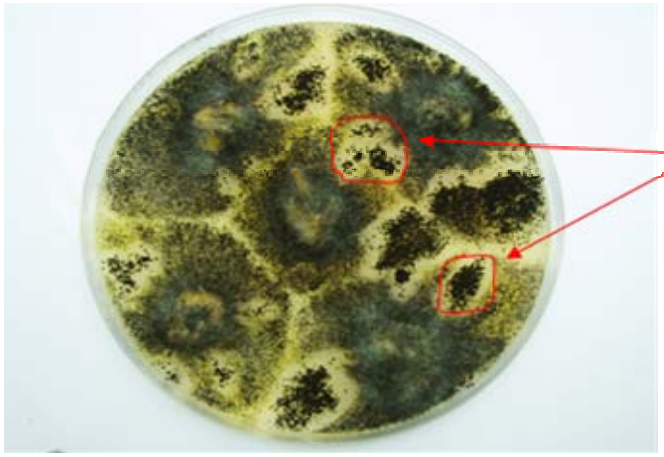
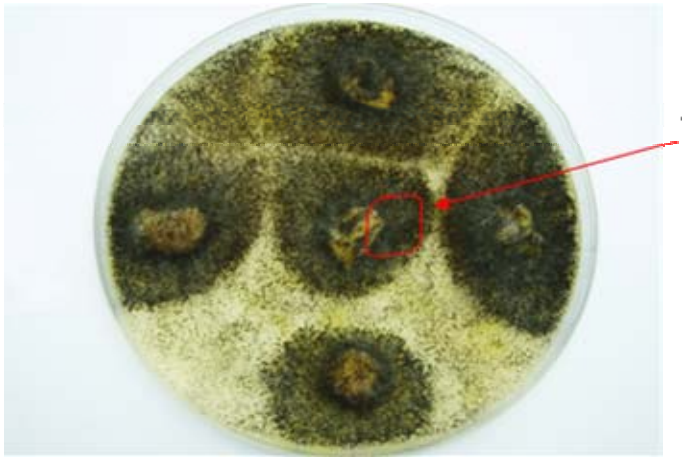


Figure 4.14. Population dynamics of black aspergilli in 3 "types" of sultanas, modified to 0.882 a_w, under controlled atmospheres (air, 25% CO_2 and 50% CO_2). Incubation time: 14 days. Isolation medium: MEA97. Keys; A: pre-industrial sultanas, B: sultanas shortly after SO_2 treatment, C: final industrial product (with heat and SO_2 treatment).



(a)

"A" type, Od 0.928a



(b)

"C" type, Od 0.884a

(c)

"B" type, control 14d 0.928a

Plate 4.3. Plates (a) and (b) show black aspergilli isolated by direct plating and on MEA97, incubating at, for a week. Plate (c) shows sultanas type "B" (in air), after 14 days of incubation, at 25° C.

Statistical analysis

♦ Serial dilution method

The abiotic factors studied in the current survey significantly influenced black aspergilli population dynamics (see Tables 4.20(a,b) - 4.23).

Table 4.20(a,b). Statistical tables of significance (Anova) show the effects of a_w, x controlled conditions (CO_2) and their interaction on (a) *A. carbonarius* and (b) *A. niger* aggregate using the serial dilution method after 7 days and 14 days of incubation, at 25°C. Three "types" of sultanas were used as substrates.

(a)

7 days	Df	F	Sig.	**14 days**	F	Sig.
a_w	1	2.890	0.095	a_w	24.988	0.000*
type	2	1.158	0.322	type	3.072	0.049*
CO_2	2	1.070	0.350	CO_2	20.396	0.000*
a_w * type	2	1.158	0.322	a_w * type	6.224	0.004*
a_w * CO_2	2	1.070	0.350	a_w * CO_2	13.952	0.000*
type * CO_2	4	0.414	0.798	type * CO_2	4.464	0.003*
a_w * type * CO_2	4	0.414	0.798	a_w * type * CO_2	8.215	0.000*

(b)

7 days	Df	F	Sig.	**14 days**	F	Sig.
a_w	1	16.626	0.000*	a_w	1.494	0.227
type	2	20.402	0.000*	type	27.479	0.000*
CO_2	2	0.087	0.917	CO_2	0.840	0.437
a_w * type	2	3.273	0.046*	a_w * type	1.224	0.302
a_w * CO_2	2	0.103	0.902	a_w * CO_2	1.261	0.291
type * CO_2	4	2.638	0.044*	type * CO_2	3.733	0.009*
a_w * type * CO_2	4	2.247	0.076	a_w * type * CO_2	0.934	0.451

(*) significant at $P<0.05$

Table 4.21. Effect of water availability (a_w) on *A. carbonarius,* isolated using the serial dilution method after 14 days of incubation time. Similar patterns are observed for *A. niger* aggregate, after 7 or 14 days incubation (see APENDIX B).

	Mean	Std. Error	95% Confidence Interval	
a_w			Lower Bound	Upper Bound
0.928	1.210	0.159	0.892	1.529
0.884	0.088	0.159	-0.231	0.406

Table 4.22(a,b). Homogenous subsets of the impact of type of sultanas on (a) *A. carbonarius* and (b) *A. niger* aggregate, isolated using serial dilution method, after 14 days of incubation.

(a)

	Type	N	Subset	
			1	2
Duncan(a,b)	B	24	0.2836	
	C	24	0.7048	0.7048
	A	24		0.9585

(b)

	Type	N	Subset		
			1	2	3
Duncan(a,b)	C	24	1.4924		
	B	24		3.4982	
	A	24			4.5408

Significance level = 0.05

Table 4.23. Homogenous subsets of the impact of CO_2 on *A. carbonarius* using serial dilutions, after 14 days of incubation time, based on the Duncan$^{(a,b)}$ test.

	CO_2	N	Subset	
			1	2
Duncan(a,b)	25%	24	0.1318	
	50%	24	0.1521	
	Air	24		1.6630

Significance level = 0.05

Figure 4.15 shows the effect of CO_2 on the frequency of isolation of the most dominant fungal genera grown on three 'types' of sultanas (A, B, C). Water activity was modified to two levels: 0.928 and 0.884 a_w. At 0,928 a_w, the frequency of isolation of *A. carbonarius* was stimulated (23% and 40%) by elevated CO_2 (25%), in comparison to the control samples (2%, 25%) in A and C "types" of sultanas, respectively. Similarly, the population loads of *A. niger* group were facilitated by medium concentrations of CO_2 (25%), in A "type" of sultanas.

Under 50% CO_2, no isolation of *A. carbonarius* was observed, regardless of "type" of sultanas. At a lower level of a_w (0.884), the fungus was frequently isolated in about 5%. (C type). 25% CO_2 did not stimulate growth. *A. niger* aggregate was more frequently isolated than *A. carbonarius,* mostly in "A" type of sultanas. In the control samples, *A. niger* aggregate were the dominant species, while 50% CO_2 resulted in effective inhibition (60%). Other fungal genera also frequently isolated were white yeasts. Occasionally, *Penicillium* sp. and *Eurotium* sp. were also isolated.

After 14 days incubation (Figure 4.16), a different pattern for *A. carbonarius* was observed. At the highest level of a_w (0.928), as CO_2 was increased, populations of *A. carbonarius* were strongly inhibited, in comparison to control samples, in "A" and "C" types of sultanas. In "B" type sultanas, no *A. carbonarius* were isolated, regardless of a_w levels. At the lowest a_w level (0.884), a complete absence of *A. carbonarius* was observed, regardless of the "type" of sultanas. On the contrary, populations of *A. niger* aggregate were stimulated by elevated CO_2 concentrations. Overall, no fungi were isolated from 'B type" of sultanas, regardless of a_w and incubation time. Furthermore, there may be an antagonism between the most dominant fungal species, such as black aspergilli and/or yeasts.

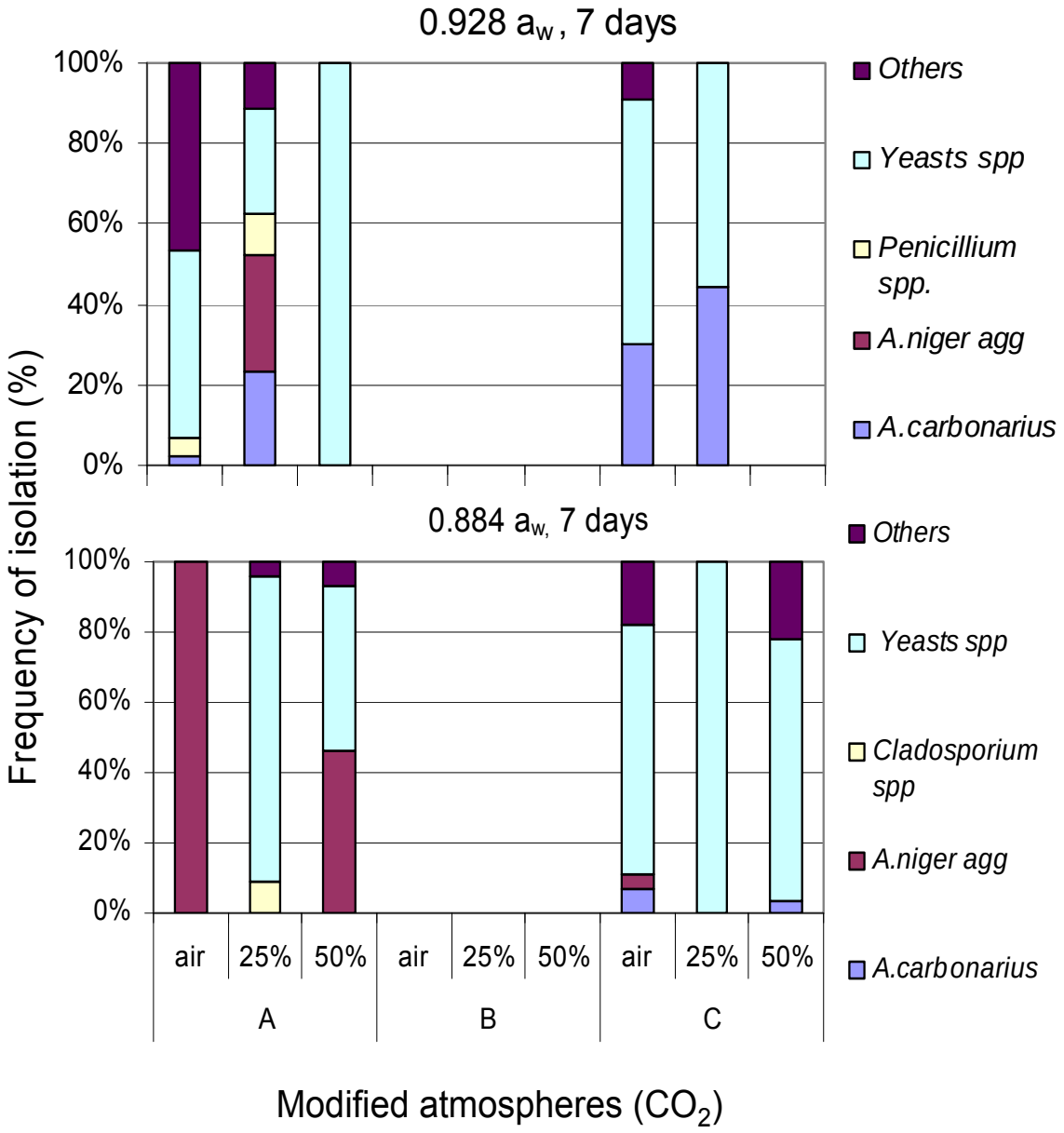


Figure 4.15. Effects of modified atmospheres (air, 25% CO_2 and 50% CO_2) x a_w, (0.928 and 0.884 a_w,) on (%) frequency of isolation of the most dominant fungal genera/species, grown on 3 "types" of sultanas. Keys; A: pre-industrial sultanas, B: sultanas shortly after SO_2 treatment, C: final industrial product (with heat and SO_2 treatment). Incubation time: 7 days. Isolation medium: MEA97.

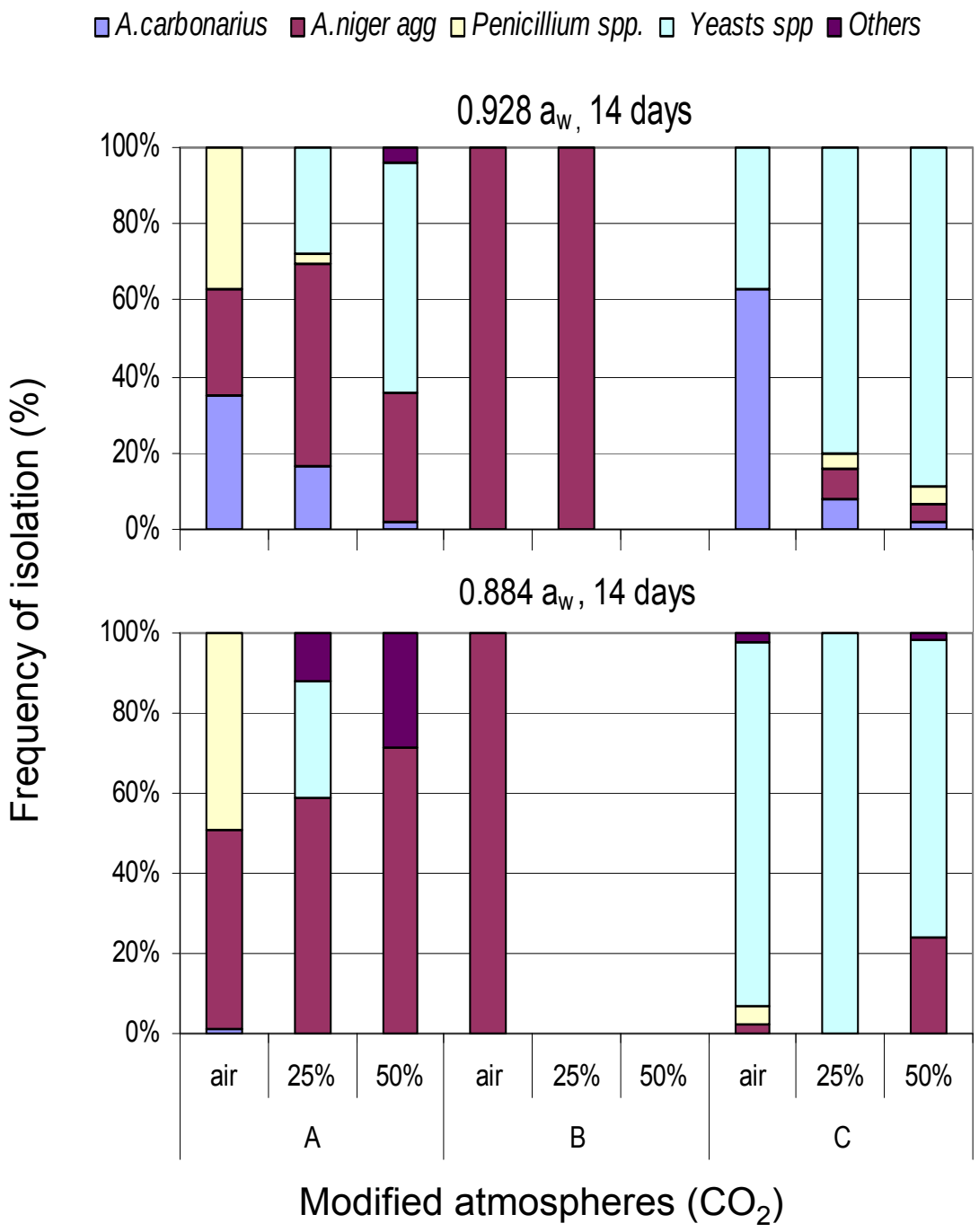


Figure 4.16. Effects of modified atmospheres (air, 25% CO_2 and 50% CO_2) x a_w, (0.928 and 0.884 a_w,) on frequency of isolation (%) of the most dominant fungal genera and species isolated from 3 "types" of sultanas. Keys; A: pre-industrial sultanas, B: sultanas shortly after SO_2 treatment, C: final industrial product (with heat and SO_2 treatment). Incubation time: 14 days. Isolation medium: MEA97.

Statistical analysis

♦ Direct plating isolation method

Table 4.24 (a,b). Summary statistical tables of significance (Anova) of effects of a_w, "type" of sultanas, controlled conditions (CO_2) and their interaction on (a) *A. carbonarius* and (b) on *A. niger* aggregate, using direct plating isolation method, after 7 and 14 days of incubation.

(a)

7 days	Df	F	Sig.	**14 days**	df	Sig.
a_w	1	44.825	0.000*	a_w	1	0.000*
Type	2	16.786	0.000*	Type	2	0.000*
CO_2	2	25.641	0.000*	CO_2	2	0.000*
a_w * Type	2	13.466	0.000*	a_w * Type	2	0.000*
a_w * CO_2	2	32.282	0.000*	a_w * CO_2	2	0.000*
Type * CO_2	4	13.374	0.000*	Type * CO_2	4	0.000*
a_w * Type * CO_2	4	11.714	0.000*	a_w * Type * CO_2	4	0.000*

(b)

7 days	Df	F	Sig.	**14 days**	F	Sig.
a_w	1	441.000	0.000*	a_w	1.881	0.171
Type	2	3541.000	0.000*	Type	409.771	0.000*
CO_2	2	1.000	0.369	CO_2	19.532	0.000*
a_w * Type	2	381.000	0.000*	a_w * Type	23.280	0.000*
a_w * CO_2	2	1641.000	0.000*	a_w * CO_2	10.614	0.000*
Type * CO_2	4	1.000	0.408	Type * CO_2	25.022	0.000*
a_w * Type * CO_2	4	1581.000	0.000*	a_w * Type * CO_2	2.480	0.043*

(*) significant at $P<0.05$

Table 4.25. Effect of water availability (a_w) on *A.carbonarius* after 7 days of incubation time. The same pattern is observed after 14 days (see APPENDIX B).

a_w	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0.928	0.167	0.016	0.136	0.198
0.884	0.017	0.016	-0.014	0.048

Table 4.26(a,b). Homogenous subsets of the impact of the "type" of sultanas on *A. carbonarius*, isolated using direct plating isolation method, after (a) 7 days and (b) 14 days of incubation.

(a)

	Type	N	Subset	
Duncan(a,b)	B	120	0.0000	
	C	120		0.1333
	A	120		0.1417

(b)

	Type	N	Subset		
			1	2	3
Duncan(a,b,c)	B	240	0.0000		
	C	230		0.1696	
	A	230			0.2217

Table 4.27(a,b). Homogenous subsets of the impact of CO_2 on *A. carbonarius*, isolated using direct plating isolation method, after (a) 7 days and (b) 14 days of incubation.

(a)

	CO_2	N	Subset		
			1	2	3
Duncan(a,b)	50%	120	0.0083		
	air	120		0.0667	
	25%	120			0.2000

(b)

	CO_2	N	Subset		
			1	2	3
Duncan(a,b,c)	50%	230	0.0087		
	25%	230		0.0652	
	Air	240			0.3042

Table 4.28. Effect of water availability (a_w) on *A. niger* aggregate, isolated using direct plating method, after 7 days of incubation time.

a_w	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0.928	0.111	0.004	0.103	0.119
0.884	0.228	0.004	0.220	0.236

Table 4.29(a,b). Homogenous subsets of the impact of type of sultanas on *A.niger* aggregate, using direct plating isolation method, after (a) 7 days and (b) 14 days of incubation.

(a)

	Type	N	Subset	
Duncan(a,b)	B	120	0.0000	
	C	120	0.0083	
	A	120		0.5000

(b)

	Type	N	Subset		
			1	2	3
Duncan(a,b,c)	C	230	0.0652		
	B	240		0.1500	
	A	230			0.8043

Table 4.30. Homogenous subsets of the impact of CO_2 on *A. niger* aggregate, after 14 days of incubation time, based on Duncan$^{(a,b)}$ test.

	CO_2	N	Subset		
			1	2	3
Duncan(a,b,c)	50%	230	0.2478		
	25%	230		0.3391	
	Air	240			0.4208

4.3.3.2. Effects on OTA contamination

According to the statistical analysis (Univariate analysis, SPPS version 10), no abiotic factor (CO_2, a_w or "type" of sultanas) examined alone or in interaction with others, significantly affected OTA production (see Figure 4.17).

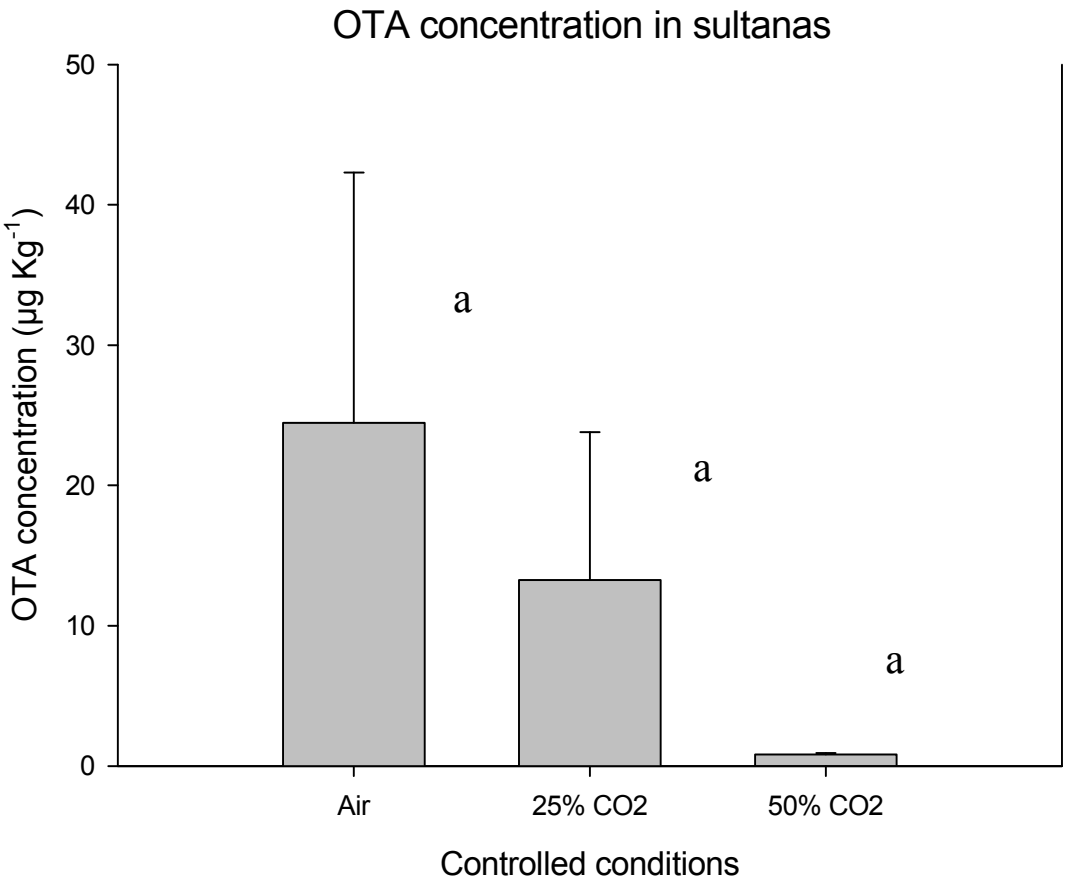


Figure 4.17. Effect of CO_2 on OTA production (µg Kg^{-1}) by *A. carbonarius*, grown on three "types" of sultanas (A, B, C), modified to two a_w levels (0.928, 0.884), after 14 days of incubation, at 25° C. The same letters indicate no significant difference between treatments.

4.3.4. INTERACTIONS OF *A. CARBONARIUS* AGAINST COMMON VINEYARD FUNGAL GENERA IN *VITRO* GRAPE-JUICE-BASED MEDIUM

4.3.4.1. Effects of fungal interaction on mycelial growth of *A. carbonarius*

In this experiment interactions between *A. carbonarius in vitro* against other common fungal genera (eight) and species (eleven), isolated from grapes and sultanas of the Cretan ecosystem were studied.

Table 4.31 shows the interaction scores and Index of Dominance (I_D) for *Aspergillus carbonarius* (GRE105) against some common vineyard fungi, on WGJM, after 10 days incubation at 25^o C. In almost all cases examined *A. carbonarius* was the dominant species on contact (4/0) against other species. When it was inoculated with *A. niger* aggregate, it was mutually antagonistic on contact (2/2), at higher (≥ 0.95) a_w levels. In drier conditions (0.93 a_w) *A.niger* aggregate competed with *A. carbonarius.* *Rhizopus* sp. was only dominant over *A. carbonarius* on contact (0/4), at ≥ 0.954 a_w.

Figures 4.18–4.24 show some examples of the temporal effect of fungal interaction x a_w on radial growth rates. Although, in general *A. carbonarius* is dominant over other fungi, depending on a_w level, the growth rate decreased after fungal intermingling.

Table 4.31. Index Dominance score (I_D) for *Aspergillus carbonarius* (GRE105) against some common vineyard fungi, on WGJM, after 10 days of incubation, at 25° C.

a_w	Antagonistic fungal species/genera	Score	I_D
0.985	x *Aspergillus niger* ag. (GRE0119)	2/2	
0.954	x *Aspergillus niger* ag. (GRE0119)	2/2	5/6
0.934	x *Aspergillus niger* ag. (GRE0119)	1/2	
0.985	x *Penicillium* sp (GRE0636)	5/0	
0.954	x *Penicillium* sp (GRE0636)	5/0	14/0
0.934	x *Penicillium* sp (GRE0636)	4/0	
0.985	x *Penicillium* sp (GRE0657)	4/0	
0.954	x *Penicillium* sp (GRE0657)	4/0	12/0
0.934	x *Penicillium* sp (GRE0657)	4/0	
0.985	x White *Yeast* sp. (GRE0686)	1/1	
0.954	x White *Yeast* sp. (GRE0686)	4/0	9/1
0.934	x White *Yeast* sp. (GRE0686)	4/0	
0.985	x Red *Yeast* sp. (GRE0689)	1/1	
0.954	x Red *Yeast* sp. (GRE0689)	4/0	9/1
0.934	x Red *Yeast* sp. (GRE0689)	4/0	
0.985	x *Botrytis cinerea* (GRE0624)	1/1	
0.954	x *Botrytis cinerea* (GRE0624)	2/1	7/2
0.934	x *Botrytis cinerea* (GRE0624)	4/0	
0.985	x *Eurotium* sp. (GRE0659)	4/0	
0.954	x *Eurotium* sp. (GRE0659)	4/0	12/0
0.934	x *Eurotium* sp. (GRE0659)	4/0	
0.985	x *Cladosporium* sp. (GRE0611)	4/0	
0.954	x *Cladosporium* sp. (GRE0611)	4/0	12/0
0.934	x *Cladosporium* sp. (GRE0611)	4/0	
0.985	x *Rhizopus* sp. (GRE0623)	0/4	
0.954	x *Rhizopus* sp. (GRE0623)	0/4	2/10
0.934	x *Rhizopus* sp. (GRE0623)	2/2/	
0.985	x *Alternaria* sp. (GRE111)	4/0	
0.954	x *Alternaria* sp. (GRE111)	4/0	12/0
0.934	x *Alternaria* sp. (GRE111)	4/0	
0.985	x *Aspergillus flavus* (GRE0680)	4/0	
0.954	x *Aspergillus flavus* (GRE0680)	4/0	12/0
0.934	x *Aspergillus flavus* (GRE0680)	4/0	
		I_D	96/10

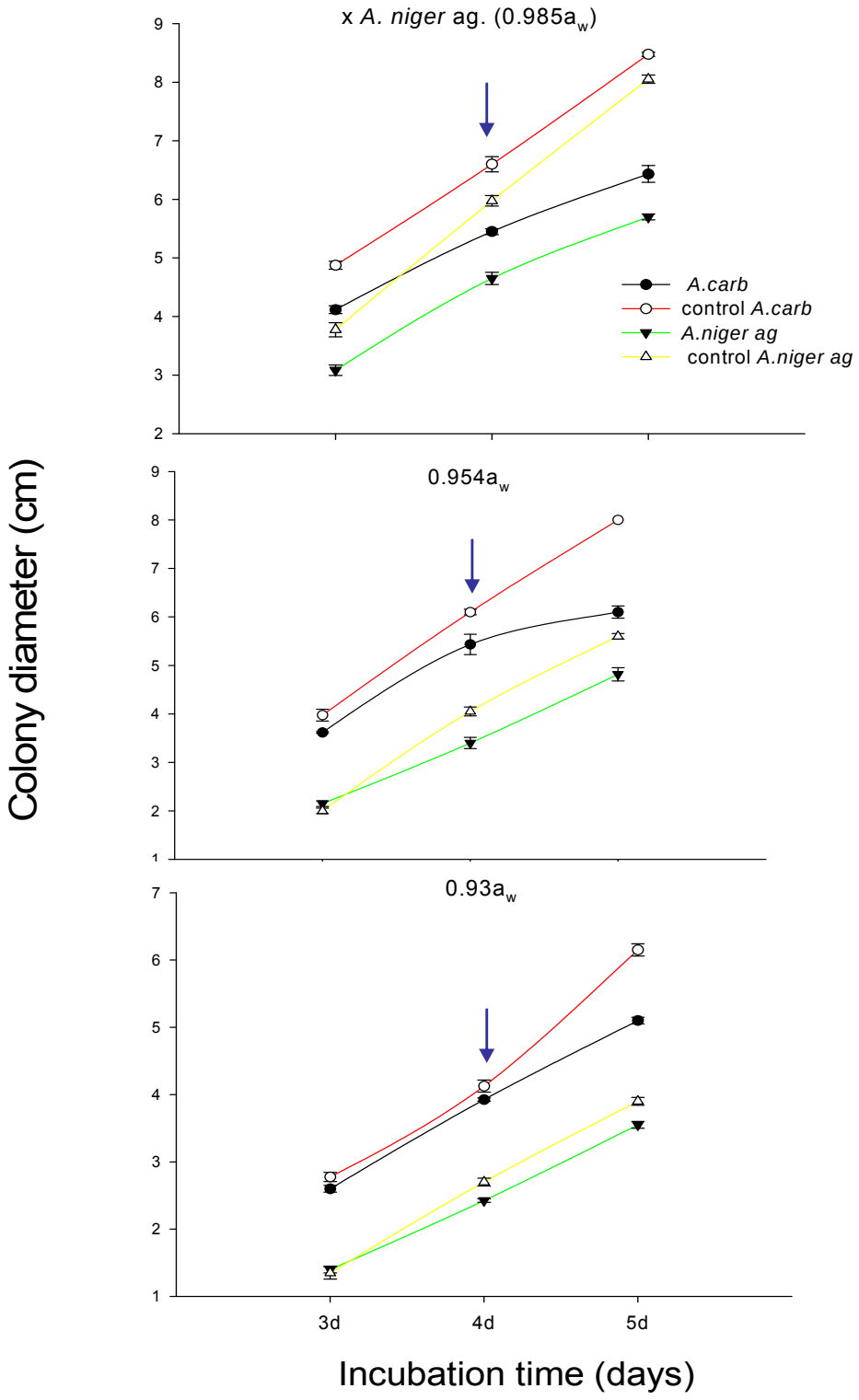


Figure 4.18. Temporal effect of fungal interaction (*A. carbonarius* vs *A.niger* aggregate) x a_w level, colony diameter (cm) of fungi examined. WHJM was used as subrate, at 25° C. Arrows indicate day of contact. Bars indicate standard error of mean.

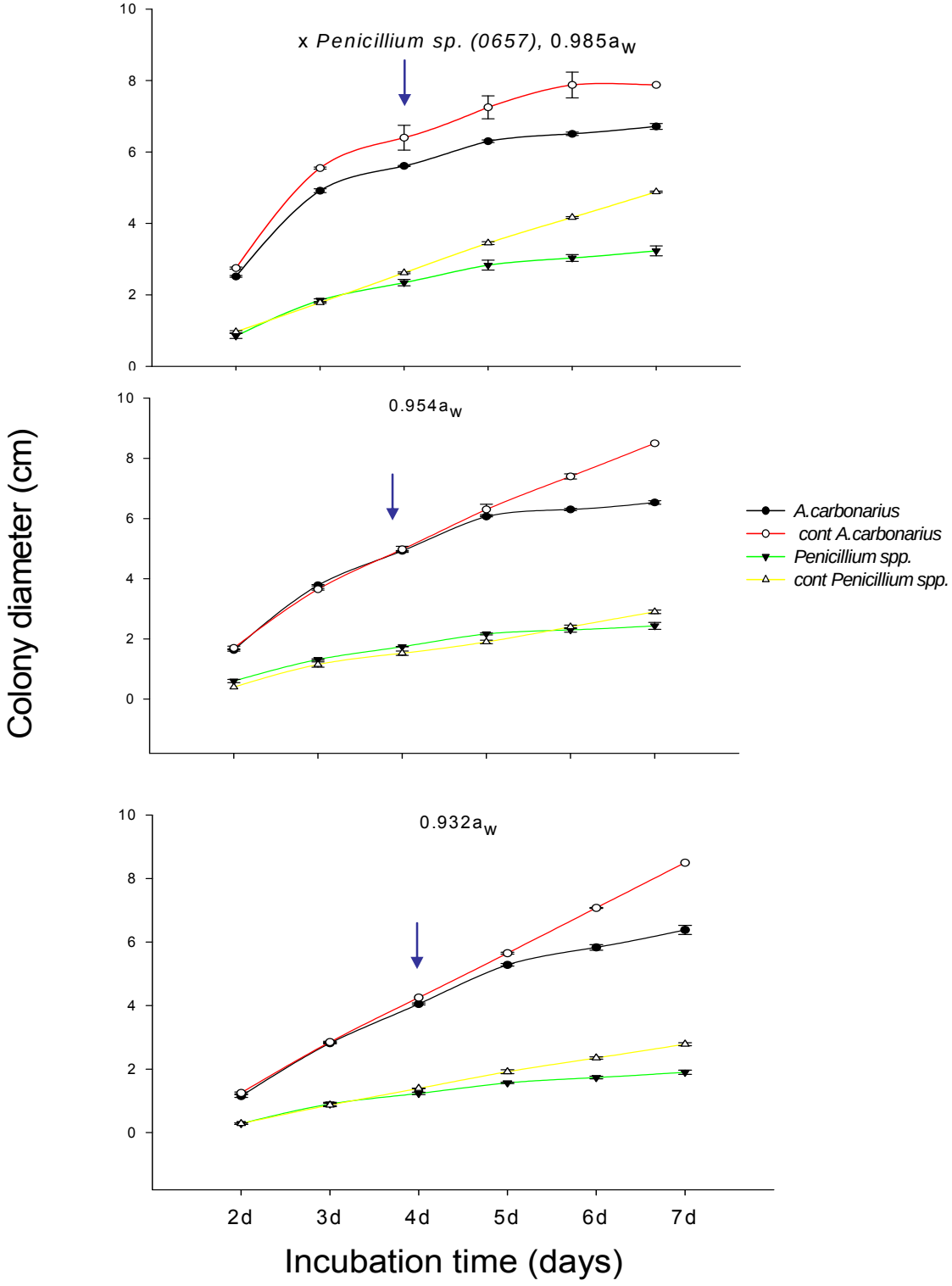


Figure 4.19. Temporal effect of fungal interactions (*A. carbonarius* vs *Penicillium* sp (GRE0657) x level on colony diameter (cm) of fungi examined. WHJM was used as subrate, at 25° C. Arrows indicate day of contact. Bars indicate standard error of mean.

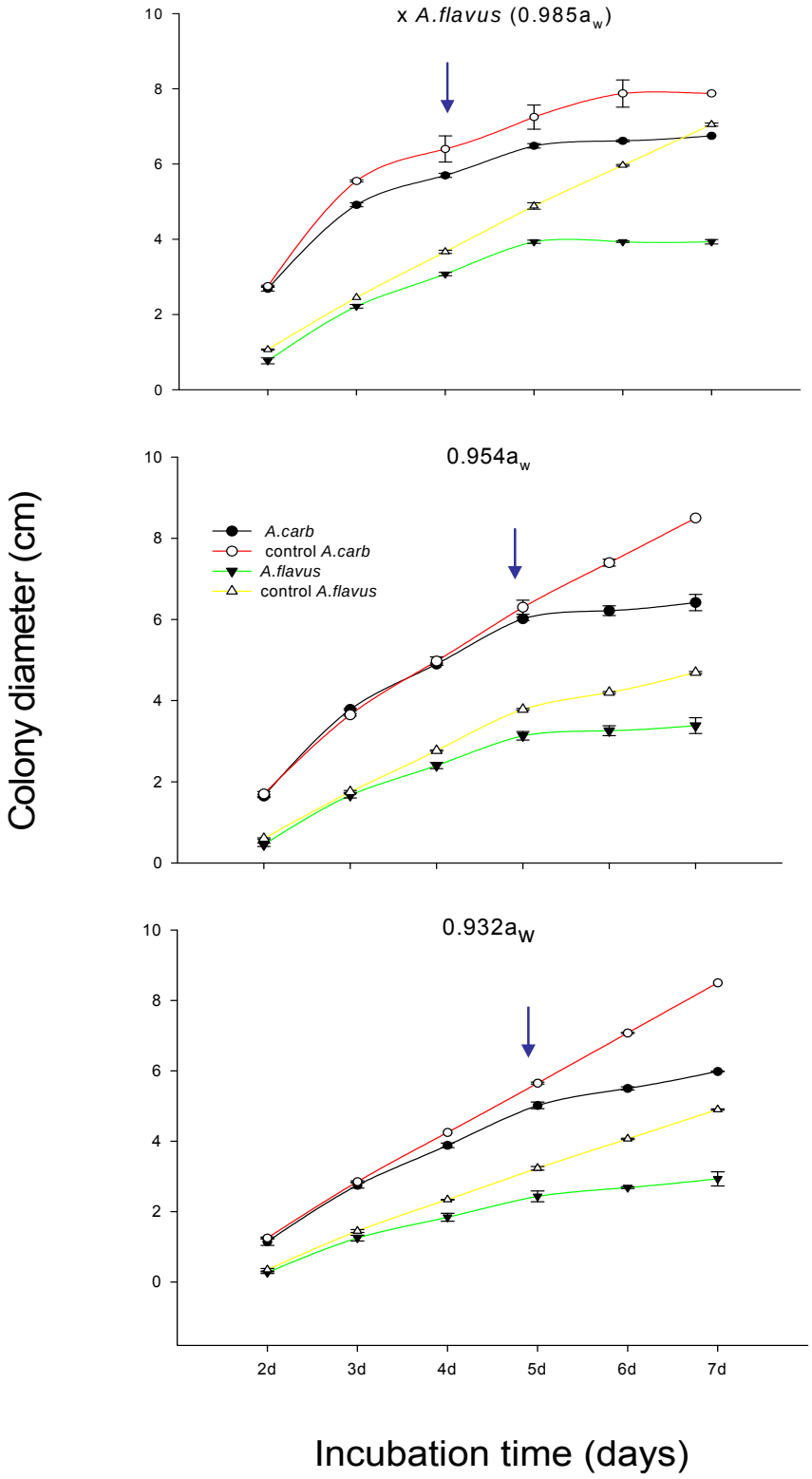


Figure 4.20. Temporal effect of fungal interactions (*A. carbonarius* vs *A. flavus*) x a_w on colony diameter (cm) of fungi examined. WHJM was used, at 25° C. Arrows indicate day of contact. Bars indicate standard error of mean.

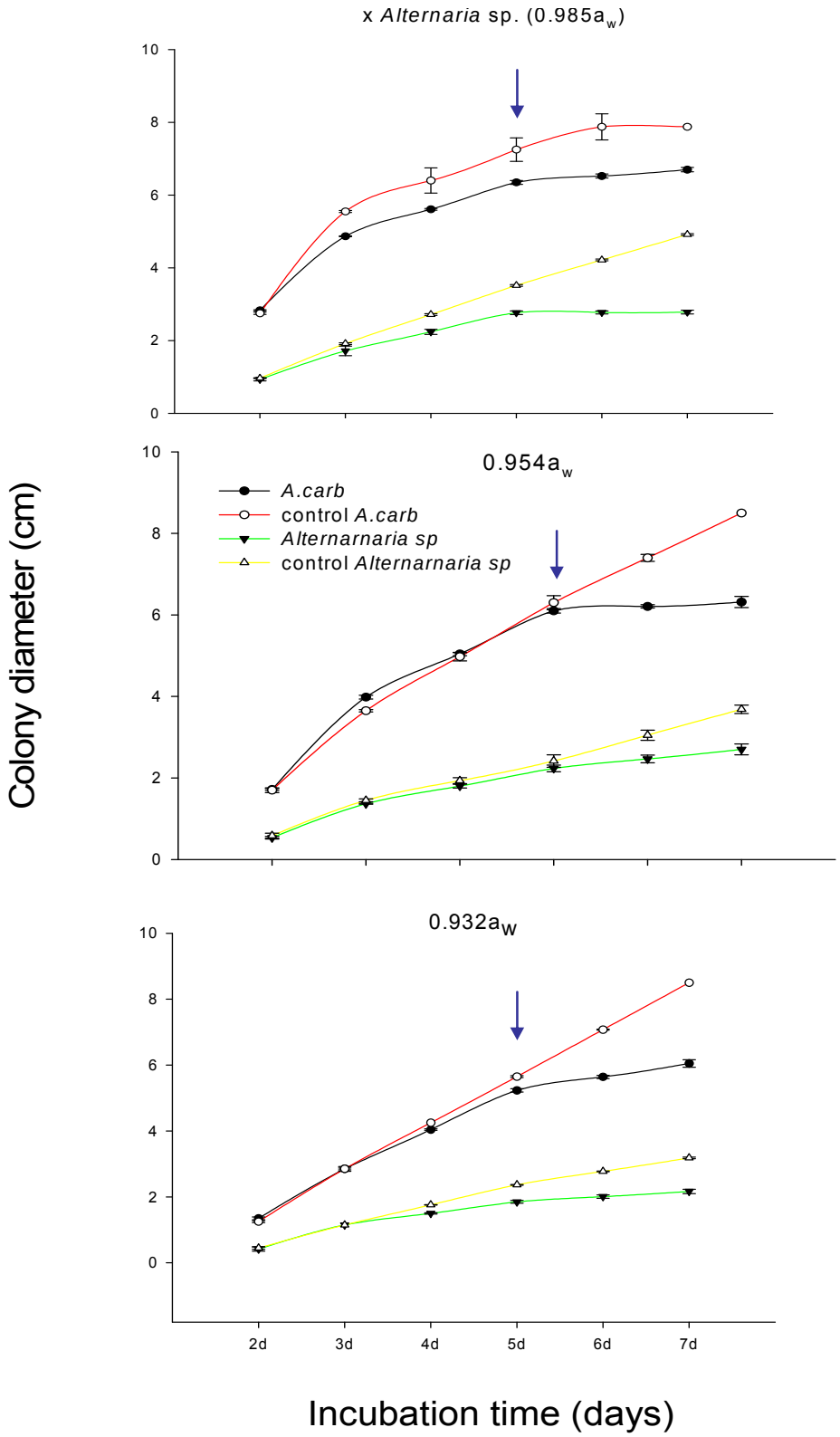


Figure 4.21. Temporal effect of fungal interactions (*A. carbonarius* vs *A.alternata*) x a_w on colony diameter (cm) of fungi examined. WHJM was used, at 25° C. Arrows indicate day of contact. Bars indicate standard error of mean.

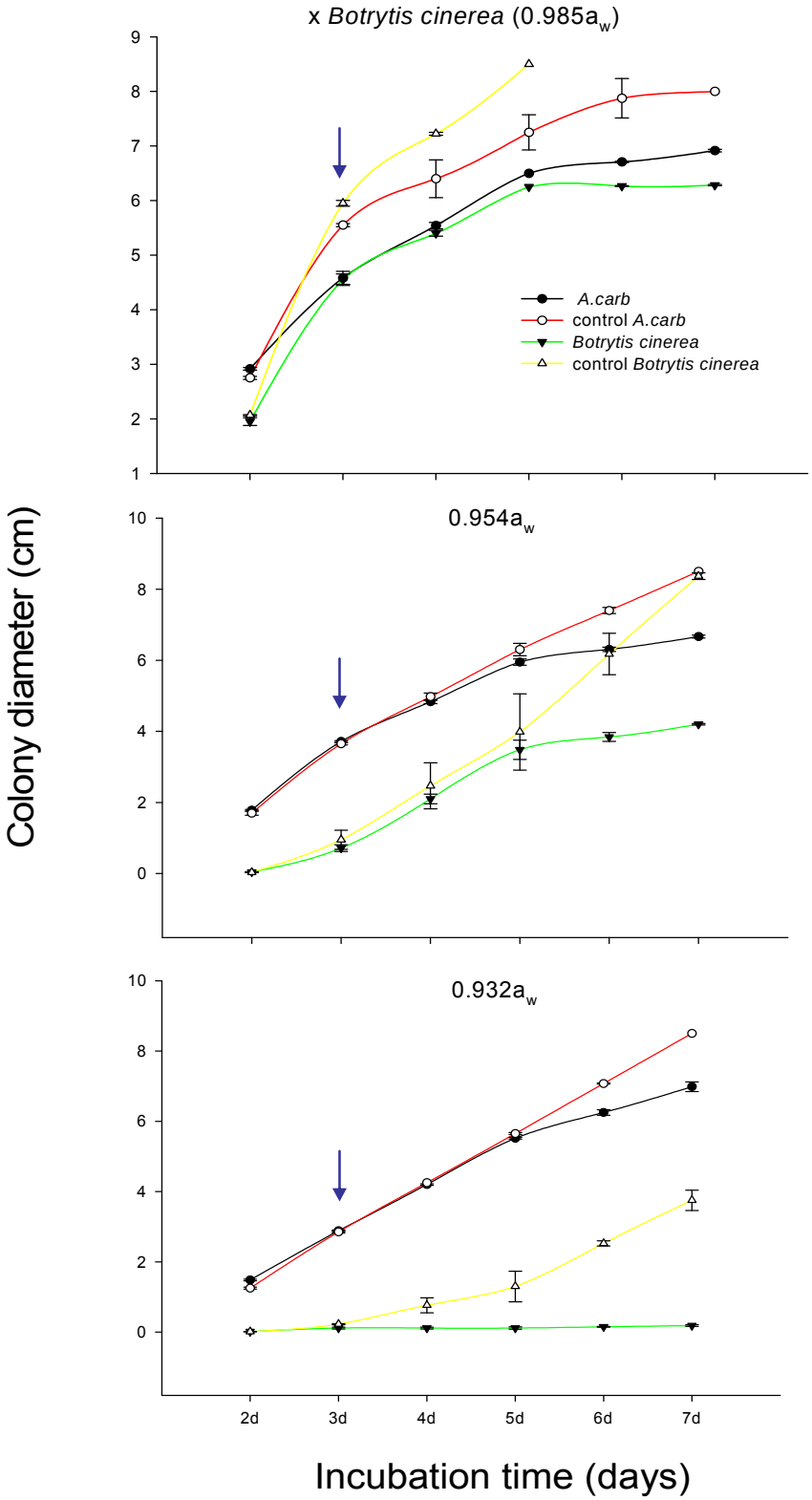


Figure 4.22. Temporal effect of fungal interactions (*A. carbonarius* vs *B. cinerea*) x a_w on colony diameter (cm) of fungi examined. WHJM was used, at 25° C. Arrows indicate day of contact. Bars indicate standard error of mean.

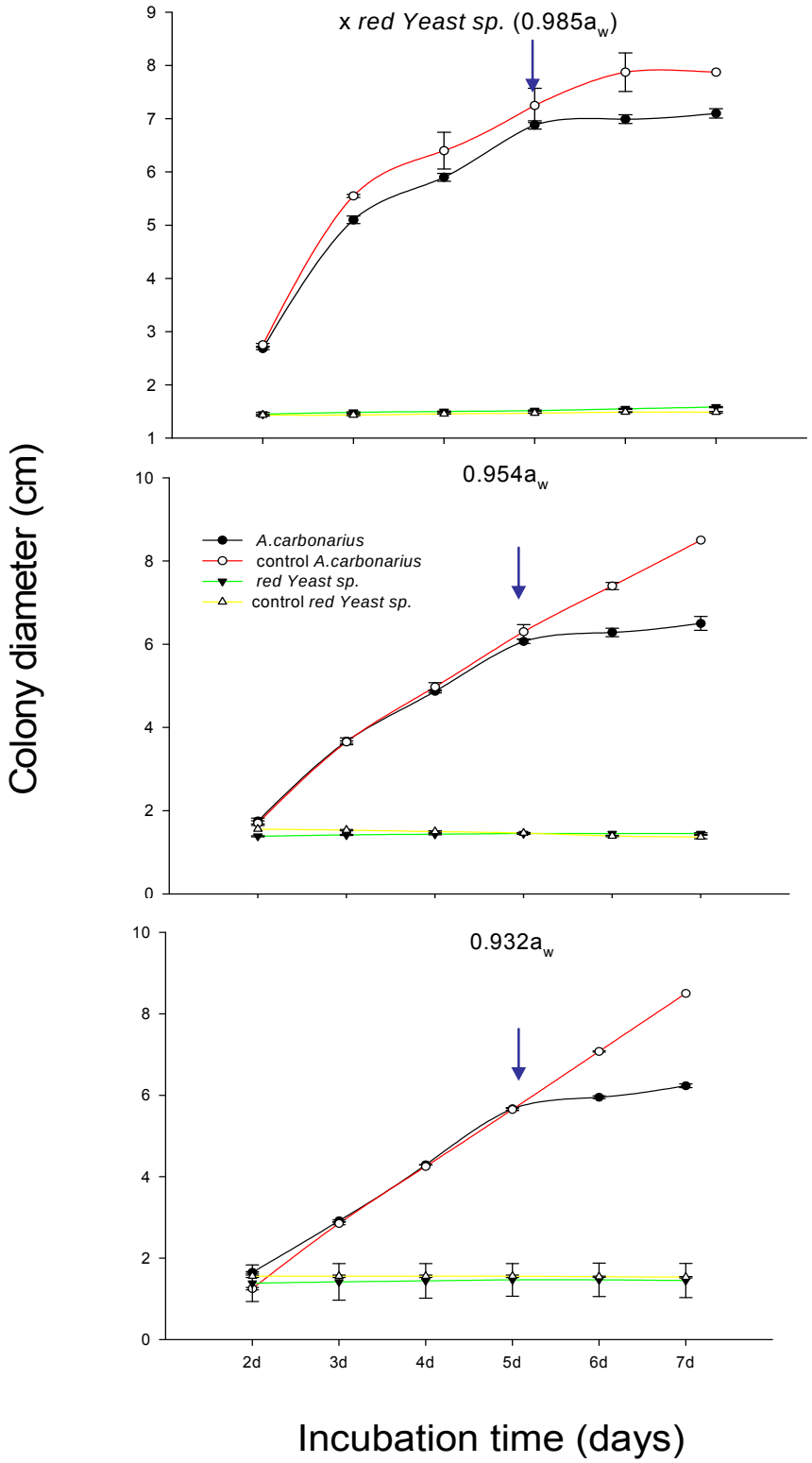


Figure 4.23. Temporal effect of fungal interactions (*A. carbonarius* vs yeast sp.) x a_w on colony diameter (cm) of fungi examined. WHJM was used, at 25° C. Arrows indicate day of contact. Bars indicate standard error of mean.

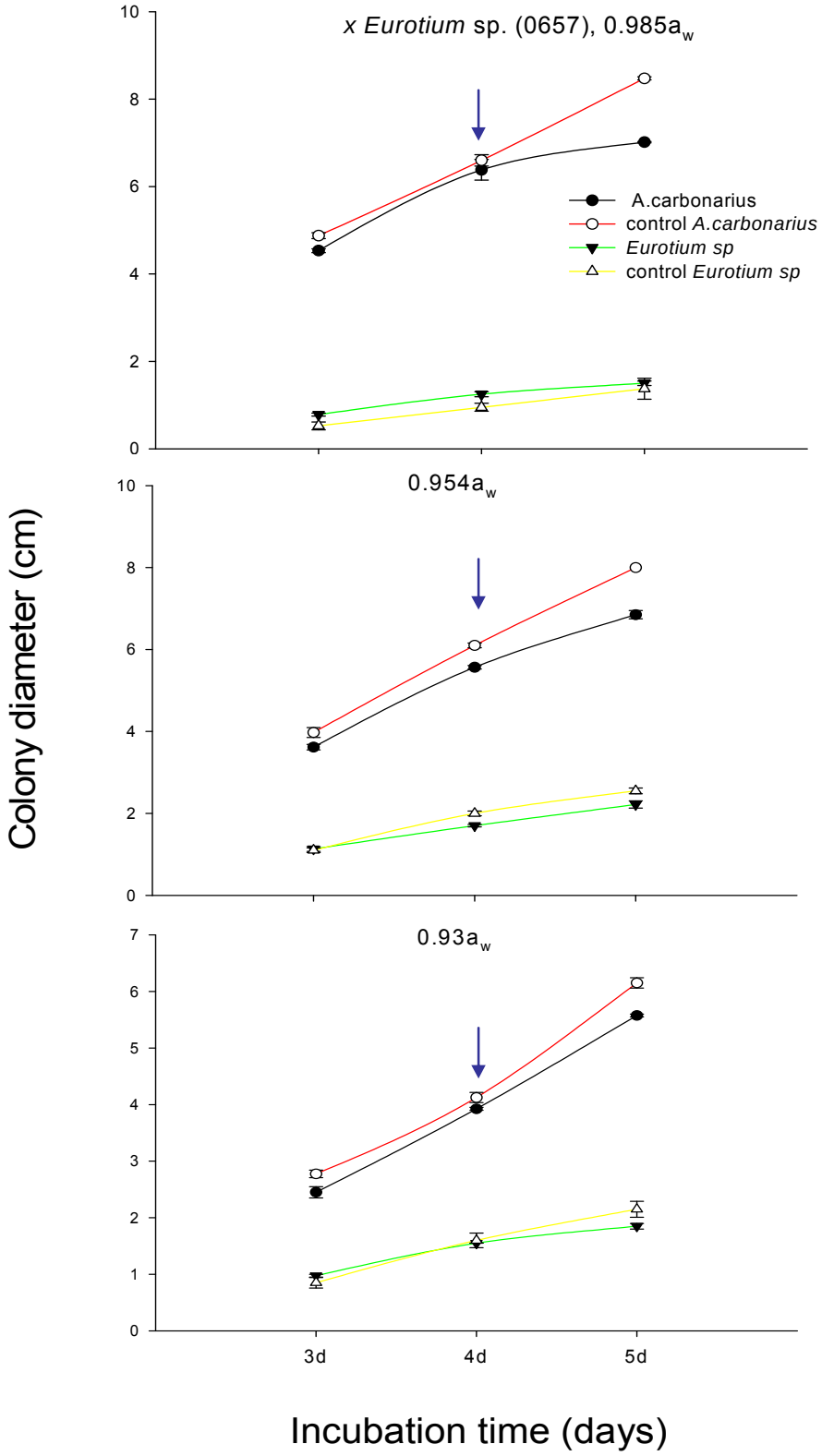


Figure 4.24. Temporal effect of fungal interactions (*A. carbonarius* vs *Eurotium sp.*) x a_w on colony diameter (cm) of fungi examined. WHJM was used, at 25° C. Arrows indicate day of contact. Bars indicate standard error of mean.

4.3.4.2. Effect of fungal interaction x a_w levels on ochratoxin A production by *A. carbonarius* isolates, on white grape juice medium

Figures 4.25-4.27 show the impact of fungal interaction x a_w on OTA production by *A. carbonarius* (GRE105 strain). Overall results demonstrate that in all treatments, OTA production by *A. carbonarius* was strongly inhibited (55%-98%) by the presence of other fungal species, at the highest water availability (0.985 a_w) examined, in comparison to control samples (*A. carbonarius* grown alone). The highest inhibition of OTA production was observed when *A. carbonarius* was grown with *Botrytis cinerea,* and *A. flavus*. Moreover, 95% reduction in OTA concentration occurred when *A. carbonarius* was grown with *Rhizopus* spp. However, when *A. carbonarius* was grown with a non ochratoxigenic *A. niger* aggregate strain (GRE0119) levels of OTA accumulation were not significantly different (300 μg Kg^{-1}) from the control samples (280 μg Kg^{-1}).

At lower levels of water activity (0.954 and 0.934 a_w), patterns are similar to the previous ones. Overall, a considerable reduction in OTA concentration was observed as the level of water availability (a_w) was decreased. In general, inhibition in OTA production ranged from 30-50%, at 0.954 a_w, while in the drier conditions (0.934 a_w), a more restricted inhibition was observed (*ca* 10%). This indicates that, as higher water availability level is, stronger inhibition on ochratoxin A production occurs, under specific experimental conditions.

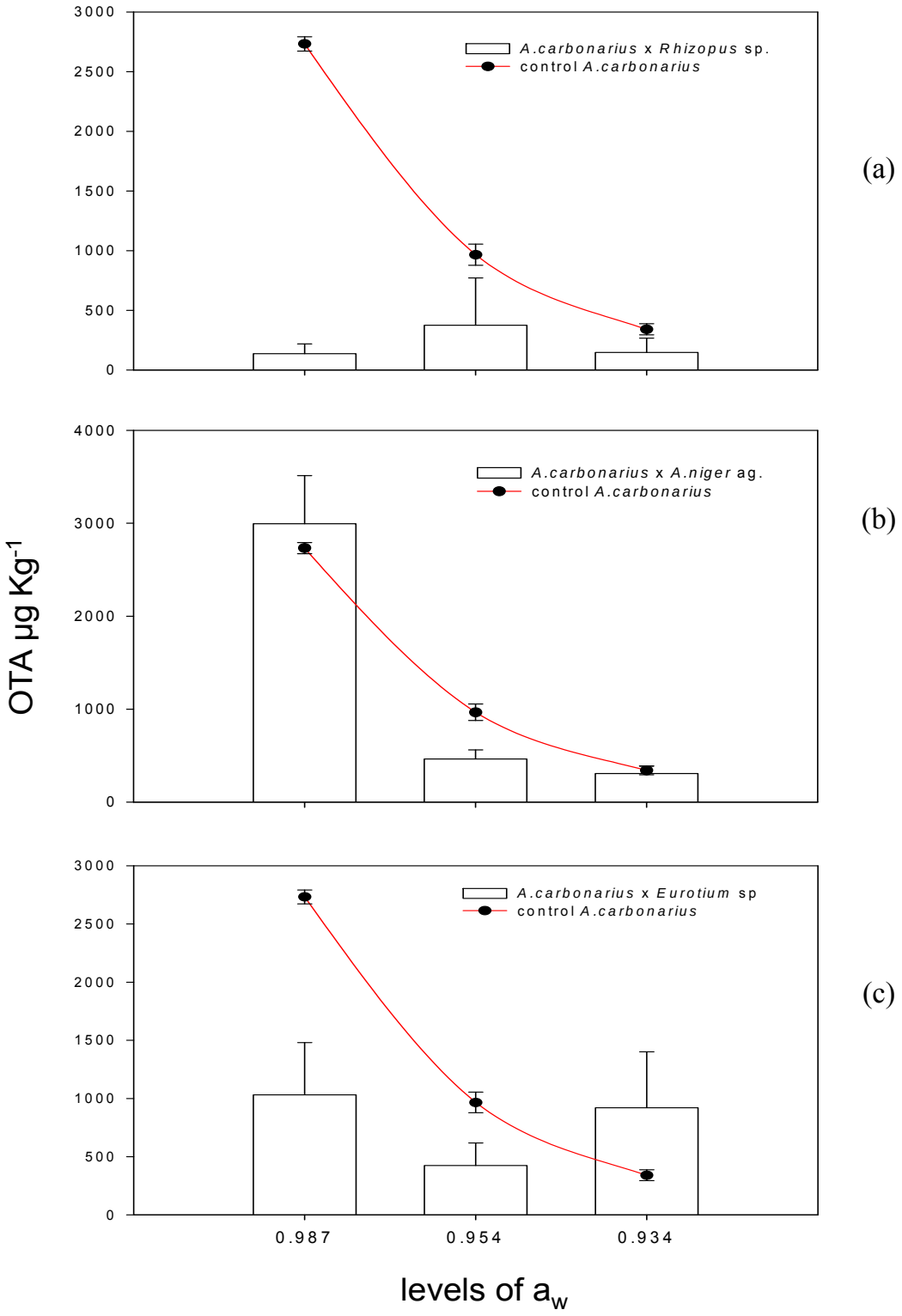


Figure 4.25. Effect of fungal interaction (*A. carbonarius* vs *Rhizopus sp (a), A. carbonarius* vs *A. niger* ag. *(b).* and *A. carbonarius* vs *Eurotium sp.(c)*) x a_w on OTA accumulation, on WHJM, after 7 days, at 25° C. Bars indicate standard error of mean.

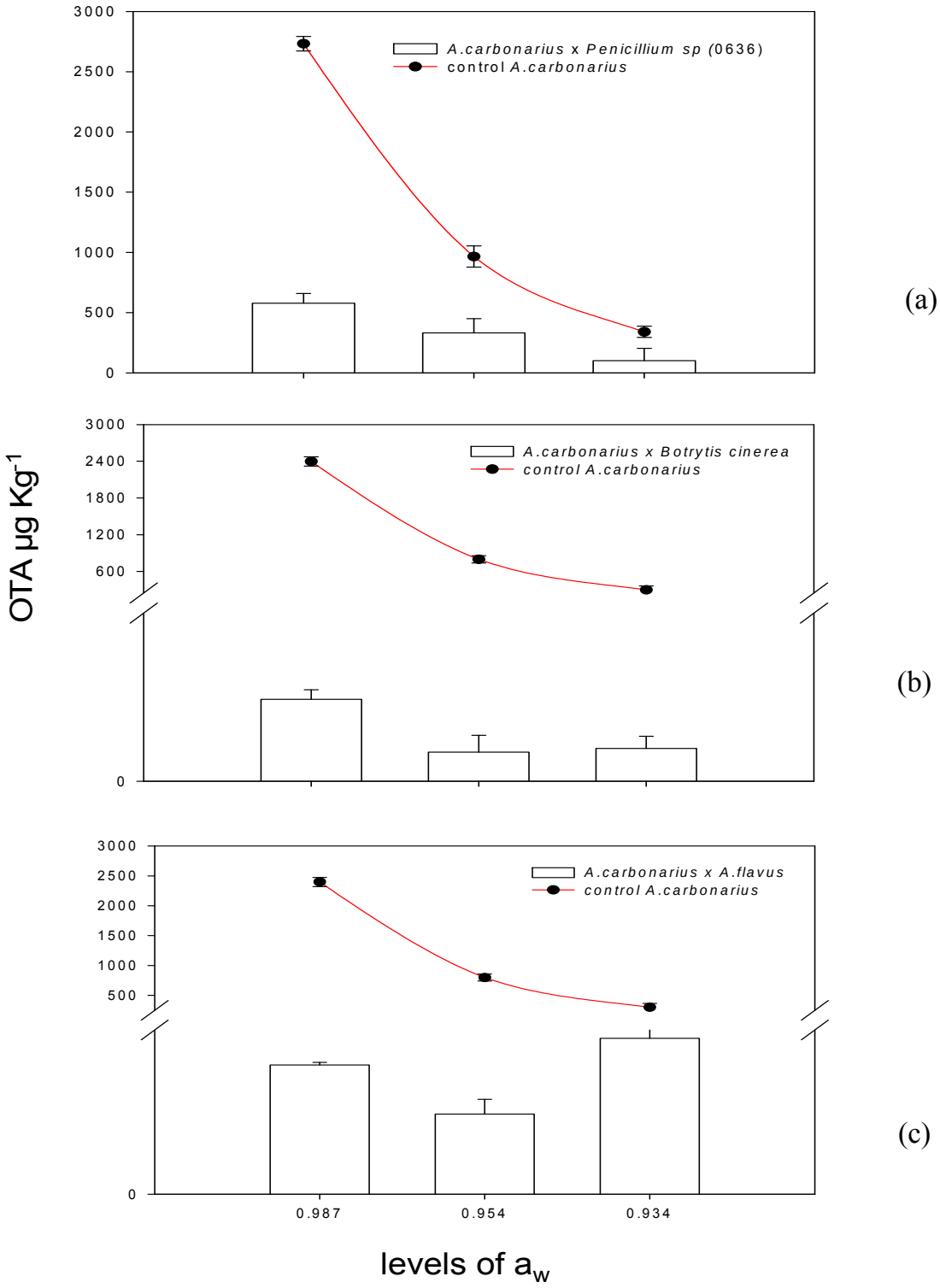


Figure 4.26. Effect of fungal interaction (*A. carbonarius* vs *Penicillum sp (a), A. carbonarius* vs *Botrytis sp. (b).* and *A. carbonarius* vs *A. flavus (c)*) x a_w on OTA accumulation, on WHJM, after 10 days, at 25° C. Bars indicate standard error of mean.

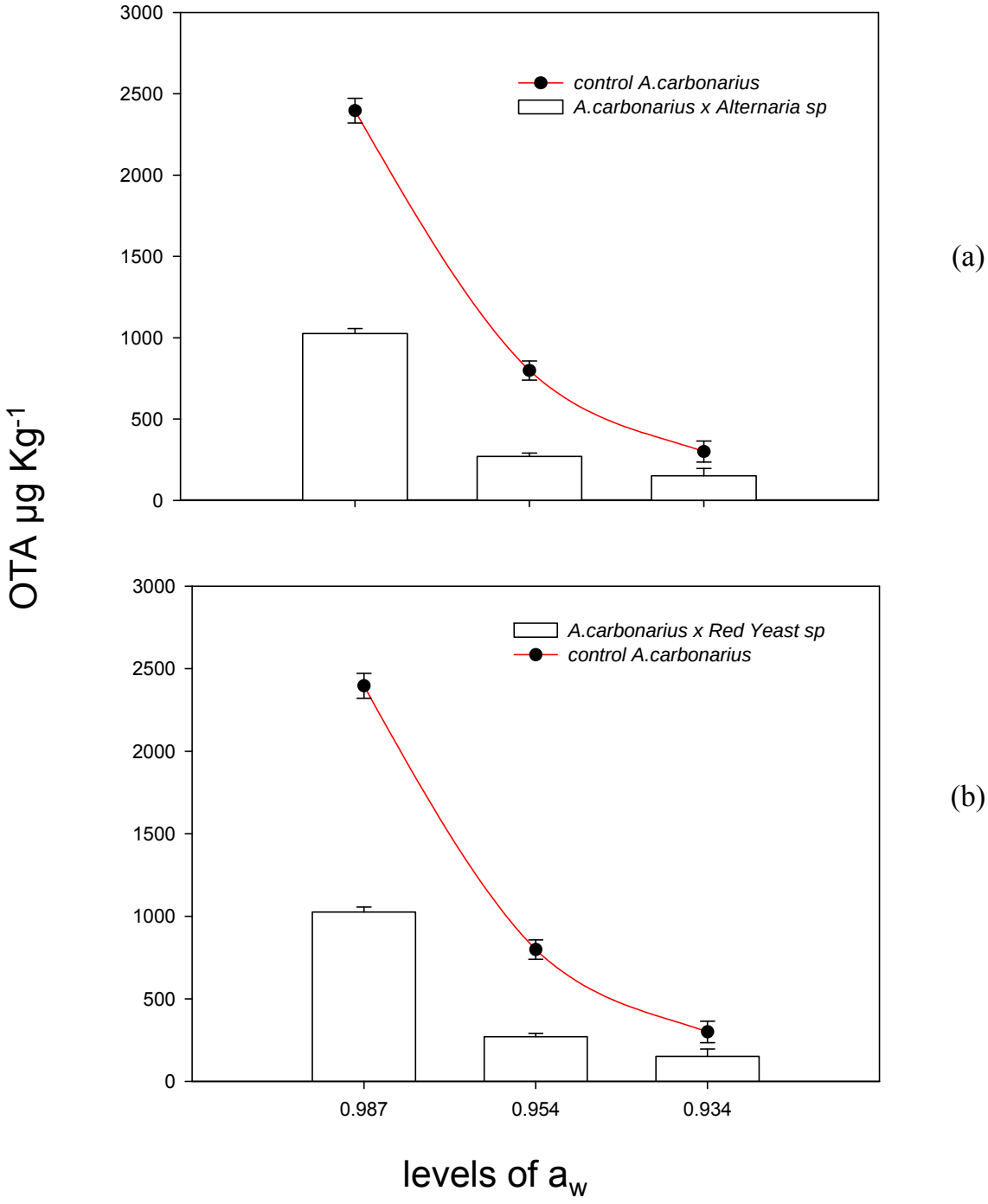


Figure 4.27. Effect of fungal interaction (*A. carbonarius* vs *Alternaria spp* (a), *A. carbonarius vs* red yeasts (b)*)* x a_w on OTA accumulation, on WHJM, after 10 days, at 25° C. Bars indicate standard error of mean.

4.3.4.3. Effect of fungal interaction x fungal spore concentration on ochratoxin A production by *A. carbonarius* isolates in sultanas.

In this study, four fungal genera were employed; an isolate of *A. carbonarius* (GRE0105), an isolate of *A.niger* aggregate, (GRE0119 strain), an isolate of *Penicillium* sp. (GRE0657) and one red yeast (GRE0689). Three different final concentrations of spore suspensions were prepared for each fungus. Both *A. niger* aggregate and *Penicillium* sp isolates were used in concentrations of approximately 10^2, 10^4 and 10^6 spores mL^{-1} while the red yeast isolate was used at spore concentrations of 10^3, 10^5 and 10^7 spores mL^{-1}. Inocula of *A. carbonarius* were modified to 10^2 spores mL^{-1}. Industrially processed sultanas with both heat and SO_2 treatment were used as substrate.

Figure 4.28 shows that at drier conditions (0.934 a_w) OTA accumulation, produced by *A. carbonarius* (GRE105), was strongly inhibited by *A. niger* aggregate with higher spore concentrations. Hence, at low spore concentration (10^2 spores mL^{-1}) of *A. niger* aggregate, toxin was produced in about 40 μg g^{-1}, while at higher concentrations (10^4 and 10^6 spores mL^{-1}) of *A. niger* group, OTA was detected in about 9 and 3 μg g^{-1}, respectively.

In contrast, when *A. carbonarius* interacted with the other two fungal species tested (*Penicillium sp*. and the red yeast), the opposite phenomenon was observed. There was a tendency for OTA production to slightly increase when higher concentrations of spores of the competitor fungi were applied. At the highest spore concentrations (10^6 spores mL^{-1}) of the *Penicillium* sp., OTA was produced in about of 12.5 μg g^{-1}. At the lower spore suspensions (10^2 and 10^4 spores mL^{-1}), OTA was detected at about 3.4 μg

g^{-1}. Interactions with the red yeast showed a similar pattern. At the lower spore concentrations (10^3 and 10^5 spores mL^{-1}), OTA was detected in about 1.6 – 1.8 µg g^{-1}. The highest OTA accumulation (3.3 µg g^{-1}) was been achieved when the highest cell concentration of the yeast (10^7 spores mL^{-1}) was applied.

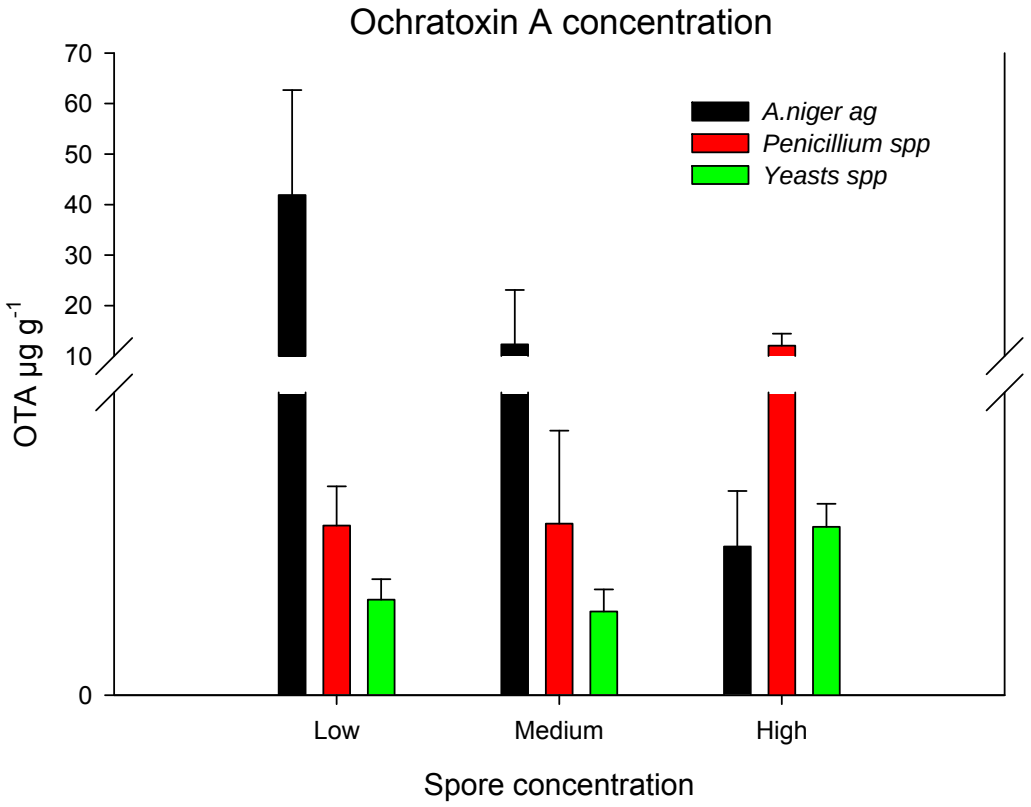


Figure 4.28. Effect of fungal interaction x fungal spore concentration on OTA production by *A. carbonarius,* on sultanas exposed to heat and SO_2 treatment, after 7 days incubation at 25° C. Key-words; Low: low spore concentration, 10^2 spores mL^{-1} for *A. niger* ag. and *Penicillium sp* , 10^3 spores mL^{-1} for red yeast; Medium: medium spore concentration, 10^4 spores $mL^{-1}$for *A. niger* ag. and *Penicillium* sp , 10^5 spores mL^{-1} for red yeast; High: high spore concentration, 10^6 spores mL^{-1} for *A. niger* ag. and *Penicillium* sp, 10^7 spores mL^{-1} for red yeast. Bars indicate standard error of mean.

Statistical analysis

Statistical analysis showed that fungal species as well as their interaction with fungal spore concentrations could significantly affect OTA production by *A. carbonarius* (10^2 spores mL^{-1}), when sultanas are used as substrate, after 7 days incubation, at 25° C. In contrast, the examined fungal spore concentrations (10^2 – 10^7 spores mL^{-1}) alone may not be adequate to impact on OTA accumulation, under the above experimental conditions (Table 4.32). The presence of both *A.niger* aggregate and *Penicillium* sp, resulted in stimulation of OTA biosynthesis by *A. carbonarius*, in relation to OTA production, when black aspergilli was intermingling with red yeast sp. (Table 4.33).

Table 4.32. Summary statistical table of significance (Anova) shown the effects of fungal species, fungal spore concentrations and their interaction on OTA production by *A. carbonarius* (10^2 spores mL^{-1}), on sultanas.

OTA log_{10}	df	F	Sig.
Fungal species	2	11.515	0.001**
Spore concentration	2	2.322	0.127
fungi * spores	4	8.480	0.000**

(**) significant at P<0.01

Table 4.33. Homogenous subsets of OTA produced by *A. carbonarius* (10^2 spores mL^{-1}), affected by fungal species, on sultanas, after 7 days of incubation, at 25° C.

OTA log10	Fungal species	N	Subset	
			1	2
Tukey HSD(a,b)	Yeasts spp	9	0.3169	
	Penicillium spp	9	0.6600	0.6600
	A.niger ag	9		1.0289

Significance level = 0.01.

4.4. DISCUSSION

4.4.1. POTENTIAL OF A_W AND NaMBS TO CONTROL GERMINATION, GERM TUBE EXTENSION, FUNGAL GROWTH AND OTA PRODUCTION BY *A. CARBONARIUS,* ON A GRAPE-LIKE MEDIUM (WGJM)

The present study examined the effect of Sodium Metabisulfite (NaMBS) x a_w on fungal spore germination, germ tube extension, fungal growth and OTA production by two isolates of *A. carbonarius.* This is critical in defining the capacity of contaminant spores to become established, and subsequently grow and produce OTA, on dried vine fruits which have undertaken SO_2 treatment. Results show that spore germination occurred over a wide range of sodium metabisulphite concentrations (0-500 mg L^{-1}) and it was very rapid at ≥0.965 a_w (6 hours, control sample). At ≥750 mg L^{-1} concentrations of NaMBS, no spore germination, fungal growth or OTA production was observed, regardless of a_w. Medium level of NaMBS concentrations (100-500 mg L^{-1}) acted more like a short-time (≤12 hours) stimulator than an inhibitor agent. However, the NaMBS concentration, when stimulation of OTA occurs, differs according to the fungal strain.

Previously, Magan (1993a) examined the effect of different concentrations of SO_2 in relation to different temperatures (15° and 25° C) and a_w levels *in vitro* and *in situ* on grain for control of *Penicillium* spp and *Aspergillus* spp. The growth of *Aspergillus* species (*Aspergillus flavus*, *A. ochraceus* and *Aspergillus terreus*) was inhibited by 50 mg L^{-1} dissolved SO_2 on a Malt extract-based medium, at 0.995 a_w and 0.95 a_w. Some *Penicillium* species and *Aspergillus niger* aggregate were tolerant of up to 250 mg L^{-1}. By contrast, growth of *Penicillium* spp. was stimulated by 100 mg L^{-1}.

Moreover, phyllosphere fungi such as *Cladosporium herbarum* and *Epicoccum nigrum* were tolerant of up to 200 mg L^{-1} while *Aureobasidium pullulans* and *B. cinerea* were inhibited by this level. Overall, at 0.96 and 0.92 a_w up to 2,000 mg L^{-1} was needed to obtain a 1–2 Log_{10} decrease in populations of spoilage fungi, on cereal grain.

Studies on onions showed *A. niger* aggregate growth, even after exposure to 0.3% SO_2 for 48 hours (Thamizharasi *et al.*, 1992). Generally, previous studies suggest that the threshold SO_2 concentration may vary considerably between fungi. These differences could be due to the fact that several factors influence the efficacy of SO_2. 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 and Segel, 1970). Furthermore, King *et al.* (1981) showed that SO_2 binding substances enabled yeasts to be tolerant to higher concentrations of SO_2. In the present study it is possible that a percentage of SO_2 was absorbed and bound to the glucose, including into 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 of SO_2 products in water varies with different pH levels (Babich and Stotzky, 1974). The significant point is that the toxicity of these products also differs, with the 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. It is important to point out that the total amount of NaMBS added was different from the final amount of SO_2 generated into

the substrate (100 g of NaMBS releases about 67.4 g SO_2). 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.

4.4.2. POTENTIAL OF CONTROLLED ATMOSPHERES TO CONTROL FUNGAL GROWTH AND OTA PRODUCTION BY STRAINS OF *A. CARBONARIUS* ON GRAPE-LIKE JUICE MEDIUM AND IN SULTANAS

The current study demonstrated that only a combination of 50% CO_2 with the lowest water availability examined (0.93 a_w) was capable of delaying germination of spores, up to 48 hours, at 25° C. A 0.965 a_w x 25% CO_2, combination stimulated spore germination. Spore germination of *A. niger* aggregate was totally inhibited when 100% CO_2 was applied, at 0.88-0.92 a_w (El Halouat *et al.*, 1997b). However, as the level of a_w decreased, germ tube extension was similarly inhibited. Moreover, 25% CO_2 concentration resulted in about 70% mycelial extension inhibition, at 0.985 a_w, the first days of incubation. This comes in aggreement with previous studies where elevated CO_2 concentrations (≥ 40%) could efficiently prevent *A. niger* aggregate growth on high moisture prunes and raisins (El Halouat *et. al.,* 1997). By contrast, OTA production was not significantly affected by elevated concentrations of carbon dioxide. It was clear that the effect of a_w on OTA production was stronger than the effect of controlled atmospheres.

This severe impact of low water availability level combined with higher concentrations of CO_2, on fungal spore germination, germ tubes extension and potential inhibition of growth rate of *A. carbonarius*, which was observed on grape-

juice like medium experiments, was carried on experiments with sultanas as substrate. However, CO_2 concentration levels examined did not significantly affect OTA production by *A. carbonarius,* in real-sample conditions. This is in agreement with findings obtained from grape-like juice medium experiments of the current thesis.

Brown (1922) suggested that the concentration of CO_2 and O_2 required to inhibit spore germination can be 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 the presence of some nutrient. Furthermore, 10-20% CO_2 was necessary for inhibition of spore germination. It has been reported that the percentage of O_2 could influence spore germination. Thus, 1% O_2 or less resulted in a total inhibition of spore germination, growth and sporulation of many fungi (El Goorani, 1981; Ellis *et al.*, 1994; El Halouat *et al.,* 1997). Recently, Cairns-Fuller *et al.* (2005) demonstrated 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. Many workers have mentioned the major role of a_w level to OTA production by *A. carbonarius.* A a_w level of 0.93 significantly reduced OTA production, regardless of CO_2 concentration. There are a number of studies in the literature that point to the crucial role of a_w in influencing mycotoxin production (Sanchis and Magan, 2004*;* Belli *et al.,* 2004; Mitchell *et al.*, 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 1,000 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 fruit 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 and 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% CO2/5% O_2 treatment resulting in only trace amounts. Production of zearalenone by *Fusarium 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_2 and 3% O_2. Moreover, *Alternaria 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 and Uota, 1970; Magan and Lacey, 1984). 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.

Previous studies have observed stimulation in terms of growth. For example, Stotzky and Goos (1965) showed that the *Fusarium 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 effective control regimes.

4.4.3. EFFECTS OF INTERACTION BETWEEN FUNGI ON *A. CARBONARIUS* GROWTH AND OTA ACCUMULATION ON GRAPE-LIKE JUICE MEDIUM AND ON SULTANAS

Fungi usually occur as a mixed consortium of several yeasts, filamentous fungi, inspects or bacteria and therefore inter-specific and intra-specific interactions are taking place depending on the prevailing environmental conditions or nutritional status of the habitat. Subsequent interaction between spoilage fungi result in combat, antagonism and niche overlap which all influence secondary resource capture (Magan *et al.*, 2003; Bennett *et al.*, 2003; Magan and Aldred, 2007).

In this experiment, the interactions between *A. carbonarius* against other common vine fungal genera/species grown on WGJM, were investigated. In almost all examined pairs, *A. carbonarius* was the dominant fungi on contact (4/0) or at distance (5/0) (Index of Dominance of Magan and Lacey, 1984). This is in aggrenment with previous findings (Mitchell, 2005). *A. carbonarius* was mutual antagonistic on contact (2/2), at higher (≥0.95) a_w levels, when it was paired with *A. niger* aggregate. But this changed when a_w level was decreased to 0.93 a_w, where *A.niger* aggregate was more competitive, since it is more tolerant to the specific environmental conditions (Esteban *et al*., 2006). Overall, *A. niger aggregate* isolates present higher growth rates and shorter lag phase for growth in comparison to *A. carbonarius*, at several levels of a_w (0.90-0.99) (Belli *et. al.,* 2004). Valero *et al.* (2007) demonstated that growth rate of *A. niger* aggregate isolates were more affected by fungal interactions while *A. carbonarius* growth was less influenced and sometimes stimulated. Findings from the same study also pointed out *A. alternata*, *P. decumbens* and *T. harziamum* as inhibitors of *A. carbonarius* under 0.97 a_w x 30^o C conditions (Valero *et al.*, 2007). Our findings showed a dominance of contact of *A. carbonarius* over *Alternaria* sp. and *Penicillium* spp isolates studied, at 25^o C, regardless of a_w level. Moreover, *Rhizopus* sp. was dominant over *A. carbonarius* on contact (0/4), at ≥ 0.954 a_w.

In the present study and in terms of OTA production the results showed it was strongly inhibited (55%-98%), at 0.985 a_w x 25^o C, in comparison to control samples. A considerable reduction in OTA concentration was observed as the level of water availability (a_w) was also decreased. However, at higher levels of water availability (0.985 a_w), a stronger inhibition in ochratoxin A production, by *A. carbonarius* occurred, under the specific experimental conditions. Previously, Mitchell (2005)

found that OTA production was stimulated at 20° C x 0.99 a_w while at lower a_w levels (0.95 a_w), OTA biosynthesis was suppressed by fungal interactions with almost all species examined. Furthermore, Valero *et al.* (2007) demonstrated that OTA production was reduced at 30° C x 0.97 a_w and it was almost negligible at drier conditions (0.92 a_w) when *A. carbonarius* was grown in paired cultures. This is in agreement with our findinds.

For the first time, the effect of interactions between fungi on OTA accumulation, in relation to several spore concentrations, was examined in real-samples conditions (sultanas). Findings showed that the fungal species could significantly affect OTA production after 7 days incubation at 25° C. Studies on SNM (Valero *et al.*, 2006) show a significantly higher OTA production when *A. carbonarius* was grown with and *E. amstelodami*. Furthermore, fungi can stimulate both OTA production and *A. carbonarius* growth (i.e. *Candida* sp) under specific conditions or could negatively affect one of the above parameters (i.e. *T.harzianum* reduced OTA production but stimulated fungal growth when it grew paired with *A. carbonariu*s). An effort to compare interactions between groups of field fungi with their Indices of Dominance (Magan & Lacey, 1984b) was taken place by Magan and Lacey (1985). A copacetic correlation between the Dominant Indices with interactions on sultanas was observerd in some fungi (i.e. *F. culmorum*) but in some other cases, this correlation was very low (i.e. *A. alternata*). The above demostrates that, under field conditions fungal activity, and concequently fungal interactions, may be different from *in vitro* studies.

4.4.4. CONCLUSIONS OF THE CURRENT SURVEYS

1. Spore germination occurred over a wide range of sodium metabisulphite concentrations (0-500 mg L^{-1}) and it was very rapid at ≥0.965 a_w.
2. At ≥750 mg L^{-1} concentrations of NaMBS, no spore germination was observed while mycelial growth was completely inhibited.
3. Medium concentrations of NaMBS (≤250 m gL^{-1}) optimized spore germination or fungal growth.
4. Ochratoxin A production was inhibited by up to 750 mg L^{-1}, depended on levels of water activity.
5. Medium concentrations of NaMBS (100-500 m gL^{-1}) stimulated OTA production, at ≥0.965 a_w, depending on the fungal strain.
6. A combination of 50% CO_2 with the lowest water availability examined (0.93 a_w) was capable of delaying germination of spores.
7. At 0.965 a_w x 25% CO_2, spore germination stimulation was evident.
8. As the level of a_w was decreased, germ tubes extension were similarly decreased.
9. A 25% CO_2 concentration resulted in about 70% mycelial extension inhibition, at 0.985 a_w.
10. OTA production was not significantly affected by elevated concentrations of carbon dioxide.
11. The effect of a_w on OTA production was stronger than the effect of controlled atmospheres.

12. The severe impact of low water availability level, combined with higher concentrations of CO_2, was carried on sultanas.

13. *A. carbonarius* was the dominant fungi on contact (4/0) in most cases examined, when paired with other fungal species.

14. *A. carbonarius* was mutually antagonistic on contact (2/2), at higher (≥0.95) a_w levels, with *A. niger* aggregate. But this changed when a_w level was decreased to 0.93 a_w, where *A.niger* aggregate was more competitive.

15. Moreover, *Rhizopus* sp. was dominant over *A. carbonarius* on contact (0/4), at ≥ 0.954 a_w.

16. OTA production by *A. carbonariu*s was strongly inhibited (55%-98%), at 0.985 a_w x 25^o C under conditions of fungal interaction.

17. A considerable reduction in OTA concentration was observed as the level of water availability (a_w) was also decreased.

18. On sultanas, OTA production by *A. carbonarius* potential was significantly influenced by the kind of the fungal competitor.

Chapter Five

SUMMARY OF CONCLUSIONS OF THE THESIS AND FUTURE CONSIDERATIONS

This chapter presents a bullet point list of conclusions of the present thesis and suggests some future considerations.

The main motivation for the current study was the detection of ochratoxin A (OTA) in grapes and their derivatives, as well as the interest of European Union for the effective control of OTA contamination in the early stages of the production and therefore, set up Critical Control Points (CCP) during the production process.

The Aim of the study was to investigate the ecology of the ochratoxigenic fungus *A. carbonarius* in relation to ochratoxin A (OTA) production and search for potential control approaches for minimal OTA contamination in vine fruits (Cretan sultanas), before harvest time (in the field), after harvesting and in the production chain. An effort of potential Critical Control Points to be idendified was taken place.

5.1. SUMMARY OF THE OUTCOMES OF THIS PROJECT

(a) In the field and after harvesting, during sun-drying process:

1. *Aspergillus niger* aggregate was the most predominant fungal species, followed by yeasts, and *Penicillium* species in pre-harvest samples. No *P. verrucosum* was isolated. Very low populations of *A. carbonarius* were isolated.

2. Grapes touching the soil were more prone to contamination by black aspergilli while grapes exposed to the direct sunshine had lower population dynamics.

3. Populations of *A. niger* aggregate gradually decreased as the altitude increased.

4. *A. carbonarius* was most frequently isolated from samples originating from intermediate altitudes (*ca* 200-500 m). Less *A. carbonarius* was isolated at sea level, at pre-harvest studies.

5. As *A. niger* aggregate populations increased, fungal dynamics of *A. carbonarius* was more restricted.

6. OTA accumulation appeared to be influenced by bunch position and not by the altitude. Bunches touching the soil had lower ochratoxin A contamination.

7. Water loss from sultanas was very rapid during the first five days of sun-drying.

8. On grapes, fungal richness was high (at least 7 identified genera), but during drying this significantly decreased (3 genera, at 10^{th} day of drying).

9. Fungal populations of black aspergilli (*A. niger* aggregate and *A. carbonarius*) increased over time. *A. niger* aggregate was the dominant fungal species during drying.

10. *A. carbonarius* was more frequently isolated at higher altitudes, at post-harvest studies. In contrast, *A. niger* aggregate was more commonly isolated at sea level.

11. Fungal diversity decreased when a_w level was more restricted (0.90 a_w). More fungal richness was observed at sea level. However, black aspergilli (*A.niger* group) were more frequently isolated at higher altitudes.

12. Ochratoxin A was detected in very low concentrations. It was not significantly affected by the altitude and time of drying.

(b) During industrial processing of sultanas:

1. The fungal populations of untreated sultanas were mainly composed of black aspergilli (dominant fungi), *Penicillium,* yeasts and *Rhizopus* species.

2. *A. carbonarius* was isolated in low levels (1.5-2 Log_{10} CFUs gr^{-1}) and frequently isolated in about 20% of untreated sultanas, regardless of the type of raisins. After heat treatment, *A. carbonarius* populations were almost eliminated.

3. *A. niger* aggregate were the predominant fungal species among black aspergilli, regardless of type of sultanas. They were isolated at about of 5.5 Log_{10} CFUs gr^{-1} and frequently isolated from up to 45% in untreated sultanas. After heat treatment, these fungi were detected in about 45-85%, in both type of sultanas and on both a_w isolation media but were not eliminated.

4. Types of sultanas (No22 and No2) significantly influenced the black aspergilli population dynamics. In general, higher contamination levels were found in No22 sultanas.

5. Heat treatment (up to 90^{o} C) was the key-procedure for the elimination of fungal population loads. Several parameters, such as duration of the treatment, might improve the efficiency of the method.

6. SO_2 treatment using specific considerations (saturated solutions of sulphur dioxide) does not seem to statistically alter the fungal population loads, and more specifically affect black aspergilli populations. However, interactions with several abiotic factors (i.e. formulation of SO_2, duration, pH of the sulphate solution, temperature, water availability should be taken into consideration to optimize the conditions of the aforementioned process.

7. The levels of ochratoxin A contamination are not statistically different in untreated and in final industrially processed sultanas, regardless of the type of the raisins (No22 and No2 sultanas) and the method used (with and without SO_2) in these two examined seasons.

8. It is important that the purity of the rinsing water is ensured to prevent cross-contamination.

(c) OTA contamination in relation to *A. carbonarius* ecology

1. Spore germination occurred over a wide range of sodium metabisulphite concentrations (0-500 mg L^{-1}) and was very rapid at ≥0.965 $a_{w.}$

2. At ≥750 mg L^{-1} concentrations of NaMBS, no spore germination was observed while mycelial growth was completely inhibited.

3. Medium concentrations of NaMBS (≤250 mg L^{-1}) facilitated rapid spore germination or fungal growth.

4. Ochratoxin A production was inhibited by up to 750 mg L^{-1}, depending on the levels of a_w.

5. Medium concentrations of NaMBS (100-500 mg L^{-1}) stimulated OTA production, at ≥0.965 a_w, depending on the fungal strain used.

6. A combination of 50% CO_2 with the lowest water availability examined (0.93 a_w) was capable of delaying germination of spores.

7. At 0.965 a_w x 25% CO_2, stimulation of spore germination was evident.

8. As the level of a_w was decreased, germ tube extension were similarly decreased.

9. A 25% CO_2 concentration resulted in about 70% inhibition of mycelial extension at 0.985 a_w.

10. OTA production was not significantly affected by elevated concentrations of carbon dioxide.

11. The a_w had a greater effect on OTA production than the effect of controlled atmospheres (up to 50% CO_2).

12. The efficacy of low water availability in combination with up to 50% CO_2 in sultanas inoculated with *A. carbonarius* confirmed the *in vitro* results.

13. *A. carbonarius* was the dominant fungus on contact (4/0) in most examined cases, when interacting with other sultana mycofloral species.

14. *A. carbonarius* was mutually antagonistic on contact (2/2) at higher (≥0.95) a_w levels, when interacting with *A. niger* aggregate. However, this changed when the a_w level decreased to 0.93 a_w, where *A. niger* aggregate was more competitive.

15. OTA production by *A. carbonarius* was strongly inhibited (55%-98%), at 0.985 a_w x 25° C, under conditions of fungal interaction.

16. A considerable reduction in OTA concentration was observed as the level of water availability (a_w) was decreased.

17. In sultanas, potential OTA production by *A. carbonarius* was significantly influenced by the kind of fungal competitor.

5.2. FURTHER CONSIDERATIONS

☞ Field sampling and fungal isolate identification from grapes and dried vine fruits should be carried on for more consecutive years in conjunction with climatic data in the specific Cretan ecosystem. Moreover, information relative to farming methods should also be taken into account. The above will lead to the establishment of a monitoring system able to confirm the potential risk of *A. carbonarius* contamination and/or OTA presence.

☞ Molecular approaches for tracking *A. carbonarius* in the field and during production of vine fruits also needs to be employed for definitive and accurate information on OTA production kinetics.

- ☞ Furthermore, investigation of the effect of chemical and non-chemical means of inhibiting fungal growth and OTA production would provide useful data for an effective control strategy of OTA contamination, during the production process.

- ☞ Perhaps data based on the correlation of spore concentrations and population dynamics of black aspergilli and/with other vine fungal and bacterial species, could be a useful "tool" for OTA prevention, in early stages of vine fruits production.

- ☞ It would also be interesting to compare berries of sultanas which are visually and non-visually contaminated by fungal species and assessment of potential OTA accumulation in them.

- ☞ A more detailed investigation on the effect of CO_2 x SO_2 x a_w on black aspergilli growth and OTA production is needed.

PUBLICATIONS

Refereed papers:

- 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. (2007). Influence of sulphur dioxide, controlled atmospheres and water availability on *in vitro* germination, growth and ochratoxin A production by strains of *Aspergillus carbonarius* isolated from grapes. *Postharvest Biology and Technology*, **44**, 141-149.

Oral presentations:

- M. Pateraki, A. Dekanea, N. Magan, D. Lydakis, 2005. Ecological studies of ochratoxigenic fungus *Aspergillus carbonarius,* isolated from dried vine fruits (Sultanina). 22nd National Conference of Greek Society for Agricultural Science (EEEO), Patra, Greece.

- Marianthi Pateraki, D. Lydakis and N. Magan, 2007. Population dynamics of *A. carbonarius* and ochratoxin A during drying of vine fruits in Crete. XII International IUPAC Symposium on Mycotoxins and Phycotoxins, Istanbul, Turkey.

- Pateraki M., Magan N., Fisarakis J., Lydakis D., 2007. Investigating the influence of the Cretan industrial process of sultanas on population's dynamic of

black aspergilli and OTA production. 23rd National Conference of Greek Society for Agricultural Science (EEEO), Chania, Greece.

Poster presentations:

- 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, 20–21 October, 2005, Italy.

- 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, Italy.

- A. Dekanea, M. Pateraki, D. Lydakis, N. Magan. Mycoflora population's dynamics and OTA contamination during drying on vine fruits. Ochratoxin A in grapes and wine: Prevention and control. International workship, 20-21, October, Marsala-Sicily, Italy.

- Pateraki M., Dekanea A., Lydakis D., Magan N., 2005. Mycoflora dynamics in drying currants and sultanas and effects on ochratoxin production. Ochratoxin A in grapes and wine: Prevention and control. International workship, 20-21, October, Marsala-Sicily, Italy.

- A. Dekanea, M. Pateraki, N. Magan, D. Lydakis, 2005. Studies on changes in fungal populations of *Aspergillus carbonarius* and *A. niger* aggregate, at pre

harvest and during sun-drying of Corinth raisins and detection of OTA presence. 22nd National Conference of Greek Society for Agricultural Science (EEEO) Patra, Greece.

- Pateraki M., Lydakis D., Fysarakis J., Magan N., 2006. Black aspergilli population dynamics and ochratoxin A accumulation in fresh grapes (Sultanina) and dried vine fruits (sultana) originated from Cretan ecosystems. 13rd National Conference of Phytopathology, Athens, Greece.

- M. Pateraki, D. Lydakis, N. Magan, 2007. Effect of industrial processing of sultanas on *Aspergillus carbonarius* and ochratoxin contamination. XII International IUPAC Symposium on Mycotoxins and Phycotoxins, Istanbul, Turkey.

REFERENCES

Abarca M.L., Accensi F., Bragulat M.R., Cabanes F.J., 2001. Current importance of ochratoxin A-producing *Aspergillus spp.* Journal of Food Protection 64 (6): 903-906.

Abarca M.L., Accensi F., Bragulat M.R., Cabanes F.J., 2004. Taxonomy and significance of black aspergilli. Antonie van Leeuwnhoek 86 (1): 33-49.

Abarca M.L., Accensi F., Bragulat M.R., Castella G., Cabanes F.J., 2003. *Aspergillus carbonarius* as the main source of ochratoxin A contamination in dried vine fruits form the Spanish market. Journal of Food Protection 66: 504-506.

Abarca M.L., Bragulat M.R., Castella G., Cabanes F.J., 1994. Ochratoxin A production by strains of *Aspergillus niger* var. *niger.* Applied and Environmental Microbiology 60 (7): 2650-2652.

Aboul-Enein H., Kutluk O.B., Altriokka G., Tuncel M., 2002. A modified HPLC method for the determination of ochratoxin A by fluorescence detection. Biomedical Chromatography 16 (7): 470-474.

Abrunhosa L. and Venancio A., 2007. Isolation and purification of an enzyme hydrolyzing ochratoxin A from *Aspergillus niger*. Biotechnology Letter 29 (12): 1909-1914.

Abrunhosa L., Paterson R.R.M., Kozakiewicz Z., Lima N., Venancio A., 2001. Mycotoxin production from fungi isolated from grapes. Letters in Applied Microbiology 32 (4): 240-242.

Abrunhosa L., Serra R. and Venancio A., 2002. Biodegradation of ochratoxin A by fungi isolated from grapes. Journal of Agricultural and Food Chemistry 50 (25): 7493-7496.

Accensi F., Abarca M.L. Cano J., Figuera L., Cabanes F.J., 2001. Distribution of ochratoxin A producing strains in the *A. niger* aggregate. Antonie van Leeuwenhoek 79 (3-4): 365-370.

Accensi F., Abarca M.L., Cabanes F.J., 2004. Occurrence of *Aspergillus* species in mixed feeds and component raw materials and their ability to produce ochratoxin A. Food Microbiology 21 (5): 623-627.

Ahmad R., Magan N., 2003. Monsooned coffee: impact of environmental factors on fungal community structure, enzyme production profiles and ochratoxin. In: Mycotoxins in food production systems (Bryson R.J., Kennedy R., Magan N., Scudarnore KA., eds), Association of applied Biologists, Warwick, United Kingdom. Annals of Applied Biology 68: 161-168.

Al-Musallam A., 1980. Revision of the Black *Aspergillus* species. Phd Thesis, Ultrecht University, The Netherlands.

AOAC International, 1995. Official Methods of Analysis, 15th edn. Edited by K. Helrich (Gaithersburg: AOAC International) 976.22, 975.35.

Arroyo M., Aldred D., Magan N., 2005. Environmental factors and weak organic acid interactions have differential effects on control of growth and ochratoxin A production by *Penicillium verrucosum* isolates in bread. International Journal of Food Microbiology 98 (3): 223-231.

Australia and New Zealand Food Authority, 1996. Food Standards Code, incorporating amendments up to and including Amendment 32, December.

Ayest G., 1969. The effects of moisture and temperature on growth and spore germination in some fungi. Journal of Stored Products Research 5 (2): 127-141.

Babich H. and Stotzky G., 1974. Gaseous and heavy metal pollutants. In: Experimental Microbial Ecology, (Burns, R.G. and Slater, J.H., eds.), Blackwell, Oxford: 631-638.

Bae S., Fleet G.H., Heard G.M., 2004. Occurrence and significance of *Basillus thuringiensis* on wine grapes. International Journal of Food Microbiology 94 (3): 301-312.

Baker J.L., Doster M.A., Michailides T.J., Mahoney N., 2002. Ochratoxin Production by the *Aspergillus ochraceus* group and *Aspergillus alliaceus.* Applied and Environmental Microbiology 68 (5): 2326-2329.

Batillani P., Giorni P., Bertuzzi T., Formenti S., Pietri A., 2006. Black *Aspergilli* and ochratoxin A in grapes in Italy. International Journal of Food Microbiology 111 (1): 55-560.

Battilani P., Giorni A., Giorni P., Bertuzzi T., Barbano C., 2003. Growth and ochratoxin A of *Aspergillus* section *Nigri* isolates from Italian grapes. Aspects of Applied Biology 68: 175-180.

Battilani P., Giorni P., Pietri A., 2003a. Epidemiology of toxin-producing fungi and ochratoxin A occurrence in grape. European Journal of Plant Pathology 109 (7): 715-722.

Battilani P., Pietri A., 2002. Ochratoxin A in grapes and wine. European Journal of Plant Pathology 108 (7): 639-643.

Battilani P., Pietri A., Bertuzzi T., Languasco L., Giorni P., Kozakiewicz Z., 2003. Occurrence of ochratoxin A-producing fungi in grapes grown in Italy. Journal Food Protection 66 (4): 633-6.

Battiliani P., Logrieco A., Giorni P., Cozzi G., Bertuzzi T., Pietri A., 2004. Ochratoxin A production by *Aspergillus carbonarius* on some grape varieties grown in Italy. Journal of the Science of Food and Agriculture 84 (13): 1736-1740.

Bau M., Bragulat M.R., Abarca M.L., Minguez S., Cabanes F.J., 2005. Ochratoxigenic species from Spanish wine grapes. International Journal of Food Microbiology 98 (2): 125-130.

Bauer J., Gedek M., 1986. Incidence of ochratoxin A in blood serum and kidneys of man and animals. Proceeding of 2nd World Congress, Foodborne Infections and Intoxications : 907-910.

Bejaoui H., Mathieu F., Taillandier P., Lebrihi A., 2004. Ochratoxin A removal in synthetic and natural grape juice by selected oenological *Sacharomyces* strains. Journal of Applied Microbiology 97 (5): 1038-044.

Bejaoui H., Mathieu F., Taillandier P., Lebrihi A., 2005. Conidia of Black Aspergilli as new biological adsorbents for Ochratoxin A in grape juices and musts. Journal of Agricultural and Food Chemistry 53 (21): 824-8229.

Bejaoui H., Mathieu F., Taillandier P., Lebrihi A., 2006. Biodegradation of ochratoxin A by Aspergillus section Nigri species isolated from French grapes: a potential means of ochratoxin A decontamination in grape juices and musts. FEMS Microbiological Letter 225 (2): 203-208.

Belli N., Marin S., Duaigues A., Ramos A.J., Sanchis V., 2003. Ochratoxin A in wines, musts and grape juices form Spain. Journal of the Science of Food and Agriculture 84 (6): 591-594.

Belli N., Marin S., Sanchis V., Ramos A.J., 2004b. Influence of water activity and temperature of growth of *Aspergillus* section Nigri strains isolated from grapes. International Journal of Food Microbiology 96 (1): 19-27.

Belli N., Mitchell D., Marin S., Alegre I., Ramos A.J., Magan N., 2005. Ochratoxin A-producing fungi in Spanish wine grapes and their relationship with meteorological conditions. European Journal of Plant Pathology 113 (3): 233-239.

Belli N., Ramos A.J., Coronas I., Sanchis V., Marin S., 2005. *Aspergillus carbonarius* growth and ochratoxin A production on a synthetic grape medium in relation to environmental factors. Journal of Applied Microbiology 98 (4): 839-844.

Belli N., Ramos A.J., Sanchis V., Marin S., 2004c. Incubation time and water activity effects on ochratoxin A production by Aspergillus section Nigri strains isolated from grapes. Letters in Applied Microbiology 38 (1): 72-77.

Belli N., Ramos, Corronas, Sanchis and Marin S., 2005. *Aspergillus carbonarius* growth and ochratoxin A production on a synthetic grape medium in relation to environmental factors. Journal of Applied Microbiology 98 (4): 839-844 (6).

Bennett. W., Klich M., 2003. Mycotoxins. Journal of Clinical Microbiology. Clinical Microbiology Reviews 16: 497-516.

Beuchat L.R., 1984. Survival of *Aspergillus flavus* conidiospores and other fungi on cowpeas during long-term storage under various environmental conditions. Journal of Stored Products Research 20 (3): 119-123.

Beuchat L.R., Hocking A.D., 1990. Some consideration when analyzing foods for the presence of xerophilic fungi. Journal of Food Protection 53: 984-989.

Bigelis R., Lasure L.L., 1987. Fungal enzymes and primary metabolites used in food processing. In: Food and Beverage Mycology – 2nd ed., (Beuchat L.R., ed.), Von Nostrand Reinhold, New York: 472-516.

Birzele B., Prange A., Kramer J., 2000. Deoxynivalenol and ochratoxin A in German wheat and changes of level in relation to storage parameters. Food Additives and Contaminants 17 (12): 1027-1035.

Blandamer M. J., Engberts J. B. F. N., Gleeson P. T. and Reis J. C. R., 2005. Activity of water in aqueous systems; A frequently neglected property. Chemical Society Reviews 34 (5): 440-458.

Blank G., Nwoko U., Frohlich A., Marquardt R., 1998. Ochratoxin A production in relation to the growth morphology of *Aspergillus alutaceus*. Lebensmittel Wissenschaft und Technologie 31 (3): 210-214.

Boddy L., Frankland J. C., Dursun S., Newsham K.K., Ineson P. (1996) Effects of dry-deposited SO_2 and sulphite on saprotrophic fungi and decomposition of tree leaf litter. In: Fungi and Environmental Change, (Frankland J.C., Magan N, Gadd G, eds), Cambridge University Press. Cambridge, UK.

Böhs B., Seidel V., Lindner W., 1995. Analysis of selected mycotoxins by capillary electrophoresis. Chromatographia 41 (11-12): 631-637.

Boudra H., Bars P. L., Bars J.L., 1995. Thermostability of ochratoxin A in wheat under two moisture conditions. Applied and Environmental Microbiology 61 (3): 1156-1158.

Breihtoltzemanuelssson A., Olsen M., Oskarsson A., Palminger I., Hult K., 1993. Journal of Food Protection 76: 842-846.

Brown W., 1992. On the germination and growth of fungi at various temperatures and in various concentrations of oxygen and of carbon dioxide. Annals of Botany 36 (2): 257-283.

Bucheli P., Taniwaki M.H., 2002. Research on the origin, and on the impact of post-harvest handling and manufacturing on the presence of ochratoxin A in coffee. Food Additives and Contaminants 19 (7): 655-665.

Burdaspal P.A. and Legarda T.M., 1999. Ochratoxin A en vinos, mostos y zumos de uva elaborados en Espana y en otros paises europeos, Alimentaria, January – Febrary : 107-113.

Burrington J. Kimberlee, 1998. More than just a milk. Food Product Design.

Cabanes F.J., Accensi F., Bragulat M.R., Abarca M.L., Castella G., Minguez S., Pons A., 2002. What is the source of ochratoxin A in wine? International Journal of Food Microbiology 79 (3): 213-125.

Cabanes J.F. and Rosa Bragulat, 2008. Ochratoxin a in profiling and speciation. In: *Aspergilus* in the genomic era, (Varga J. and Samson R., eds.), Wageningen Academic Publishers, The Netherlands: 57-70.

Cairns-Fuller V., 2004. Dynamics and control of ochratoxigenic strains of Penicillium verrucosum and Aspergillus ochraceus in the stored grain. Phd Thesis. Cranfield University, Silsoe, Bedford MK45 4DT, U.K.

Cairns-Fuller V., Aldred D., Magan N., 2005. Water, temperature and gas composition interactions affect growth and ochratoxin A production by isolates of *Penicillium verrucosum* on wheat grain. Journal of Applied Microbiology 99 (5): 1215-1221.

Clark D.S., Takacs J., 1980. Gases as preservatives. In: Microbial Ecology Foods, International Commission on Microbiology Specifications for Foods. Academic Press, New York, 1: 170-192.

Codex Alimentarius Commission, 18th May 1998. Codex Committee on Food Additives and Contaminants. Document CX/FL 98/5-CRD: 16.

Codex Alimentarius Commission, 22-26 March 1999. Codex Committee on Food Additives and Contaminants, 31st session, The Hague, The Netherlands.

Codex Alimentarius Committee, 2007. Codex Committee on Food Additives and Contaminants, Document CAC/GL 36-1989, Class Names and The International Numbering System For Food Additives.

Codex Standard for raisins,1981. Codex Stan 67.

Commission Directive 2002/26/EC, 13 March 2002. Sampling methods and the methods of analysis for the official control of the levels of ochratoxin A in foodstuffs. Official Journal of European Communities.

Commission of the European Communities, November 1997. Assessment of dietary intake of ochratoxin A by te population of EU Memper States. Reports on tasks on Scientific Co-operation, EUR 17523 EN, Official Journal of European Communities. Revised edition.

Commission of the European Communities, 14 September 1998. Directorate General XXIV, Scientific Committee on Food. Official Journal of European Communities. Outcome of discussions.

Congressional Hearing on Sulfites, 1985. 89th Congress, March 27, 1985. Publication 99-3. US Government Printing Office, Washington, DC.

Cooke R. and Whipps J., 1993. Ecophysiology of Fungi. Blackwell, Oxford.

Corneli S. and Maragos CM., 1998. Capillary Electrophoresis with Laser-Induced Fluorescence: Method for the Mycotoxin Ochratoxin A Journal of Agricultural and Food Chemistry 46 (8): 3162-3165.

Creppy E.E., 2002. Update of survey, regulation and toxic effects of mycotoxins in Europe. Toxicology Letters 127 (1-3): 19-28.

Creppy E.E., Baudrimont I., Betbeder A.M., 1995. Prevention of nephrotoxicity of ochratoxin A, a food contaminant. Toxicology Letters 82/83: 869-877.

Da Rocha Rosa C.A., Palacios V., Combina M., Fragat M.E., De Oliveira Rekson A., Magnoli C.E., Dalcero A.M., 2002. Potential ochratoxin A producers from wine grapes in Argentina and Brazil. Food Additives and Contaminants 19 (4): 408-414.

Dallyn H. and Fox A., 1980. Spoilage of materials of reduced water activity by xerophilic fungi. In: Microbial Growth and Survival Extremes of Environment, (Guld G. H. & J.E.L. Corry, eds), Academic Press, London, 129-139.

Davis N.D. Iyer S.K., Diener U.L., 1987. Improved method of Screening for Aflatoxin with a Coconut Agar Medium. Applied and Environmental Microbiology 53 (7): 1593-1595.

Deacon, J. W., 2005. Environmental conditions for growth, and tolerance of extremes. In: Fungal biology, (Deacon, J. W., ed.), Blackwell, 4th. Oxford: 142-157.

Dekanea A., 2005. Ecology and control *Aspergillus carbonarius* growth and OTA production in Corinth raisins (currants). PhD Thesis. Cranfield University, Silsoe, Bedford MK45 4DT, U.K.

Delage N., Harlingue A., Colonna Ceccaldi B., Bompeix G., 2002. Occurrence of mycotoxins in fruit juices and wine. Food Control 14 (4): 225-227.

Department of Health ACT Government Health Information, 1997. Health Services - Food Survey Reports, 1996-97.

Diener U.J. and Davis N.D., 1977. Aflatoxin formation in peanuts by *Aspergillus flavus.* Bulletin Agricultural Experimental Station, Auburn University No 493.

Dirheimer G., Creppy E.E., 1991. Mechanism of action of ochratoxin A. Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours, Castagnaro. International Agency of Research on Cancer IARC, Lyon: 171-186.

Dyer S.K.and McCammon S., 1994. Detection of toxigenic isolates of *Aspergillus flavus* and related species on coconut cream agar. Journal of Applied Bacteriology 76 (1): 75-78.

Earle M.D., Putt G.J., 1984. Microbial spoilage and use of sorbate in bakery products. Food Technology in New Zealand 19: 25-36.

EC Regulation 472/2002 of 8 March 2001 setting maximum levels for certain contaminants in foodstuffs.

El Goorani, M.A., 1981. Effect of modified atmospheres on post-harvest pathogens of fruits and vegetables. Horticultural Reviews 3: 412-461.

El Halouat A., Debevere J.M., 1997. Molds and yeasts isolated from hydrated prunes and raisins having different water activities. Science des Aliments 17 (5): 539-545.

El Halouat A., Debevere J.M., 1997b. Effect of water activity, modified atmosphere combination system. Journal Food Protection 44: 903-908.

Ellis W.O., Smith J.P., Simpson B.K., Ramaswamy, H and Doyon G., 1994. Growth of and aflatoxin production by *Aspergillus flavus* in peanuts stored under modified atmosphere packaging (MAP) conditions. International Journal of Food Microbiology 22 (2): 513-516.

Epstein E., Steinberg M.P., Nelson A.I., Wei L.S., 1970. Aflatoxin production as affected by environmental conditions. Journal of Food Science 35 (4): 389-391.

Esteban A., Abarca L.M., Bragulat M.R., Cabanes F.J., 2004. Effects of temperature and incubation time on production of ochratoxin A by black aspergilli. Research in Microbiology 155 (10): 861-866.

Esteban A., Abarca M.L., Bragulat M.R. Cabanes F.J., 2006. Effect of water activity on ochratoxin A production by *Aspergillus niger* aggregate species. International Journal of Food Microbiology 108(2): 188-195.

Esteban, A., Abarca, M. L., Bragulat, M. R. & Cabanes, F. J., 2005. Influence of pH and incubation time on ochratoxin A production by *Aspergillus carbonarius* in culture media. Journal of Food Protection 68 (7): 1435-1440.

European Commission (EC) Regulation 472/2002 of 8 March 2001 setting maximum levels for certain contaminants in foodstuffs.

European Commission regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs.

FDA/CFSAN, 1992. Food Additives, US Food and Drug Administration FDA/IFIC Brochure: http< //vm.cfsan.fda.gov/~1rd/ >.

Festas I., Herbert P., Santos L., Cabral M., Barros P., Alves A., 2000. Ochratoxin A in some portuguese wines: method validation and screening in port wine and vinho verde. American Journal of Enology and Viticulture 51 (2): 71-74.

Filali A., Ouammi L., Betbeder A.M., Baudrimont I., Soulaymani R., Benayada A., Creppy E.E., 2001. Ochratoxin A in beverages from Morocco: a preliminary survey. Food Additives and Contaminants 18 (6): 565-568.

Fousaki M., Karametsi K., Chaniotakis N.A., 2003. Method for the determination of water content in sultana raisins using a water activity probe. Food Chemistry 82 (1): 133-137.

Frisvad J.C., Thrane U., 1996. Mycotoxin production by food-borne fungi. In: Introduction to food-borne fungi, (Samson R.A., Hoekstra E.S., Frisvad J.C., eds), Ultecht, German 5: 251-261.

Gibson A.M., Baranyi J., Pitt J.I., Eyles M.I., Roberts T.A., 1994. Predicting fungal growth: the effect of water activity on *Aspergillus flavus* and related species. International Journal of Food Microbiology 23 (3-4): 419-431.

Giridhar P., Reddy S.M., 2001. Incidence of mycotoxigenic fungi on raisins. Journal of Adv. Plant. Science 14: 291-294.

Gock A. M., Hocking D. A., Pitt I.J., Poulos G.P., 2003. Influence of temperature, water activity and pH on growth of some xerophilic fungi. International Journal of Food Microbiology 81 (1): 11-19.

Gonzalez-Penas E., Leach C., Viscarret M., Perez de Obanos A., Araguas C., Lopez de Cerain A., 2004. Determination of ochratoxin A in wine using liquid-phase microextraction combined with liquid chromatography with fluorescence detection. Journal of Chromatography A 1025 (2): 163-168.

Gordon S., Fabiani A., Stockenstrom S. Ndumiso M., and Vikash S., 2003. Quantification of Ochratoxin a in South African Wines. Journal of Agricultural and Food Chemistry 51 (4): 1102-1106.

Guyonot M.E., Ramos A.J., Sala D., Sanchis V., Marin S., 2002. Combined effects of weak acid preservatives, pH and water activity on growth of *Eurotium* species on a sponge cake. International Journal of Food Microbiology 76 (1-2): 39-46.

Haggblom P., 1982. Production of ochratoxin A in barley by *Aspergillus ochraceus* and *Penicillium viridicatum*: effect of fungal growth, time, temperature and inoculum size. Applied and Environmental Microbiology 43 (5): 1205-1207.

Heenan C.N., Shaw K.J., Pitt J.I., 1998. Ochratoxin A production by *Aspergillus carbonarius* and *A. niger* isolates and detection using coconut cream agar. Journal of Food Mycology 1: 67-72.

Heilmann W., Rehfeldt A.G., Rotzoll F., 1999. Behaviour and reduction of ochratoxin A in green coffee beans in response to various processing methods. European Food Research and Technology 29 (3-4): 297-300.

Hwanga C.-A., Draughon F.A., 1994. Degradation of ochratoxin A by *Acimetobacter calcoaceticus*. Journal of Food Protection 57 (5): 410-414.

International Agency for Research on Cancer (IARC), 1993. IARC monograph on the evaluation of carcinogenic risk to humans: some naturally occurring substances: sine food, tons and constituents, heterocylic aromatic amines and mycotoxins. Ochratoxin A. IARC, Lyon 56: 489-531.

Joosten H.M.L.J., Goetz J., Pittet A., Schellenberg M., Bucheli P., 2001. Production of ochratoxin A by *Aspergillus carboanarius* on coffee cherries. International Journal of Food Microbiology 65 (1-2): 39-44.

Jorgensen K., 1998. Survey of pork, poultry, coffee, beer and pulses for ochratoxin A. Food Additives and Contaminants 15 (5): 550-554.

Jyoti S., Munimbazi C., Bullerman L.B., 2001. Relationship of mould count, ergosterol and ochratoxin A production. International Journal of Food Microbiology 71 (1): 29-34.

Kazi B.A, Emmett R.W., Clarke K., Nancarrow N., 2003. Black *Aspergillus* moulds in Australian vineyards. In Proceedings of 8th International Congress of Plant Protection, Christchurch, New Zealand, 2-4 February, 8.74, 119 (Abstract).

Kim, H.J., 1995. Filtrates and residues: inhibition of enzymatic browning reaction by sulfite. Journal of Chemical Education 72: 242-248.

King A.D. Jr., Ponting J.D., Sanshuck D.W., Jackson R., Mihara K., 1981. Factors affecting death of yeast by sulphur dioxide. Journal of Food Protection 44: 92-97.

Kovacs F., Sandor G., Vanyi A., Domany S., Zomborszky-Kovacs M., 1995. Detection of ochratoxin A in human blood and colostrums. Acta Veterinaria Hungarica 43: 393-400.

Kozakiewicz Z., 1989. Aspergillus species on stored products. Mycological Papers, 161, C.A.B. International, UK.

Kozakiewicz Z., Battilani P., Cabanes J., 2003. Making wine safer: the case of ochratoxin A. In: Meeting the mycotoxin menace, (Barug D., Egmond H., Lopes-Carsia R., Osenbruggen T., Visconti A., eds), Wageningen Academic Publishers, Netherlands.

Kusters-van Someren M.A., Samson R.A. and Visser J., 1991. The use of RLFP analysis in classification of the black Aspergilli: reinterpretation of the *Aspergillus niger* aggregate. Current Genetics 19 (1): 21-26.

Lacey J. and Magan N., 1991. Fungi in cereal grains, their occurrence and water and temperature relationships. In: Cereal Grain, Mycotoxins, Fungi and Quality in "Drying and Storage" (Chelkowski, J., ed.), Elsevier, Amsterdam: 77-118.

Larson K.and Moller T., 1996. Liquid chromatographic determination of ochratoxin A in barley, wheat bran, and rye by the AOAC/IUPAC/NMKL method: NMKL collaborative study. Journal AOAC International 79 (5): 1102-1105.

Lau B.P.Y., Scott P.M., Lewis D.A., Kanhere S.R., 2000. Quantitative determination of ochtratoxin A by liquid Chromatography/electrospray tandem mass spectrometry. Journal of Mass Spectrometry 35 (1): 23-32.

Leake L. I., 2006. Water activity and food quality. Food Technology 60: 62-67.

Lee H.B. and Magan N., 2006b. Impact of environment and inter-specific interactions between spoilage fungi and *Aspergillus ochraceous* and ochratoxin production on maize. International Journal of Microbiology 61 (1): 11-16.

Legan J.D., 1993. Mould spoilage of bread: the problem and some solutions. International Biodeterioration & Biodegradation 32 (1-3): 33-53.

Leong S.L., Hocking A. D., Pitt J.I., 2004. Occurrence of fruit rots fungi (*Aspergillus* section *Nigri*) on some drying varieties of irrigated grapes. Australian Journal of Grape and Wine Research 10 (1): 83-88.

Leong Su-lin L., Hocking D., Ailsa, Scott S. Eileen, 2006. Survival and growth of *Aspergillus carbonarius* on wine grapes before harvest. International Journal of Food Microbiology 111 (1): 83-86.

Leong Su-lin L., Hocking D., Ailsa, Scott S. Eileen, 2007. *Aspergillus* species producing ochratoxin A: isolations from vineyard soils and infection of Semilon bunches in Australia. Journal of Applied Microbiology 102 (1): 124 -133.

Leoni L.A.B., Soares L.M.V. and Oliveira P.L.C., 2000. Ochratoxin A in Brazilian roasted and instant coffees. Food Additives and Contaminants 17 (10): 867-870.

Lindblad M., Johnsson P., Jonsson N., Lindqvist P., Olsen M., 2004. Predicting noncompliant levels of ochratoxin A in cereal grain form *Penicillium verrucosum* counts. Journal of Applied Microbiology 97 (8): 609-616.

Lo Curto R., Pellicano T., Vilasi F., Munafo P., Dugo G., 2004. Ochratoxin A occurence in experimental wines in relationship with different pesticide treatments on grapes. Food Chemistry 84 (1): 71-75.

Lobeau M., Saeger S. De., Sibanda L., Barna-Vetro I., Van Peteghem C., 2005. Development of a new clean-up tandem assay column for the detection of ochratoxin A in roasted coffee. Analytica Chimica Acta 538 (1-2): 57-61.

Lombaert G.A., Pellaers M., Chettiar D., Lavalee P.M., Scott B.P., 2002. Survey of Canadian retail coffees for ochratoxin A. Food Additives and Contaminants 19 (9): 869-877.

Lopez De Cerain A., Gonzales-Penas E., Jimenez A.M., Bello J., 2002. Contribution to the study of ochratoxin A in Spanish wines. Food Additives and Contaminants 19 (11): 1058-1064.

MAFF (Ministry of Agriculture, Fisheries and Food), 1997. Survey of aflatoxins and ochratoxin A in cereals and retail products. MAFF Food Surveillance Information Sheet 130, London.

MAFF (Ministry of Agriculture, Fisheries and Food), 1999. Survey of Retail Products for Ochratoxin A. Ministry for Agriculture, Fisheries and Food, London.

Magan N. & Olsen M., 2004. Mycotoxins in food, Detection and control. Woodhead Publishing Ltd, Oxford, England.

Magan N. and Aldred D. C.N, 2005. Conditions of formation of ochratoxin in drying, transport and in different commodities. Food Additives and Contaminants IS: 10-16.

Magan N. and Aldred D.C.N., 2007. Post-harvest control strategies: Minimizing mycotoxins in the food chain. International Journal of Food Microbiology 119 (1-2): 131-139

Magan N. and Lacey J., 1984. Effects of gas composition and water activity on growth of field and storage fungi and their interactions. Transactions of the British Mycological Society 82 (2): 305-314.

Magan N. and Lacey J., 1985. Interactions between field and storage fungi on wheat grain. Transactions of the British Mycological Society 85 (1): 29-37.

Magan N. and McLeod, A.R., 1991. Effect an open-air fumigation with sulphur dioxide on the occurrence of phylloplane fungi on winter barley. Agriculture, Ecosystems and Environment 33 (3): 245-261.

Magan N., 1988. Effects of water potential and temperature on spore germination and germ-tube growth in vitro and on straw leaf sheaths. Transactions of the British Mycological Society 90: 97-107.

Magan N., 1992. Use of sulphur dioxide to control fungi in stored grain. In Proceedings of International Conference on Controlled Atmospheres and Fumigation in Grain Storages, (Navarro, S. and Donahaye E., eds), Caspit Press Jerusalem: 163-171.

Magan N., 1993b. Tolerance of fungi to sulphur dioxide. In: Stress Tolerance of Fungi, (Jennings D.H., ed), Marcell Dekker: 173-187.

Magan N., 1997. Fungi in extreme environments. In: the Mycoto IV, Environmental and Microbial Relationships, Berlin Heidelberg.

Magan N., 2006. Mycotoxin contamination of food in Europe: Early detection and prevention strategies. Mycopathologia 162 (3): 245-253.

Magan N., Hope R., Cairns V., Aldred D., 2003. Post-harvest fungal ecology: Mycotoxin Accumulation in Stored Grain. European Journal of Plant Pathology 109 (7): 723-730.

Magan N., Lacey J., 1984b. The effect of water activity, temperature and substrate on interactions between field and storage fungi. Transactions of the British Mycological Society 82: 305-314.

Magan N., Lacey J., 1985. Interactions between field, and storage fungi on wheat grain. Transactions of the British Mycological Society 85 (1): 29-37.

Magan N., Lacey J., 1986. The phylloplane microbial populations of wheat and effect of late fungicide applications. Annals of Applied Biology 109: 117-128.

Magan N., McLeod A.R., 1986. In vitro growth and germination of phyllosphere fungi in atmospheric sulphur dioxide. Transactions of the British Mycological Society 90: 571-582

Magan N., Smith M.K., Kirkwood I. A., 1994. Effects of atmospheric pollutants on phyllosphere and endophytic fungi. Symposium of British Mycological Society Held, Cranfield University, UK.

Magan, N., Hope R., Cairns V., Aldred D., 2003. Post-harvest fungal ecology: Impact of fungal growth and mycotoxin accumulation in stored grain. European Journal of Plant Pathology 109 (7): 723-730.

Magnoli C., Astoreca A., Ponsone L., Combina M., Palacio G., Rosa C.A.R., Dalcero A.M., 2004. Survey of mycoflora and ochratoxin A in dried vine fruits from Argentina markets. Letters in Applied Microbiology 39 (4): 326-331.

Magnoli C., Violante M., Combina M., Palacio G., Dalcero A., 2003. Mycoflora and ochratoxin-producing strains of *Aspergillus* section *Nigri* in wine grapes in Argentina. Letters in Applied Microbiology 37 (2): 179-184.

Majerus P., Bresch H., Otteneder H., 2000. Ochratoxin A in wines, fruits juices and seasonings. Archiv fur Lebensmittel Hygiene 51: 95-97.

Majumber S.K., Raghunathan A.N., Rangaswamy J.R., 1973. Control of mycoflora on moist grain. In: Preservation of Wet Harvested Grains, (Multon, J.L.,

Guilbot, A., eds), Institut National de la Recherche Agronomique, Paris, France: 343-352.

Mantle P.G., 2002. Risk assessment and the importance of ochratoxins. International Biodeterioration and Biodegration 50 (3-4): 143-146.

MAPA (Ministerio de Agricultura, Pesca y Alimentacion), 1998. Loas parasitos de la vid. Estategias de proteccion razonada, 4th edn. Mundi-Prensa, Madrid 189-194.

Marin S., Belli N., Lasram S., Chebil S., Ramos A.J., Ghorbel A., Sanchis V., 2006. Kinetics of Ochratoxin A production and accumulation by *Aspergillus carbonarius* on synthetic grape medium at different temperature levels. Journal of Food Science 71 (6): 196-200.

Marin S., Sanchis V., Ramos A.G. and Magan N., 1998b. Environmental factors, interspecific interactions, and niche overlap between *Fusarium nonilofórme* and *F. proliferatum* and *Fusarium graninearum, Aspergillus* and *Penicillium* spp. Isolated from maize. Mycological Research 102: 831-837.

Markaki P., Delpont Binet, Grosso C., Dragacci S., 2001. Determination ochratoxin A in red wine and vinegar by immunoaffinity high-pressure liquid chromatography. Journal of Food Protection 64 (4): 533-537(5).

Matissek R. and Raters M., 2000. Ochratoxin A in cocoa and human health aspects. 13th International Cocoa Research Conference, Kota Kinabalu, Malaysia, 9-14 October.

McDonald S., Wilson P., Barnes K., Damant A., Massey R., Mortby E., Shepherd J., 1999. Ochratoxin A in dried vine fruit: method development and survey. Food Additives and Contaminants 16 (6): 253-260.

Medica A., Mateo R., Lopez-Ocana L., Valle-Algarra F.M., Jimenez M., 2005. Study of Spanish grape mycobiota and Ochratoxin A production by isolates of *Aspergillus tubignensis* and other members of *Aspergillus* section *Nigri*. Applied and Environmental Microbiology 71: 4696-4702.

Micco C., Grosst M., Miraglia M., Brera C., 1989. A study of the contamination by ochratoxin A of green and roasted beans. Food Additives and Contaminants 6: 33-339.

Ministry of Agriculture, Fisheries and Food, 1999.

Mitchell D., Parra R., Aldred D., Magan N., 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 (2): 439-445.

Molnar O. Scahtzmayr G., Fuchs E., Prillinger H., 2004. *Trichosporon mycooxivorans* sp. Nov., a new yeast species useful in biological detoxification of various mycotoxins. Systematic and Applied Microbiology 27: 661-671.

Morters A., Hald B., Lillehoj E., Mortensen H.R., 1982. Feeding experiments with ochratoxin A contaminated barley for bacon pigs. 5 Ochratoxin in a pig blood. Acta Agriculturae Scandinavica 33 (3): 235-239.

Official Journal of the European Union (OJEU), 2003. Directive 2003/89/EC of the European Parliament and of the Council of 10 November 2003-amending

Directive 2000/13/EC as regards indication of the ingredients present in foodstuffs L309: 15-18.

Otteneder H., Majerus P., 2000. Occurrence of ochratoxin A (OTA) in wines: influence of the type of wine and its geographical origin. Food Additives and Contaminants 17 (9): 793-798.

Ough C.S., Corison C.A., 1980. Measurement of patulin in grapes and wine. Journal of Food Science 43 (3): 476-478.

Palacios-Cabrera H., Taniwaki M.H., Hashimoto J.M., Menezes H.C, 2005. Growth of *Aspergillus ochraceus, A. carbonarius* and *A. niger* on culture media at different water activities and temperatures. Brazilian Journal of Microbiology 36 (1): 24-28.

Pardo E., Marin S., Sachis V., Ramos A.J., 2004. Prediction of fungal growth and ochratoxin A production by *Aspergillus ochraceus* on irradiated barley grain as influenced by temperature and water activity. International Journal of Food Microbiology 95 (1): 79-88.

Parenicova L., Benen J.A., Samson R.A. and VisserJ., 1997. Evaluation of RLFP analysis the classification of selected black aspergilli. Mycological Research 101 (7): 810-814.

Paster N., Barkai-Golan, Calderon M., 1986. Control of T-2 toxin production using atmospheric gases. Journal of Food Protection 49 (8): 615-617.

Paster N., Blumenthal – Yonassi J., Barkai – Golan R., Menasherov M., 1991. Production of zearalonone in vitro and in corn grains stored under modified atmospheres. International Journal of Food Microbiology 12 (2-3): 157-166.

Paster N., Lisker N., Chet I., 1983. Ochratoxin production by *Aspergillus flavus* grown under controlled atmospheres. Applied and Environmental Microbiology 45 (3): 1136-1139.

Paster N., Menasherov N., 1988. Inhibition of T-2 toxin production on high moisture conrn kernels by modified atmospheres. Applied and Environmental Microbiology 54 (2): 540-543.

Pateraki M., Dekanea A., Mitchell D., Lydakis D., and Magan N., 2007. Influence of sulphur dioxide, controlled atmospheres and water availability on in *vitro* germination, growth and ochratoxin A production by strains of *Aspergillus carbonarius* isolated from grapes. Postharvest Biology and Technology 44 (2): 141-149.

Perrone G., Mule G., Susca A., Battilani P., Pietri A., Logrieco A., 2006. Ochratoxin A production and Amplified Fragment Length Polymorphism Analysis of *Aspergillus carbonarius, Aspergillus tubingensis,* and *Aspergillus niger* strains isolated from grapes in Italy. Applied and Environmental Microbiology 72 (1): 680-685.

Peteri Z., Teren J., Vagnolgyi C., Varga J., 2007. Ochratoxin degradation and adsorption caused by astaxanthin-producing yeasts. Food Microbiology 24 (3): 205-210.

Petzinger E., 1999. Potential relation between mercury concentrations and necropsy findings in small cetaceans in German waters of the North and Baltic seas. Marin Pollution Bull 38: 285-295.

Petzinger E., Weidenbach A., 2002. Mycotoxins in the food chain: the role of ochratoxins. Livestock Production Science 76 (3): 245-250.

Petzinger E., Ziegler K., 2000. Ochratoxin A from a toxicological perspective. Journal of veterinary Pharmacology and Therapeutics 23 (2): 91-98.

Pietri A., Bertuzzi T., Pallaroni L., Piva G., 2001. Occurrence of ochratoxin A in Italian wines. Food Additives and Contaminants 18 (7): 647-654.

Pitt J.I., 2000. Toxigenic fungi: which are important? Medical Mycology 38 (I): 17-22.

Pitt J.I., Basilico J.C., Abarca M.L., Lopez C., 2000. Mycotoxins and toxigenic fungi. Medical Mycology 38 (I): 41-46.

Pitt J.I., Hocking A., 1997. Fungi and food spoilage. Blackie Academic and Professional, London.

Pohland A. E., 1993. Mycotoxins in review. Food additives and contaminants 10 (1): 17-28.

Pohland A.E., Schuller P.S., Steyn P.S, Van Egmond H.P., 1982. Physicochemical data for some selected mycotoxins. Pure and Applied Chemistry 54: 2219-2284.

Porter S.N., Howarth G.S., Butler R.N., Jornet D., Busto O., Guasch J., 2000. Solid-phase extraction applied to the determination of ochratoxin A in wines by reversed-phase high-performance liquid chromatography. Journal of Chromatography A 882 (1): 29-35.

Rahirutula A.D., Bereziat J.C., Bussacchini-Giot V., Bartseh H., 1988. Lipid peroxidiation as a possible course for ochratoxin A toxicity. Biochem. Pharmacology 37: 4469-4475.

Raper K.B. and Fennell D.I., 1965. The genus of Aspergillus. Williams & Williams Company, Baltimore.

Rhone Diagnostics, 1999. Quantitative Detection of ochratoxin A (Rhone Diagnostics Technologies).

Ringot D., Chango A., Schneider Y.J., Larondelle Y., 2006. Toxicokinetics and toxicodynamics of ochratoxin A, an update. Chemical-Biological Interactions 159 (1): 18-46.

Rizzo A., Eskola M., Atroshi F., 2002. Ochratoxin A in cereals, foodstuffs and human plasma. European Journal of Plant Pathology 108 (7): 631-637.

Rossa C.A., Magnoli C.E., Fraga M. E., Dalcero A.M., Santana D.M., 2004. Occurrence of ochratoxin A in wine and grape juice marketed in Rio de Janeiro, Brazil. Food Additives and Contaminants 21 (4): 358-364.

Russell R. and Paterson M., 2006. Identification and quantification of mycotoxingenic fungi by PCR. Review. Process Biochemistry 41: 1467-1474.

Sage L., Garon D., Seigle-Murandi F., 2004. Fungal Microflora and Ochratoxin A Risk in French Vineyards. Journal of Agricultural and Food Chemistry 52 (18): 5764-5768.

Sage L., Krivobok S., Delbos E., Seigle-Murandt F., Creppy E.E., 2002. Fungal flora and ochratoxin production in grapes and musts from France. Journal of Agricultural and Food Chemistry 50: 1306-1311.

Samson R.A., 1994. Current systematic of the genus *Aspergillus*. In: The genus Aspergillus: From taxonomy and genetics to industrial application, (Powell K.A., Renwick A. and Pebedy J.F., eds), Plenum Press, London.

Sanchis V., Magan N., 2004. Environmental profiles for growth and mycotoxin production. In: Mycotoxin in Food: Detection and Control, (Magan N., Olsen M., eds), Woodhead Publishing Ltd.

Seiler D.A.L., 1976. The stability of intermediate moisture foods with respect to mould growth. In: Intermediate Moisture Foods, (Davies R., Birch G.G. and Parker K.J., eds), Applied Science Publishers Ltd, London: 166-181.

Serra R., Abrunhosa L., Koazakiewicz Z., Venancio A., 2003. Black *Aspergillus* species as ochratoxin A producers in Portuguese wine grapes. International Journal of Food Microbiology 88 (1): 63-68.

Serra R., Cabanes F.J., Perrone G., Castella G., Venancio A., Mule G., Koazakiewicz Z., 2006. *Aspergillus ibericus*: a new species of section *Nigri* isolated from grapes. Mycologia 98 (2): 295-306.

Sherma J., 2000. Thin-Layer chromatography in food and agricultural analysis. Review. Journal of Chromatography A 880 (1-2): 129-147.

Siddiqui, I.A., Shaukat, S.S. & Khan, A., 2004. Differential impact of some *Aspergillus* species on *Meloidogyne javanica* biocontrol by *Pseudomonas fluorescens* strain CHAO. Letters in Applied Microbiology 39 (1): 74-83.

Singh A.K. and Rai B., 1990. Effect of SO_2 and NH_3 on growth behavior of some phylloplane fungi from wheat in vitro. Water, Air and Soil Pollution 49 (3): 343-347.

Skaug M.A., 1999. Analysis of Norwegian milk and infant formulas for ochratoxin A. Food Additives and Contaminants 16 (2): 75-78.

Smith J.P., Jackson E.D., Doraikul B., 1983. Storage study of a gas-packaged bakery product. Journal of Food Science 48 (4): 1370-1371.

Snowdon A.L., 1990. A Colour of Post-Harvest Diseases and Disorders of Fruit and Vegetables. In: General Introduction and Fruits, Wolf Scientific, London: 256.

Sofos J.N., 1994. Antimicrobial Agents. In: Food Additive Toxicology, (Maga J.A and. Tu A.T., eds.), Marcel Dekker, New York: 514-517.

Soleas G., Yan J., Goldberg D., 2001. Assay of Ochratoxin A in Wine and Beer by High-Pressure Liquid Chromatography photodiode Array and Gas Chromatography Mass Selective Detection. Journal of Agricultural and Food Chemistry 49 (6): 2733-2740.

Solfrizzo M., Avvantaggaiato G., Visconti A., 1998. Use of various clean-up procedures for the analysis of ochratoxin A in cereals. Journal of Chromatography A 815 (1): 67-73.

Staurakakis E., 1998. Viniculture II (Industrial Raisins). Notes of Lectures, University of Athens: 41-64.

Stefanaki I., Foufa E., Tsatsou-Dritsa A., Dais P., 2003. Ochratoxin A concentrations in Greek domestic wines and dried vine fruits. Food Additives and Contaminants 20 (1): 74-83.

Strotzky G., Goos R.D., 1965. Effect of high CO_2 and low O_2 tensions on the soil microbiota. Canadian Journal of Microbiology 11 (5): 853-868.

Studer-Rohr J., Dietrich D.R., Schlatter J., Schlatter C., 1995. The occurrence of ochratoxin A in coffee. Food and Chemical Toxicology 33 (5): 341-355.

Sweeney M., Dobson A. D.W., 1998. Mycotoxin production by *Aspergillus, Fusarium* and *Penicillium* species. Review. International Journal of Food Microbiology 43 (3): 141-158.

Taniwaki M.H., Pitt J.I., Teixeira A.A., Iamanaka B.T., 2003. The source of ochratoxin A in Brazilian coffee and its formation in relation to processing methods. International Journal of Food Microbiology 82 (2): 173-179.

Teren J., Varga J., Hamari Z., Rinyu E., Kevel F., 1996. Immunochemical detection of ochratoxin A in black *Aspergillus* strains. Mycopathologia 134 (3): 171-176.

Thamizharasi V. and Narasimham P., 1992. Growth of *Aspergillus niger* on onion bulbs and its control by heat and sulphur dioxide treatments. Tropical Science 33: 45-55.

Thirumala D.K., Mayo M.A. Gopal R., Reddy S.V., Delfosse P. Reddy D.V.R., 2000. Production of polyclonal antibodies against ochratoxin A and tis detection in chillies by ELISA. Journal of Agricultural and Food Chemistry 48: 5079-5082.

Thom C. and Raper K.B., 1945. Manual of the *Aspergilli*. Williams and Wilkins Co., Baltimore, MD.

Tournas V.H and Katsouda E., 2005. Mould and yeast flora in fresh berries, grapes and citrus fruits. International Journal of Food Microbiolog 105(1): 11-17.

Trucksess M.W., Giler J., Young K., White K.D., Page S.W., 1999. Determination and survey of ochratoxin A in wheat, barley and coffee. Journal of AOAC International 82 (1): 85-89.

Tsubouchi H., Yamamoto K., Hisada K., Sakabe Y., Udagava S., 1987. Effect of roasting on ochratoxin A level in green coffee beans inoculated with *Aspergillus ochraceus.* Mycopathologia 97 (2):111-115.

Tweedie J.W., Segel I.W., 1970. Specificity of transport processes for sulphur, selenium, and molybdenum anions by filamentous fungi. Biochimica et Biophysica Acta 196: 95-106.

Tzamos S.E., Antoniou P.P., Kalantzidou A., Antonopoulos D.F., Papageorgiou I., Tzamos E.C., 2004. *Aspergillus niger* and *Aspergillus carbonarius* in Corinth Raisin and Wine-Producing Vineyards in Greece: Population composition, Ochratoxin A production and Chemical control. Journal of Phytopathology 152 (4): 250-255.

Ueno Y., Maki S., Lin J., Furuya M., Sugiura Y., Kawamura O., 1998. A four-year study of plasma ochratoxin A in selected population in Tokyo by immunoassay and immunoafinity column-linked HPLC. Food and Chemical Toxicology 36 (5): 445-449.

Uota M., 1965. Inhibition of germination of *Alternaria* spores by sulphur dioxide under various moisture conditions. Phytopathology 525-527.

Valente H., 1998. Chromatographic method for the detection of ochratoxin A in animals and human tissues and fluids. Journal of Chromatography 31: 445-449.

Valero A. Farre J., R., Sanchis V., Ramos A., Marin S., 2006. Effects of fungal interaction on ochratoxin A production by *A. carbonarius* at different temperatures and aw. Int. Journal of Food Microbiology. International Journal of Food Microbiology 110 (2): 160-164.

Valero A. Farre J., R., Sanchis V., Ramos A., Marin S., 2006. Kinetics and spatial distribution of OTA in *Aspergillus carbonarius* cultures. Food Microbiology 23 (8): 753-756.

Valero A., Martin S., Ramos A.J., Sanchis V., 2004. Ochratoxin A producing species in grapes and sun dried grapes and their relation to ecophysiological factors. Letters in Applied Microbiology 41 (2): 196-201.

Valero A., Olivan S., Marin S., Sanchis V., Ramos A.J., 2007. Effect of *intra* and interspecific interaction on OTA production by *A.* section *Nigri* in grapes during dehydration. Food Microbiology 24 (3): 254-259.

Van de Merwe K.J., Steyne P.S., Fourie L.F., Scott D.B., Theron J.J., 1965. Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus* With. Letters to Nature 205: 1112-1123.

Van der Stegen, .Jorisen G.H.D.,Pittet U., Saccon M., Steiner W., Vinzenci M., Zapp J., Schlatter C., 1997. Screening of european coffee final products for occurrence of ochratoxin A (OTA). Food Additives and Contaminants 14 (3): 211-216.

Varga J., Kevei G., Fekete C., Coenen A., Kozakiewich Z., Croft J.H., 1993. Restriction fragment length polymorphisms in the mitochondrial DNAs of the *Aspergilus niger* aggregate. Mycological Research 97 (10): 1202-1207.

Varga J., Kevel E., Rinyu E., Teren J., Kozakiewicz Z., 1996. Ochratoxin production by *Aspergillus* species. Applied and Environmental Microbiology 62 (12): 4461-4464.

Varga J., Kocsubé S., Tóth B., Frisvad J. C., Perrone G., Susca A., Meijer M. and. Samson R. A., 2007. *Aspergillus brasiliensis* sp. nov., a biseriate black *Aspergillus* species with world-wide distribution. International Journal of Systematic and Evolutionary Microbiology 57 (9): 1925-1932.

Varga J., Peteri Z. Tabori K., Teren J., Vagnolgyi C., 2005. Degradation of ochratoxin A and other mycotoxins by *Rhizopus* isolates. International Journal of Food Microbiology 99 (3): 321-328.

Varga J., Rigo K., T`eren J., 2000. Degradation of ochratoxin A by *Aspergillus* species. International Journal of Food Microbiology 59 (1-2): 1-7.

Varga J., Rigo K., Teren J., Mesterhazy A., 2001. Recent advances in ochratoxin research In: Production, detection and occurrence of ochratoxins. Cereal Research Communications 29: 85-92.

Visconti A., Pascale M., Centonze G., 1999. Determination of ochratoxin A in wine by means of immunoaffinity column clean-up and high-performance liquid chromatography. Journal of Chromatography A 864 (1): 89-101.

Vos P. and Labuza T.P., 1974. A technique for measurement of water activity in the high a_w ranges. Journal of Agriculture and Food Chemistry 22: 326-327.

Vries R., Frisvad J.C., Peter J.I., van de Vondervoort, Burgers K., Kuijpers A., Samson A.R., Visser J., 2005. *Aspergillus vadensis,* a new species of the group of black *Aspergilli*. Antonie van Leeuwnhock 87 (3): 195-203.

Walker R., 2002. Risk Assessment of Ochratoxin: Current Views of the Europeian Scientific Committee on Food, the Jecfa and the Codex Committee on food additives and contaminants. In: Mycotoxins and food safety: Proceedings of and American chemical Society, (DeVries, J. W., Trucksess, M. W., Jackson, L. S., eds), Kluwer Academic Publishers 504: 249-255.

Wells J.M., Uota M., 1970. Germination and growth of five fungi in low oxygen and high carbon dioxide. Phytopathology 60 (1): 50-53.

WHO (World Health Organization), February 6-15, 2001. Report f the 56th Meeting of the Joint FAO/WHO Expert Committee on Food Additives. Geneva, Switzerland.

Wilson M., Lindow S., 1994a. Coexistence among epiphytic bacterial populations mediates through nutritional resource partitioning. Applied and Environmental Microbiology 60 (12): 4468-4477.

Wilson M., Lindow S., 1994b. Ecological similarity and co-existence of epiphytic ice-nucleating (Ice+) *Pseudomonas syringae* strains and non-ice nucleating (Ice-) biological control agent. Applied and Environmental Microbiology 60 (9): 3128-3137.

World Health Organization (WHO), 2000. Evaluation of certain food additives (fifty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Reprot Series No. 891, WHO, Geneva.

Zahavi T., Cohen L., Weiss B., Schena L., Daus A., Kaplunov T., Zutkhi J., Ben-Arie R., Droby S., 2000. Biological control of *Botrytis, Aspergillus* and *Rhizopus* rots on table and wine grapes in Israel. Postharvest Biology and Technology 20 (2): 115-124.

Zimmerli B., Dick R., 1996. Ochratoxin A in table wine and grape-juice: occurrence and risk assessment. Food Additives and Contaminants 13 (6): 655-668.

APPENDIX A

Graphs, Tables, Plates

Plate 2.1. Map of Greece. Red line indicates position of sampling area, (Iraklio Crete).

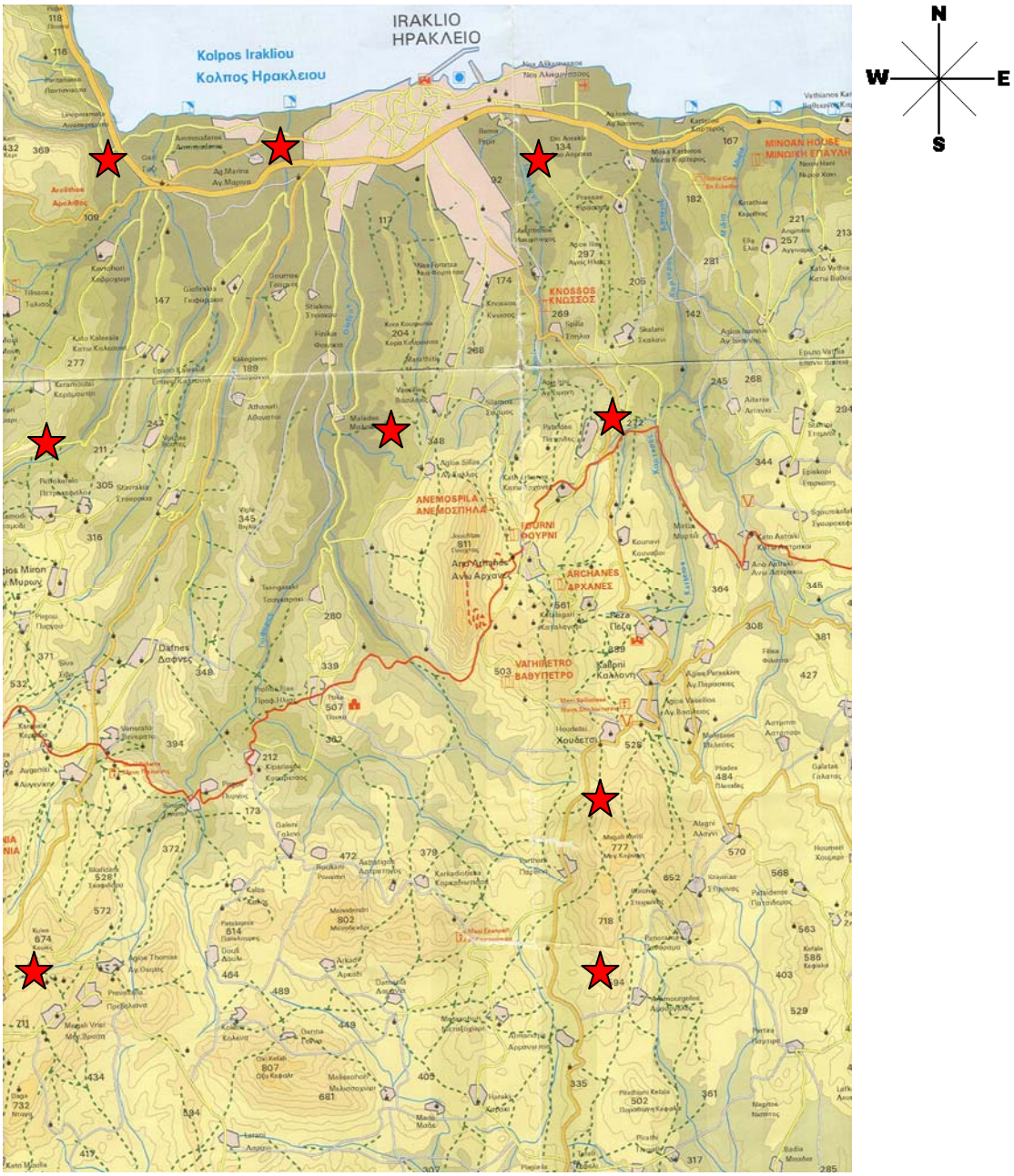


Plate 2.2. Sampling area; the nine examined vineyards situated at three different altitudes, 2005/06.

Table 2.1(a,b). Modification of water activity by adding glycerol (a) or glucose (b).

(a)

Water activity	glycerol g^{-1}
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

(b)

Water activity	glucose g^{-1}
0.995	4.66
0.99	9.76
0.98	18.73
0.96	39.81
0.94	60.89
0.92	77.65
0.90	94.4
0.88	112.96
0.86	1140.7

(source: Dallyn & Fox, 1980)

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

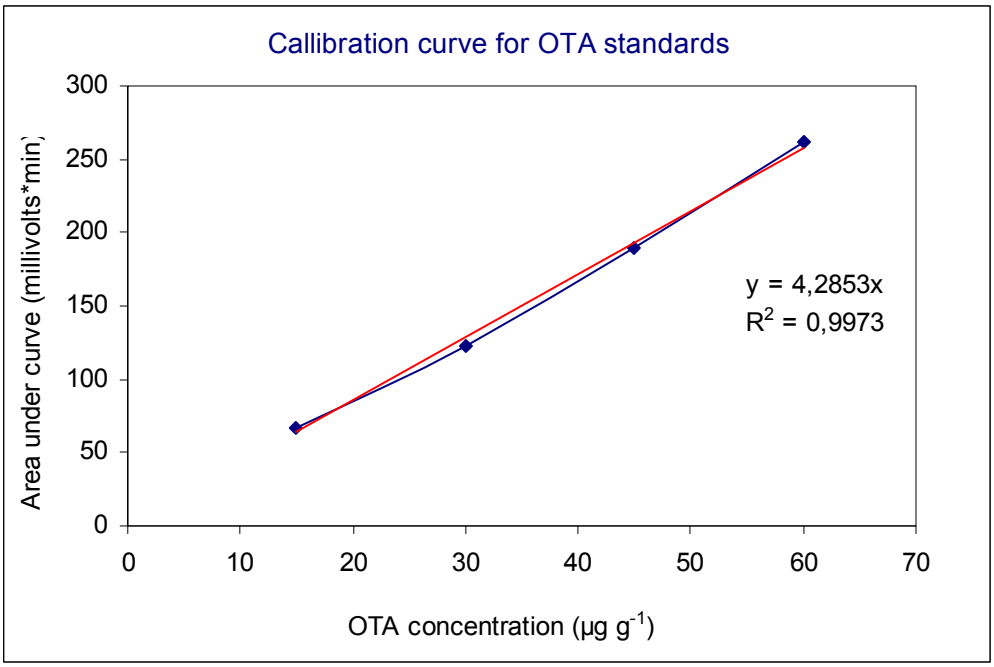


Figure 2.1. Calibration curve for ochratoxin A standards

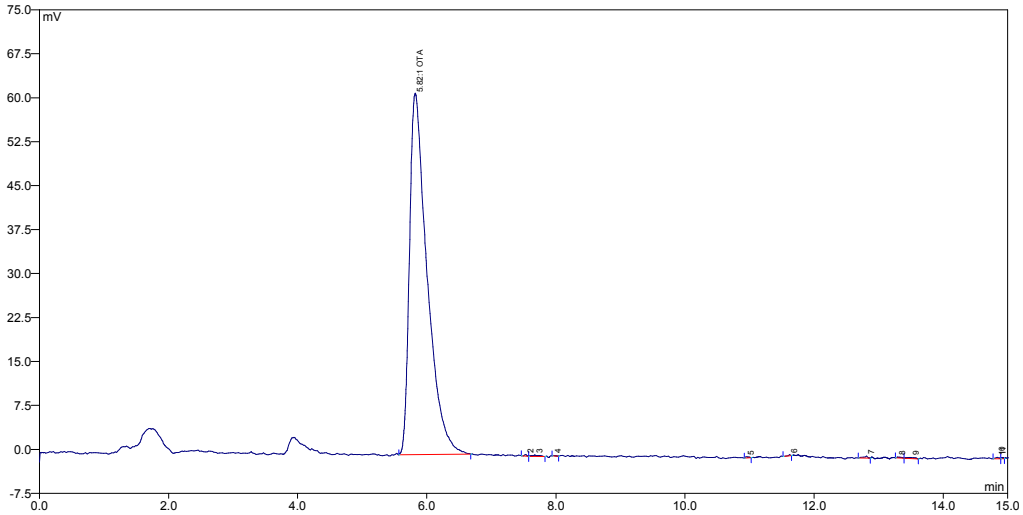


Figure 2.2. Chromatograms with Ochratoxin A detection showing ochratoxin A detection at 5.68 minutes.

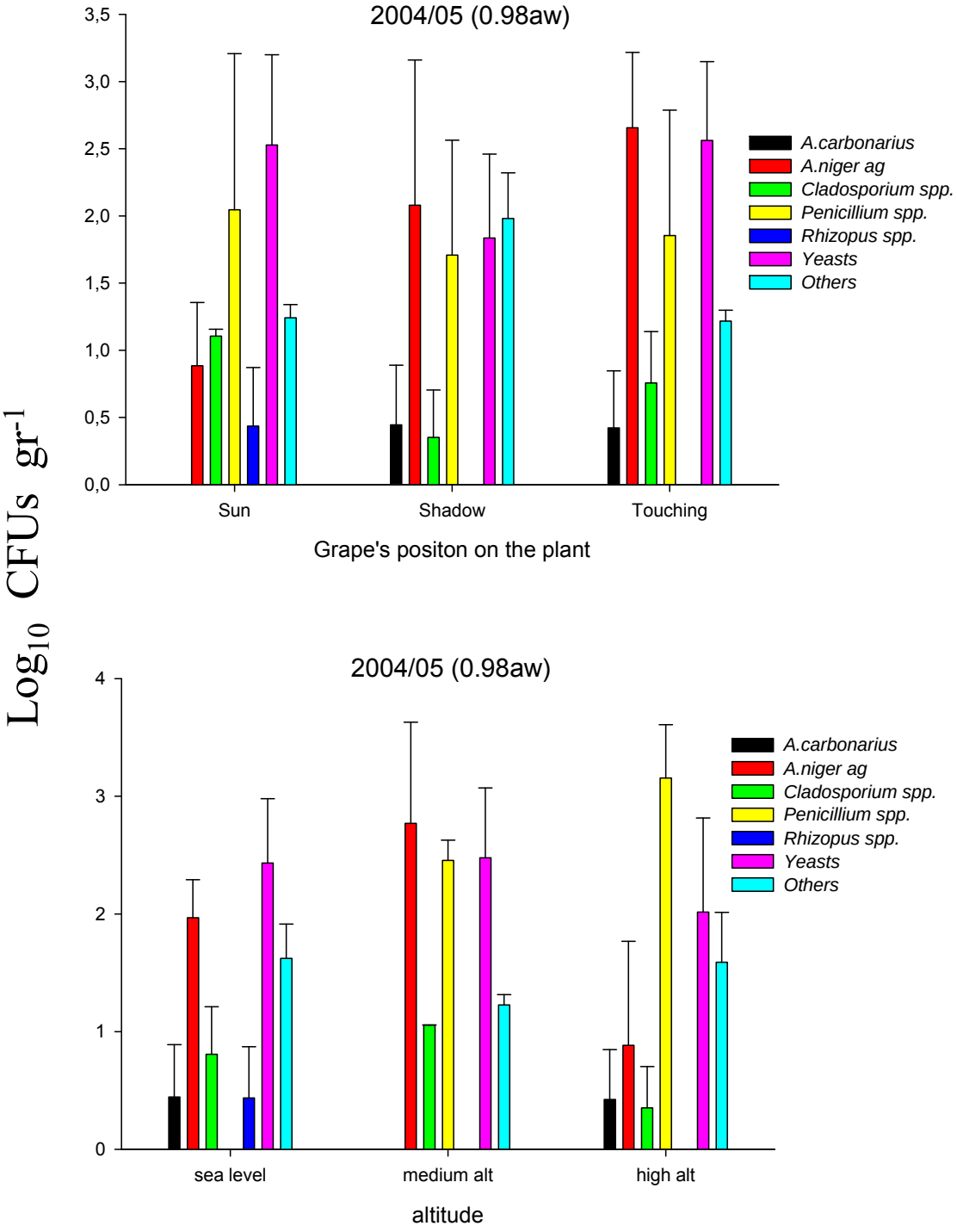


Figure 2.3. Fungal diversity according to (a) bunch position and (b) altitude of the most dominant fungal species/genera on MEA98, isolated from fresh grapes (Cretan Sultanina) 5dys prior harvesting, on 2004/05.

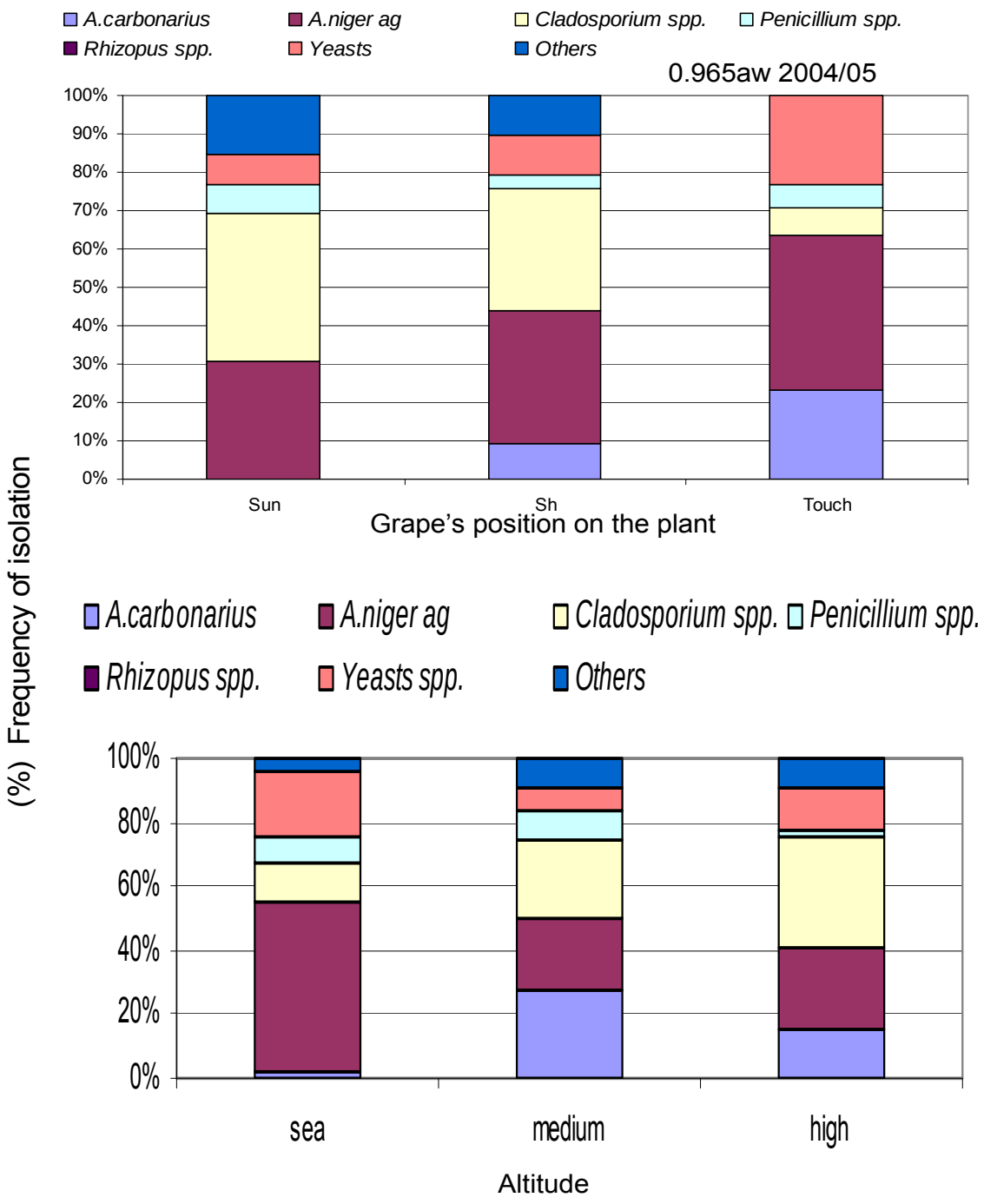


Figure 2.4. Fungal diversity (frequency of isolation) according to (a) bunch position and (b) altitude of the most dominant fungal species/genera on MEA96, isolated from fresh grapes (Cretan Sultanina) 5days prior harvesting, on 2004/05.

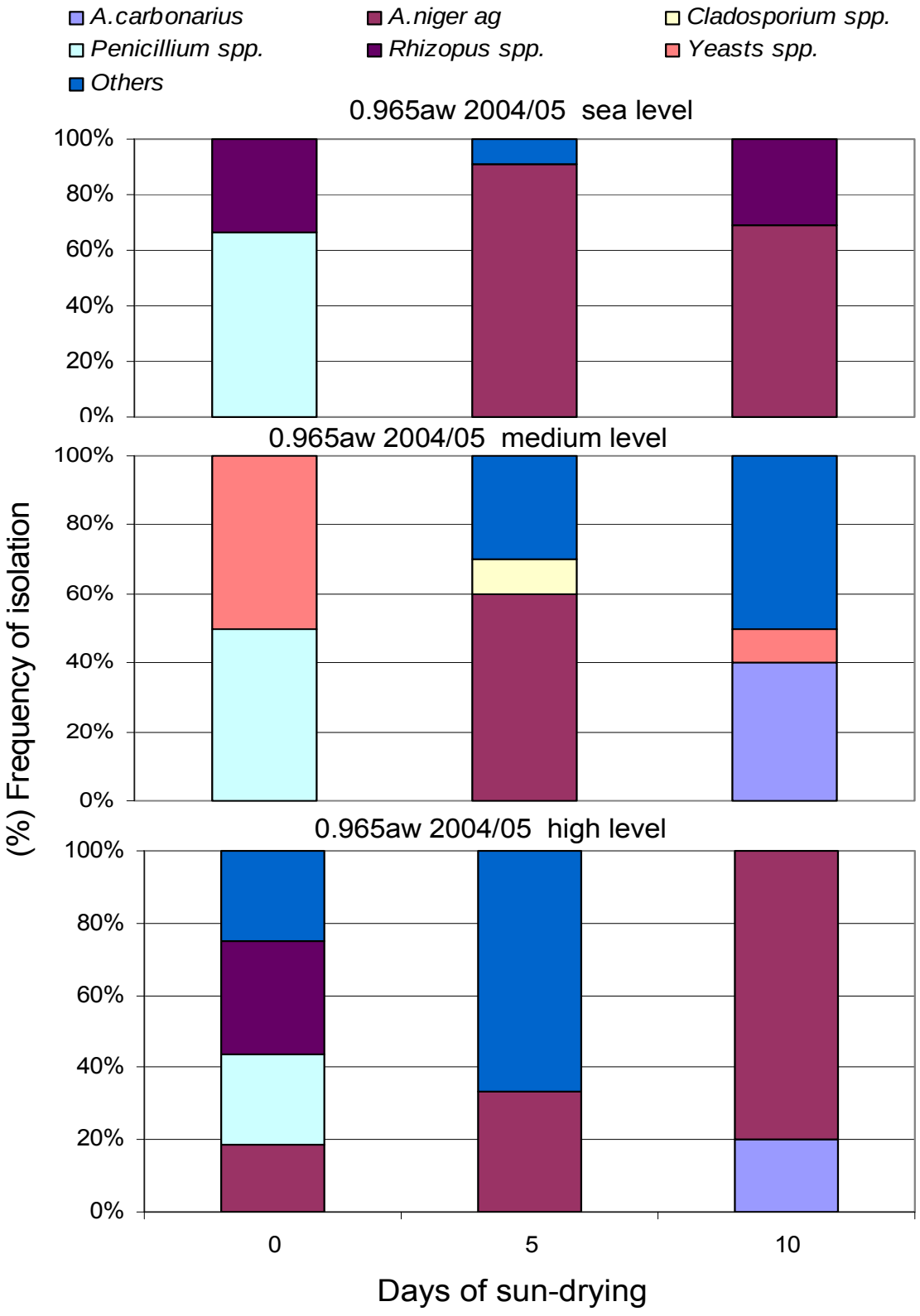


Figure 2.5. Fungal diversity (frequency of isolation) according to altitude of the most dominant fungal species/genera on MEA98, isolated from drying sultanas during sun-drying process, on 2004/05.

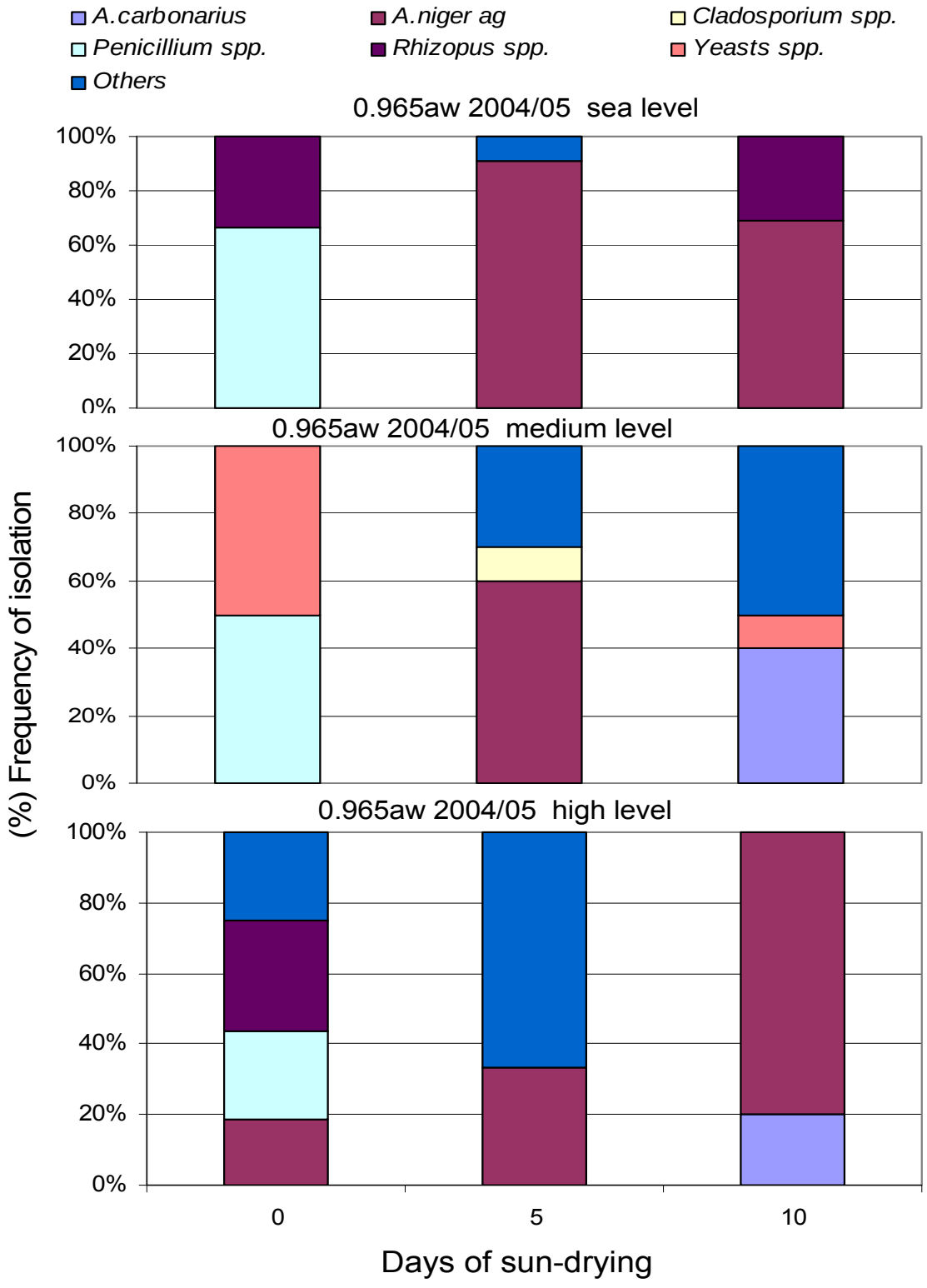


Figure 2.6. Fungal diversity (frequency of isolation) according to altitude of the most dominant fungal species/genera on MEA96, isolated from drying sultanas during sun-drying process, on 2004/05.

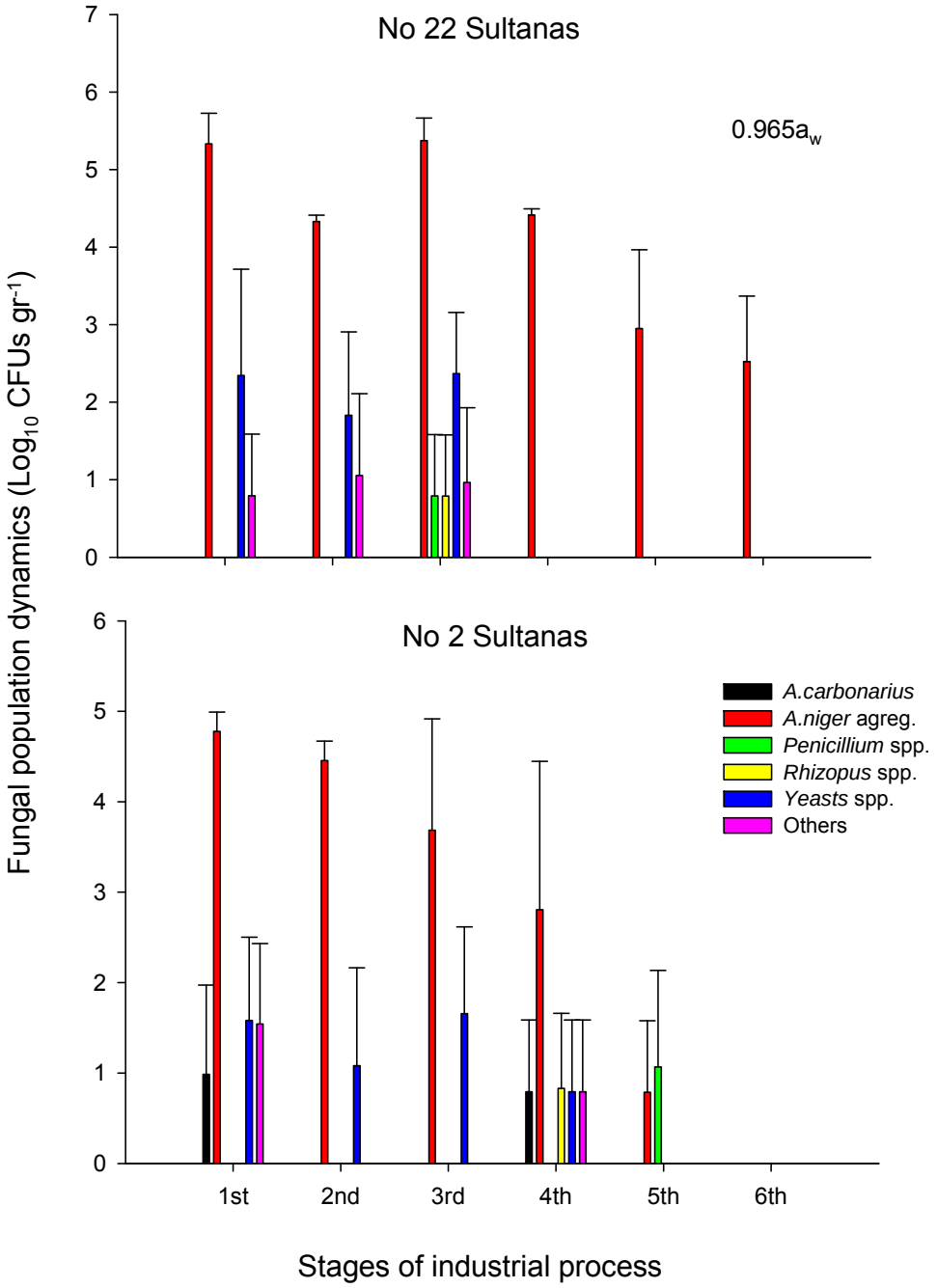


Figure 3.1. Population dynamics of the most dominant fungi, on two types of sultanas (No22 and No2 from 2004/05 vine crop) treated with SO_2, on MEA95 (0.954a_w) isolation medium, during industrial process. The values are the means of four replicates. Errors bars indicate standard errors of the mean. Keys: 1st=classification stage, 2nd=pre-washing, 3rd=SO2 treatment, 4th=final washing, 5th=heat-treatment, 6th=final product.

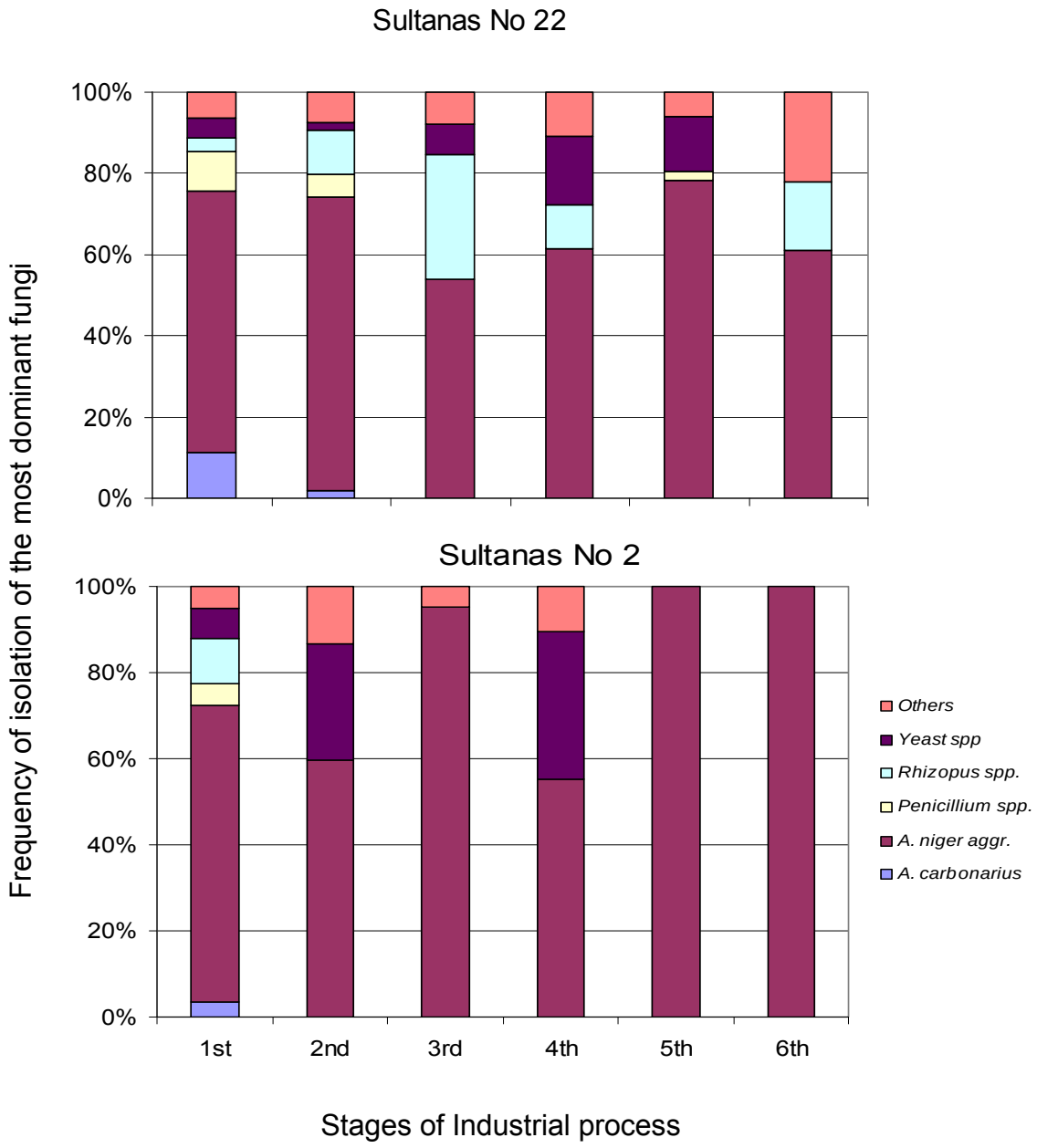


Figure 3.2. Frequency (%) of isolation of the most dominant fungal genera and species on both types of sultanas (No2 and No22 from the crop of 2004/05) treated with SO_2, on MEA95 ($0.954a_w$) isolation medium, during industrial process. The values are the means of four replicates. Keys: 1st=classification stage, 2nd=pre-washing, 3rd=SO2 treatment, 4th=final washing, 5th=heat-treatment, 6th=final product.

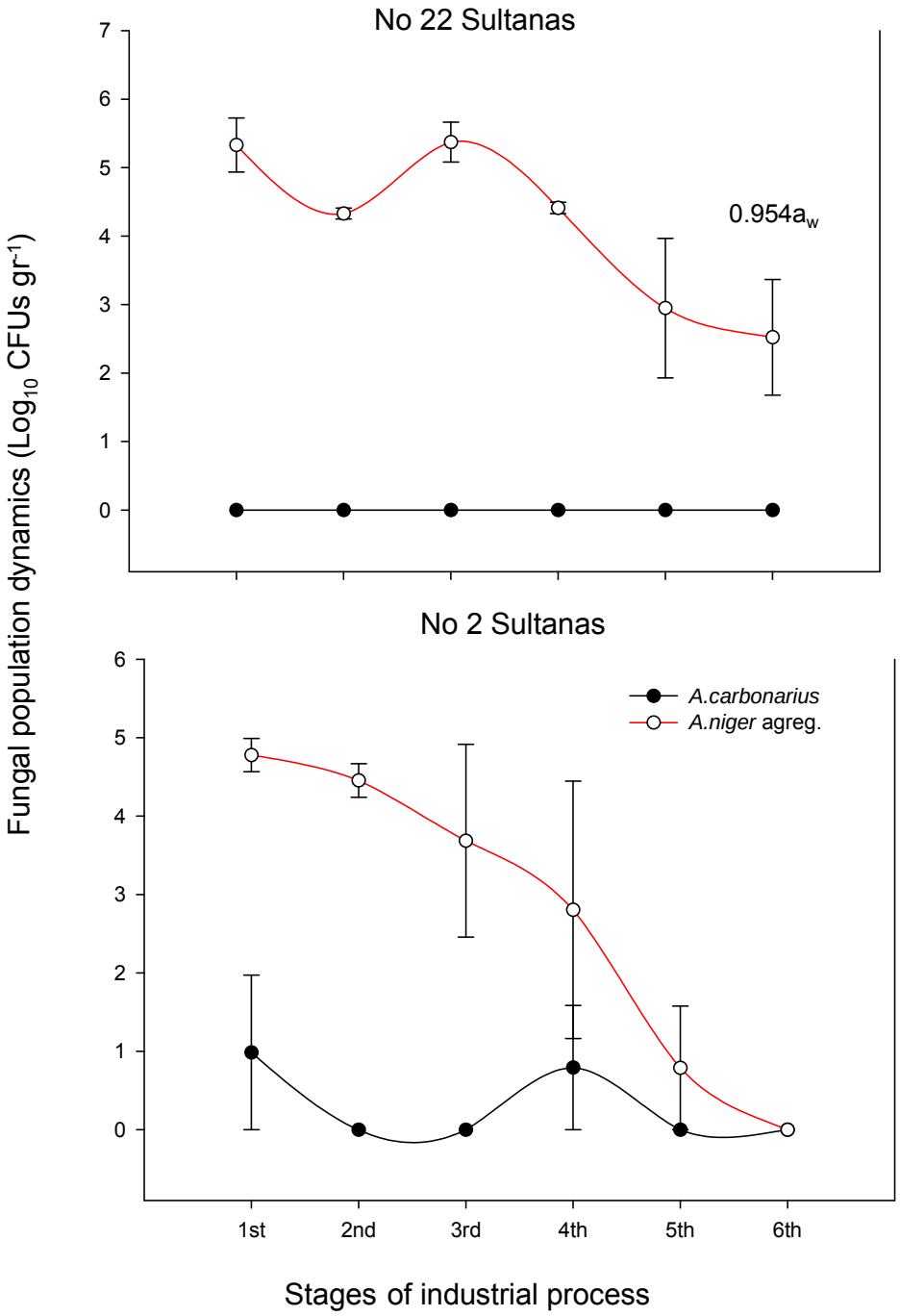


Figure 3.3. Population dynamics of black aspergilli, specifically of *A. carbonarius* and *A. niger aggregate*, on MEA95 (0.954a_w) isolation medium, during industrial process of sultanas treated with SO_2, from 2004/05 vine crop. The values are the means of four replicates. Errors bars indicate standard errors of the mean. Keys: 1st=classification stage, 2nd=pre-washing, 3rd=SO2 treatment, 4th=final washing, 5th=heat-treatment, 6th=final product.

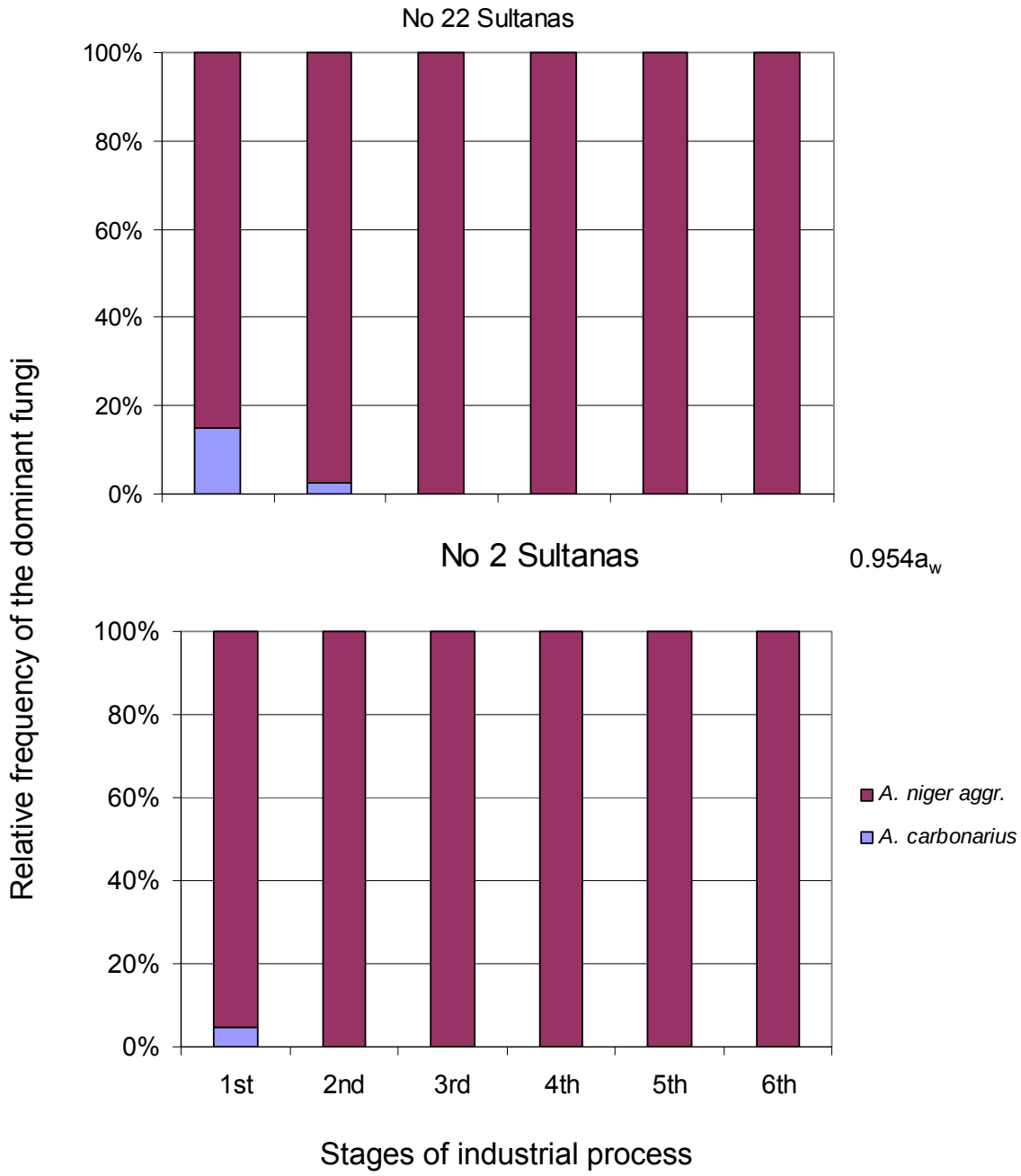


Figure 3.4. Relative frequency of isolation of black aspergilli, specifically of *A. carbonarius* and *A. niger* aggregate, on two types of sultanas (No2 and No22) from the crop of 2004/05, during industrial process with SO_2. The isolation medium used is MEA95 (0.954a_w). The values are the means of four replicates. Keys: 1st=classification stage, 2nd=pre-washing, 3rd=SO2 treatment, 4th=final washing, 5th=heat-treatment, 6th=final product.

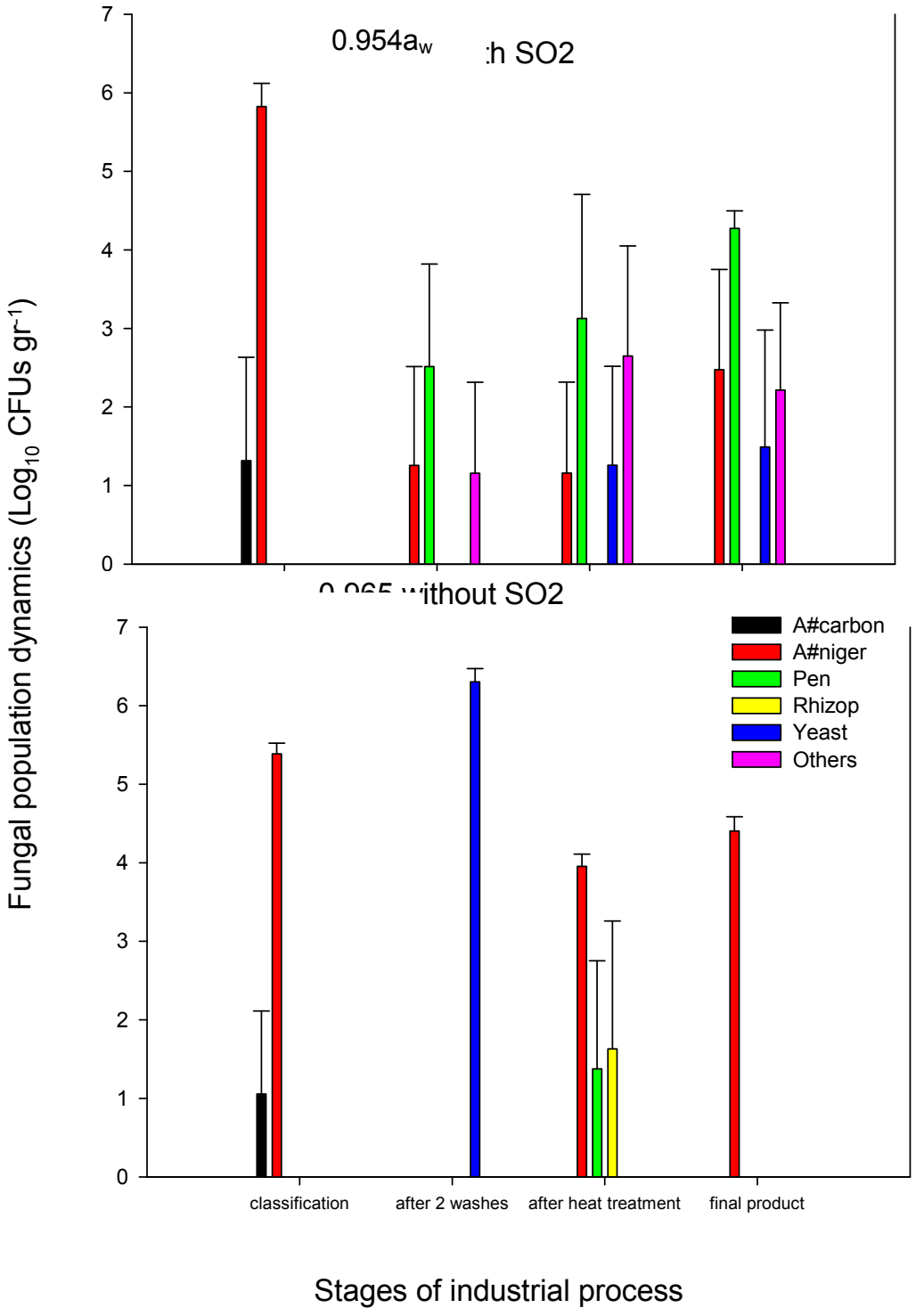


Figure 3.5. Population dynamics of the most dominant fungal species and genera on sultanas from 2005/06 crop, treated (No 2 sultanas) or not treated (No 22 sultanas) with SO_2, on MEA95 ($0.954a_w$) isolation medium, during industrial process. The values are the means of three replicates. Error bars indicate standard errors of the mean.

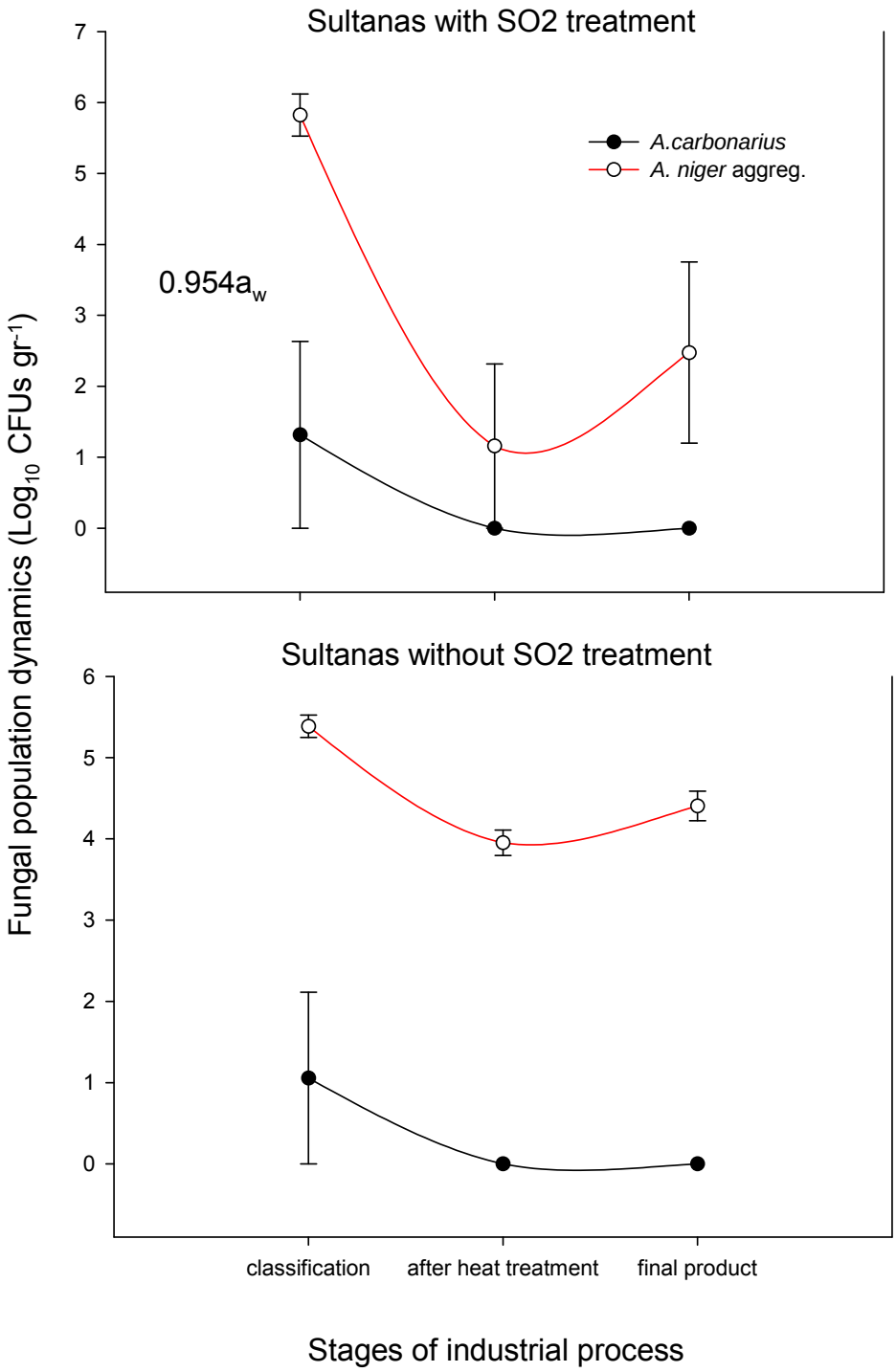


Figure 3.6. Population dynamics of black aspergilli, specifically of *A. carbonarius* and *A. niger aggregate*, on sultanas treated (No 2 sultanas) or not treated (No22 sultanas) with SO_2, on MEA95 ($0.954a_w$) isolation medium, during industrial process. The values are the means of three replicates. Error bars indicate standard errors of the mean.

Table 4.1. Nutritional composition of Sainsbury's pure white grape juice. Typical values per 100 mL of juice

Protein	0.1 gr
Carbohydrate	15.9 gr
(of which sugars)	15.9 gr
(of which starch)	Less than 0.1 gr
Fat	Less than 0.1 gr
(of which saturates)	Less than 0.1 gr
Fibre	Less than 0.1 gr
Sodium	Less than 0.1 gr
Energy	279 JK, 66 Kcal

Table 4.2. Citric acid – Na_2HPO_4 (Mcllvaine) buffer solutions, pH apprx. 2.6-7.6 (Mcllvaine, JBC 49, 183 (1921).

pH	*x* mL 0.1M Citric acid	*y* mL 0.2M Na_2HPO_4
2.6	89.10	10.90
2.8	84.15	15.85
3.0	79.45	20.55
3.2	75.30	24.70
3.4	71.50	28.50
3.6	67.80	32.20
3.8	64.50	35.50
4.0	61.45	38.55
4.2	58.60	41.40
4.4	55.90	44.10
4.6	53.25	46.75
4.8	50.70	49.30
5.0	48.50	51.50
5.2	46.40	53.60
5.4	44.25	55.75
5.6	42.00	58.00
5.8	39.55	60.45
6.0	36.85	63.15
6.2	33.90	66.10
6.4	30.75	69.25
6.6	27.25	72.75
6.8	22.75	77.25
7.0	17.65	82.35
72	13.05	86.95
7.4	9.15	90.85
7.6	6.35	93.65

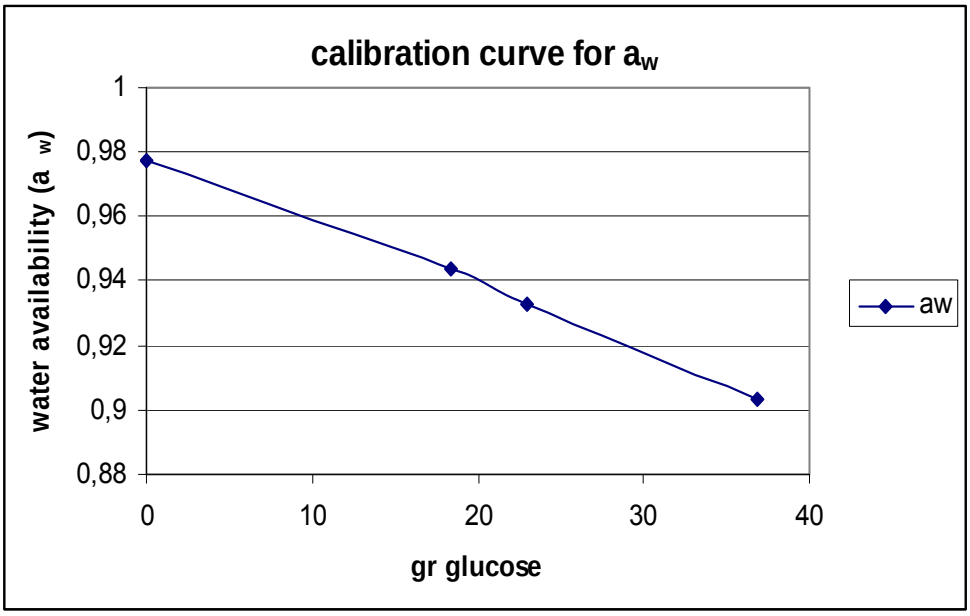


Figure 4.1. Calibration curve of water activity for white grape juice. All amounts are for those to be added to 100 mL water (Dallyn & Fox, 1980)

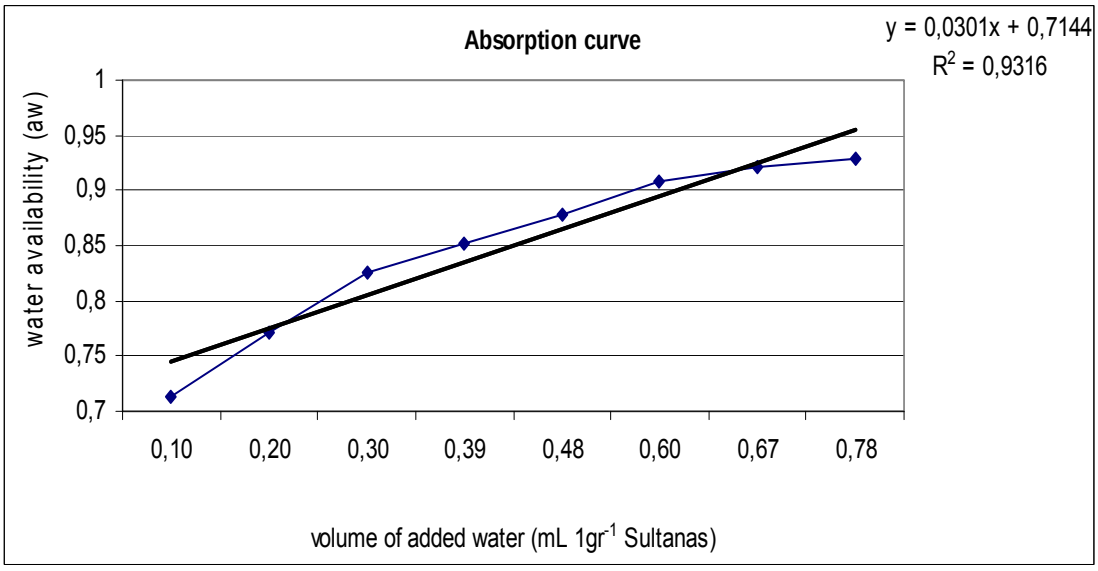


Figure 4.2. Absorption curve of sultanas

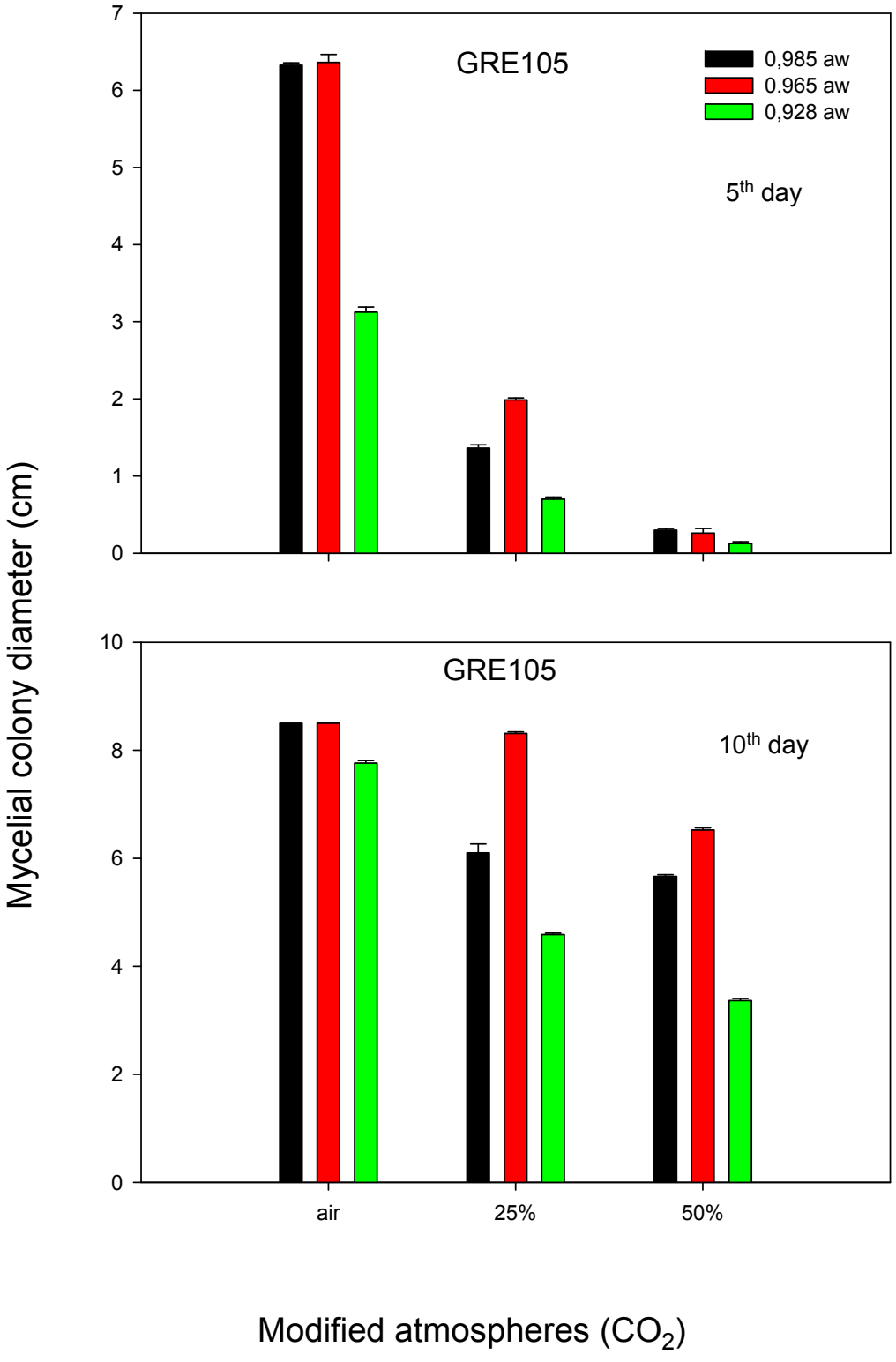


Figure 4.3. Effect of controlled atmospheres on mycelial colony diameter of *A. carbonarius* (GRE105), at three different a_w levels, the 5th and 10th day incubation, at 25^{o}C.

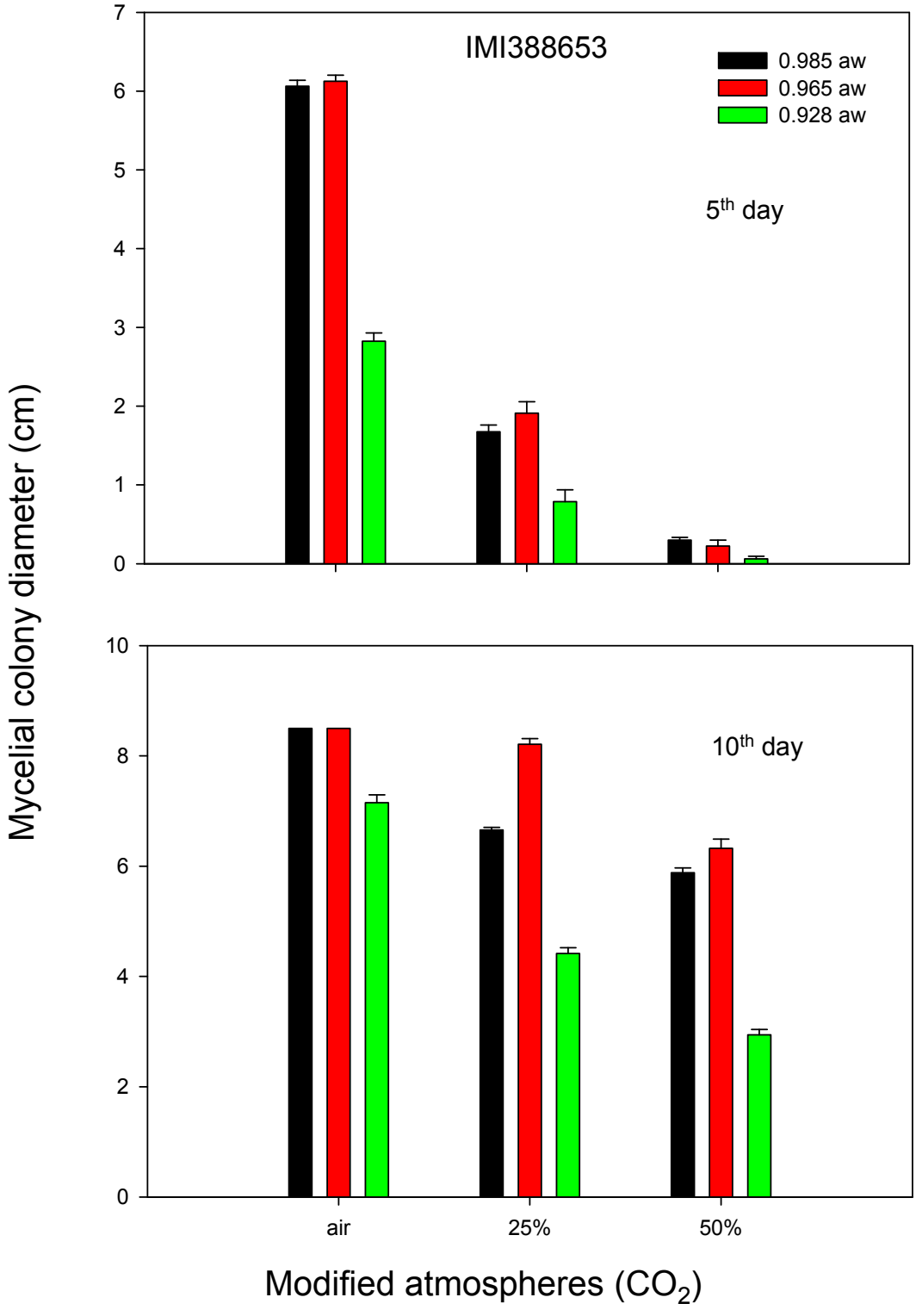


Figure 4.4. Effect of controlled atmospheres on mycelial colony diameter of *A. carbonarius* (IMI388653), at three different aw levels, the 5th and 10th day incubation at 25