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P. NATSKOULIS

Mycotoxigenic fungi and ochratoxin contamination in Greek grapes and wine

CRANFIELD HEALTH

PhD Thesis

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Supervisor:
Prof. Naresh Magan

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Abstract

Basic concept of the present study was to examine ochratoxin A (OTA) presence in Greek grapes on the field, during winemaking and at the final product, wine. Finally the project was an attempt to integrate approaches for controlling the responsible fungus (*Aspergillus carbonarius*) in Greece, together with ways to eliminate presence of OTA in wine. To this end the effect of water activity (a_w), and other important environmental factors such as temperature, pH and strains and their interactions on growth/OTA production were studied. Greek *A. carbonarius* isolates grew optimally at 30-35ºC and 0.96 a_w, while maximum OTA production occurred under suboptimal growth conditions (15-20ºC & 0.93-0.96 a_w). Greek isolates examined in the present study were more xerotolerant than others of the Mediterranean basin. Studies were performed in organic and non-organic vine grape production systems in an attempt to correlate the presence of total *Aspergillus* section *Nigri* and ochratoxigenic *A. carbonarius* strains with meteorological conditions and geographical localization. Besides the studies on ecophysiology and in the field, growth data were fitted to several predictive models in order to contribute to the evaluation of solutions that can be practically used in Greece for protecting production from OTA contamination. The effect of temperature and a_w on the growth of *A. carbonarius* strains could be satisfactorily predicted under the experimental conditions studied, and the proposed boundary models for growth/no growth and toxin/no toxin production could serve as a useful tool. Another component of the present thesis was the investigation of OTA content in Greek wines and the correlation of the results with the origin of production, the year of production, the colour and type, and other vinification techniques. This showed that Greek red, rosé and white wines had lower levels and incidences of contamination than wines from other countries of the Mediterranean basin. Moreover, Greek wines were below the European limit of 2.0 μg L^{-1}. Although contamination with the mycotoxigenic fungus was rather high, most of the samples contained low levels of OTA. The key areas of concern were the results from the southern regions of Greece, mainly Peloponnese and Aegean Islands, especially for the dessert type ones. In order to understand the

partitioning of OTA during vinification and clarification processes in Greek wines, an experimental vinification process was performed using different fermentation techniques (presence or absence of artificially inoculated OTA, *A. carbonarius*, starter yeasts). The general trend for the evolution of OTA concentration throughout the vinification process was a decrease of the initial concentration which was more significant in the first days of fermentation when acceleration of yeast population took place. This data has been integrated to produce a prevention management strategy in Greece to minimise OTA contamination in the grape-wine chain.

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MYCOTOXIGENIC FUNGI & OCHRATOXIN CONTAMINATION IN GREEK GRAPES AND WINES

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Nomenclature

a_w = water activity

$a_{w\,max}$ = a_w over which no growth occurs

$a_{w\,min}$ = a_w below which no growth occurs

$a_{w\,opt}$ = a_w at which μ_{max} is optimal

$b_w = 1 - a_w$, a_w transformation by Gibson *et al.* (1996)

CAST = Council for Agricultural Science and Technology

CFU = colony forming units

CO_2 = carbon dioxide

EFSA = European Food Safety Authority

ELISA = enzyme linked immune-sorbent assay

EMAN = European Mycotoxin Awareness Network

Eq(s) = Equation(s)

EU = European Union

FAO = Food and Agricultural Organization

HPLC = High performance liquid chromatography

IARC = International Agency for Research on Cancer

k = slope term at the point of inflexion for the rate of increasing germinated spores (d^{-1}, h^{-1})

k_i = inactivation rate (min^{-1})

N = number of spores

N(t) = number of surviving spores after treatment

N_0 = initial number of spores

ºC = degree Celsius

ºK = degree Kelvin

P = percentage of germinated spores (%)

pH_{max} = pH over which no growth occurs

pH_{min} = pH below which no growth occurs

pH_{opt} = pH at which the μ_{max} is optimal

P_{max} = maximal percentage of germinated spores (%)

r = time where $P = P_{max}/2$ (d, h)

T = temperature (ºC)

t = time (d, h)

T_{max} = temperature over which no growth occurs (ºC)

T_{min} = temperature below which no growth occurs (ºC)

T_{opt} = T at which μ_{max} is optimal (ºC)

UV = Ultra-violet

WHO = World Health Organization

y = colony diameter (or radius) (mm, μm)

y_0 = initial colony diameter (or radius), usually zero (mm, μm)

y_{max} = maximum colony diameter (or radius) attained, asymptotic value (mm, μm)

λ = lag period (defined as the intersection of the line defining the maximum specific growth rate with the x axis) (d, h)

λ_{germ} = lag phase prior germination (d, h)

μ_{max} = maximum specific growth rate (it is defined as the slope of the growth curve at the point of inflexion), (mm d^{-1}, μm h^{-1})

μ_{opt} = maximum growth rate at optimal conditions (mm d^{-1}, μm h^{-1})

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Chapter 1: Introduction and Research Objectives

1.1 Introduction

Mycotoxins are a class of highly toxic chemical compounds produced, under particular environmental conditions by several moulds (Pitt & Hocking, 1997). The presence of this diverse group of fungal secondary metabolites on feed or food has wide fluctuation from year to year mainly because of the large number of factors affecting fungal invasion and growth (Kuiper-Goodman, 2004). Some of these critical factors are: fungal strain, climate and geographical conditions, cultivation techniques and postharvest handling (JEFCA, 2001). Consumption of food contaminated with mycotoxins has been linked with carcinogenic, nephrotoxic and teratogenic potency and, generally, suppressive actions on the immune system (Creppy, 2002; Kuiper-Goodman, 2004). Mycotoxin outbreaks in developing countries have led to numerous toxicosis incidents and deaths due to the high consumption of contaminated food products (Shephard, 2008). These contaminants may occur in a wide range of agricultural products such as cereals, fresh and dried fruits, coffee beans, cocoa, coffee, and beverages such as beer and wine (Samson *et al.*, 2000). Table 1.1 summarize the most frequently occurring mycotoxins, the responsible fungal species and their toxic effects (C.A.S.T., 2003).

Table 1.1 Commodities in which mycotoxins have been found and the resulting effects on animals and humans (adapted from Bullerman 1979, 1981, 1986)

		Effects of mycotoxins	
Mycotoxin	**Contaminated commodities**	**Affected species**	**Pathological effects**
Aflatoxins (B_1, B_2, G_1, G_2, M_1, M_2)	Peanuts, corn, wheat, rice, cottonseed, copra, nuts, various foods, milk, eggs, cheese, figs.	Birds Duckling, turkey, poult, pheasant chick, mature chicken, quail Mammals Young pigs, pregnant sows, dog, calf, mature cattle, sheep, cat, monkey, human Fish Laboratory animals	Hepatotoxicity (liver damage) Bile duct hyperplasia Haemorrhage Intestinal tract Kidneys Carcinogenesis (liver tumors)
Citrinin	Cereal grains (wheat, barley, corn, rice)	Swine, dog, laboratory animals	Nephrotoxicity (tubular necrosis of kidney) Porcine nephropathy
Cyclopiazonic acid	Corn, peanuts, cheese, kodo millet	Chicken, turkey, swine, rat, guinea pig, human	Muscle necrosis Intestinal haemorrhage and edema Oral lesions
Ochratoxin A	Cereal grains (wheat, barley, oats, corn),	Swine, dog, duckling, chicken, rat, human	Nephrotoxicity (tubular necrosis of kidney)

	dry beans, mouldy peanuts, cheese, tissues of swine, coffee, raisins, grapes, dried fruits, wine		Porcine nephropathy Mild liver damage Enteritis Teratogenesis Carcinogenesis (kidney tumours) Urinary tract tumours
Patulin	Mouldy feed, rotted apples, apple juice, wheat straw residue	Birds Chicken, chicken embryo, quail Mammals Cat, cattle, mouse, rabbit, rat, human Others Brine shrimp, guppy, zebra, fish larvae	Edema Brain Lungs Haemorrhage Lungs Capillary damage Liver Spleen Kidney Paralysis of motor nerves Convulsions Carcinogenesis Antibiotic
Penicillic acid	Stored corn, cereal grains, dried beans, mouldy tobacco	Mouse, rat, chicken embryo, quail, brine shrimp	Liver damage (fatty liver, cell necrosis) Kidney damage Digitalis-like action on heart Dilates blood vessels Antidiuretic Edema in rabbit skin Carcinogenesis Antibiotic
Penitrem	Mouldy cream cheese, English walnuts, hamburger bun, beer	Dog, mouse, human	Tremors, death, incoordination, bloody diarrhea
Sterigmatocystin	Green coffee, mouldy wheat, grains, hard cheeses, peas, cottonseed	Mouse, rat	Carcinogenesis Hepatotoxin
Trichothecenes (T-2 toxin, diacetoxyscirpenol, neosolaniol, nivalenol, diacetylnivalenol, deoxynivalenol, HT-2 toxin, fusarenon X)	Corn, wheat, commercial cattle feed, mixed feed, barley, oats	Swine, cattle, chicken, turkey, horse, rat, dog, mouse, cat, human	Digestive disorders (emesis, diarrhea, refuse to eat) Haemorrhage (stomach, heart, intestines, lungs, bladder, kidney) Edema Oral lesions Dermatitis Blood disorders (leucopenia)
Zearalenone	Corn, mouldy hay, pelleted commercial feed	Swine, dairy cattle, chicken, turkey, lamb, rat, mouse, guinea pig	Estrogenic effects (edema of vulva, prolapse of vagina, enlargement of uterus) Atrophy of testicles Atrophy of ovaries, enlargement of mammary glands Abortion

Among mycotoxins, ochratoxin A (OTA) is very important. This isocoumarin derivative of the secondary metabolism of the genus *Aspergillus* (*A. ochraceus*, *Aspergillus* Section *Nigri*) and *Penicillium* (*P. verrucosum* and *P. nordicum*) has gained the attention of the scientific community due to its carcinogenic potential after consumption (WHO, 2002;

Creppy, 2002). Ochratoxin A was first reported in South Africa on 1965 as a secondary metabolite of a strain of *A. ochraceus* (Van der Merwe *et al.*, 1965). Nevertheless, only during the last two decades has the contamination of agricultural products been well documented. After cereals, wine is considered to be the major source of daily OTA intake according to several studies (Murphy *et al.*, 2006). Surveys in Europe and other wine regions worldwide, including South American countries and Australia, confirmed the frequent presence of OTA in grapes and wines. (Blesa *et al.*, 2004; Leong *et al.*, 2006b; Magnoli *et al.*, 2004; Sera *et al.*, 2004; Shephard *et al.*, 2003; Soufleros *et al.*, 2003; Stefanaki *et al.*, 2003).

OTA contamination of grape products is now widely attributed to the infection of grapes on the vine by members of *Aspergillus* Section *Nigri*. Toxigenic isolates of *Aspergillus niger* and *Aspergillus carbonarius* have been isolated from grapes in Australia (Leong *et al.*, 2006a), South America (Rosa *et al.*, 2004), South Africa (Chulze *et al.*, 2006) and many European countries (Sage *et al.*, 2002; Abarca *et al.*, 2003; Battilani *et al.*, 2006a) including Greece (Tjamos *et al.*, 2006).

There is evidence that temperature and a_w are two factors of paramount importance in influencing growth and OTA formation by mycotoxigenic spoilage fungi on wine grapes. The difference in temperature among the viticultural regions, the daily mean temperature, the reduction of water availability within the grapes due to sugar content increment and vinification techniques influence growth and OTA production by *A. carbonarius* (Pitt & Hocking, 1997; Sanchis & Magan, 2004; Belli *et al.*, 2006a; Serra *et al.*, 2006).

All these studies led to an investigation of the reasons causing this newly derived health hazard. The European Union developed legislative limits of 2 ng ml^{-1} for wine and grape juice and 10 ng ml^{-1} for dried vine fruits (123/2005/EC). Moreover, this stimulated more research on the prevention of OTA contamination of grapes and their products. In the framework of prevention strategies, research is related to both the grape culturing techniques and ecophysiology of *A. carbonarius* in the field, and moreover the clarification of the wine during vinification procedures. The present study is an attempt to integrate approaches for controlling the responsible fungus (*A. carbonarius*) in Greece, together with ways to eliminate the presence of OTA in wine.

1.2 Specific Objectives of the Research Project

The present research project was carried out to address 4 objectives (Figure 1.1). The first one was the study the mycoflora dynamics in organic/non-organic vine grape production systems in relation to the presence of ochratoxigenic *A. carbonarius* strains. For this purpose

the mycoflora population changes were determined in relation to the production system. The enumeration and isolation of the total *Aspergillus* section *Nigri* isolates, identification of the *Aspergillus carbonarius* isolates and control of their ochratoxigenic ability by both qualitative and quantitative methods were carried out.

The second objective was a detailed study of the ecology and control of *A. carbonarius* in grapes. Very little information is available on the growth tolerances to the key environmental factors and on OTA production by isolates of *A. carbonarius* from Greece and how these compare with other regions with a Mediterranean climate. As grape exhibits a wide water content gradient from the time it is set on the vineyard until processing to wine, the role of a_w/moisture content on the presence of OTA has to be clarified. In this instance, the effect of a_w, along with other important environmental factors, temperature, pH and strains and their interactions on growth/OTA production was studied. The growth studies were performed with both *in vitro* using Petri dish culture and with the "Wimpenny" gradient plate technique.

Besides the ecophysiological study, the raw growth data were fitted to several predictive models. There was a need for the development of validated predictive models that can describe the influence of environmental and preservative factors on the growth of fungi of importance in grapes. This would contribute to the evaluation of solutions that can be practically used in Greece for protecting production from OTA contamination. Several models including (i) Arrhenius type models (ii) cardinal models, (iii) Belehradrek type and (iv) polynomial models were examined to describe the trends observed.

The third objective was to understand the partitioning of OTA during vinification and clarification of Greek wines with an experimental vinification process using the different techniques of fermentation (presence or absence of OTA, *A. carbonarius*, SO_2, starter yeasts). The increasing consumer resistance to food produced with the use of chemical preservatives also force to develop techniques based on the application of natural antifungal chemicals. Thus, the final product was furthermore processed with several commercial substances for clarification of the existing OTA and their effectiveness was assessed. A survey of OTA presence in Greek commercial wines has also been conducted from the first year of the project (2004) until its end (2008). This survey contacted to help correlate the winemaking region, the type of wine and the year of production, in relation to OTA contamination and risk from contamination.

The fourth objective of the project was to integrate the gathered information to develop prevention strategies in Greece. Mycotoxin contamination of food is often a multi-stage process. Mycotoxins occur in crops when a mycotoxigenic strain is in the environment, comes

in contact with the crop, either in the field or during transportation and storage, and is able to infect and grow in that crop under conducive conditions that allow mycotoxin production. OTA must then be stable throughout the subsequent processing and the vinification fermentation, in order to present a potential health risk in the final product. These studies on ecology and OTA production must take into account the gathered knowledge for the Greek winemaking industry, which will engage the appropriate management strategies in field and during winemaking in order to minimise or even clarify the OTA content of their products.

1st AIM/OBJECTIVE: study of mycoflora dynamics in organic/non-organic vine grape production systems.

- Recordings of vine grapes' Mycoflora
- Enumeration and isolation of the total *Aspergillus* section *Nigri* isolates
- Identification of the *Aspergillus carbonarius* strains and control of their ochratoxigenic ability by both qualitative and quantitative methods

2nd AIM/OBJECTIVE: study of the ecology and the control of *A. carbonarius* in grapes.

- Effect of environmental factors (longitude, altitude, strain, a_w, T, pH) and their interactions on growth/OTA production
- Use of different methods as common Petri dishes, "Wimpenny's" gradient plates, enzyme essays.
- Predictive models to predict and control growth and OTA production.

3rd AIM/OBJECTIVE: partitioning of OTA during vinification and clarification of Greek wines.

- Experimental vinification with different techniques of fermentation (presence or absence of OTA, *A. carbonarius*, SO_2, starter yeasts)
- Clarification of the existing OTA and study of the effectiveness of the substances used
- Survey of Ochratoxin A Presence in Greek Wines.

4th AIM/OBJECTIVE: use of the gathered information for developing prevention strategies in Greece.

Figure1.1 Aims and Objectives of the research project.

Chapter 2: Literature Review

2.1 Importance of Vine Grape Production and Winemaking in Greece

2.1.1 History

Vine cultivation and wine production first appeared in Greece around 3500 BC. We have the earliest identification of wines from the period 800-1500 BC from Homer's epics. Today, the total area of vineyards with grapes for vinification amounts to roughly 70,000 hectares, of which 10% produces appellation of origin wines and the rest is for the production of table wines, including wines with a geographical indication. Total production is 4,000,000 hectolitres per year. Of the total production, 30% is intended for self-consumption, 30% is produced by privately owned wineries, and the remaining 40% is vinified by cooperatives. The wine industry has more than 350 active enterprises in both the private and cooperative sectors, the majority of which are of small or medium size and have the production of wine as their sole activity.

2.1.2 Characteristics of Greek Vineyard

The main characteristic of the Greek vineyard is the many scattered smallholdings. With regard to geographical conditions, Greece is characterized by its diversity. The soil is generally rocky, chalk with clay and lime-stone subsoil or over porous rock. There is a wide diversity of topography and aspects and vines can be found cultivated in areas from sea level up to altitudes of 1,000 meters. Climates show a similar diversity. The climate of Greece is generally classified as Mediterranean, but in the northern regions the climate can be said to be continental, while in the south it verges on the sub-tropical. This combination of soil and climatic conditions is conducive for culturing of vines. Moeover, contributes to the creation of a wide variety of microclimates, which with the many, fine local grape varieties allows for the production of a wide variety of wines. The large number of indigenous grape varieties constitutes one of the major advantages of wine production in Greece; that these varieties are unique to Greece gives the country a powerful comparative advantage on a world map that is tending toward varietal homogenization (Boutaris, 2008).

2.1.3 Ochratoxin A Presence in wine

Unfortunately, although Greece produces world-class wines, both in quality and quantity, there are numerous publications referring to contaminated wine (Stefanaki *et al.*, 2003;

Soufleros *et al.*, 2003) and products such as grapes (Tjamos *et al.*, 2004; Tjamos *et al.*, 2006; Melletis *et al.*, 2007) and dried vine fruits (MacDonalds *et al.*, 1999; Stefanaki *et al.*, 2003).

Thus, in order to protect an important economic sector such as winemaking it is essential that strategies are developed which will allow the production of wine free of any toxin contaminants.

2.2 Fungal and Mycotoxin Contamination of Vine Grape

2.2.1 Vine Grape Morphology

OTA contamination of grapes requires the presence of toxigenic fungi in the vineyard and the transfer of the fungus into the berry, together with conditions that will favour growth and toxin production. The interior of grape berry is sterile, comprising fleshy mesocarp tissue (pulp) and seeds. The berry exocarp (skin) is the primary barrier to fungal infection, consisting of a waxy cuticle, and epidermal and sub-epidermal cells. The skin can be damaged by disease, pests and environmental conditions resulting in the splitting of the fruit (Amerine *et al.*, 1972). Grapes contain high levels of sugars and other nutrients, and although they possess ideal water activity for microbial growth, their acidic pH makes them particularly susceptible to fungal spoilage, because a large bacterial component is eliminated since most bacteria prefer near neutral pH. Some fungi are plant pathogens and can start the spoilage process from the vineyard while others, although they could contaminate grapes in the field, actually proliferate and cause substantial spoilage only after harvest when the main plant defences are reduced or eliminated. Fungal spoilage of grapes will depend on cultivation, harvesting, handling, transport, and post-harvest storage and marketing conditions (Katsoudas & Tournas, 2005).

2.2.2 Mycobiota and Ochratoxin A Contamination

Moulds commonly isolated from grapes are *Botrytis cinerea*, *Alternaria* and *Cladosporium*. Less common are *Fusarium*, *Penicillium*, *Aspergillus*, *Ulocladium* and yeasts (Pit & Hocking, 1997). In a recent survey carried out in Uruguay to evaluate the mycoflora of grapes, *Aspergillus* species were isolated in low frequency. Again, the main species isolated were *A. alternata*, *Cladosporium cladosporioides* and *Epicoccum purpurascens*. Among the *Penicillium* species isolated *P. chrysogenum*, *P. minoluteum* and *P. decumbens* were most frequently isolated, while *Botrytis cinerea* was commonly isolated at harvest (Chulze *et al.*, 2006).

From the above genera only *Aspergillus* and *Penicillium* include ochratoxigenic species. The Aspergilli the section *circumdati* (yellow Aspergilli) are well known OTA producers. They have been studied extensively on cereals and cereal products where *A. ochraceus* and *Penicillium verrucosum* are considered the major cause of OTA contamination of many commodities (Ramos *et al.*, 1998; Magan, 2006; Cabañas *et al.*, 2008; Villa & Markaki, 2009). The same species were also believed to produce OTA in grapes until 1999 when *A. carbonarius* and *A. niger* were identified in dried vine fruits (Codex Alimentarius Commission, 1999). *Aspergillus* section *Nigri*, the so-called "the black Aspergilli", were first described as OTA producers by Abarca *et al.* (1994), and this was furthermore confirmed by many other researchers (Abarca *et al.*, 2001; Cabanes *et al.*, 2002; Samson *et al.*, 2004; Perrone *et al.*, 2005).

After more than a decade of research there are many published studies supporting that *Aspergillus* section *Nigri* and in particular *A. carbonarius* having a central role in OTA contamination of grape products (Cabañes *et al.*, 2002; Bellí *et al.*, 2005b; Jørgensen, 2005; Battilani *et al.*, 2006a). *A. carbonarius* is supposed to play the main role for two reasons. Mainly, because the percentage of positive strains and the amount of OTA produced in vitro are definitely higher than in other black aspergilli. Furthermore, because the frequency of *Aspergillus* Section *Nigri* that are present on bunches on early véraison, increases going towards later grape's growth stages (Cabañes *et al.*, 2002; Battiliani & Pietri, 2002; Belli *et al.*, 2004b; 2005b; Serra *et al.*, 2006b).

2.3 Mycofloral Dynamics in the Field

2.3.1 Black Aspergilli Distribution

There is lack of information in the international literature referring to black Aspergilli field dynamics. The reason is that *Aspergillus* section *Nigri* was not believed to be a classical pathogen of grapes (Battiliani *et al.*, 2004). Although, sometimes black Aspergilli can cause "black rot" disease of grape, due to high sporulation on berries, resulting in shrunken and dry fruit (Visconti *et al.*, 2008). Soon after the first reports of OTA presence in wine (Majerus & Otteneder, 1996; Zimmerli & Dick, 1996), researchers started isolating ochratoxigenic black *Aspergillus* spp. from the field of vineyards, believing that such species were the main source of contamination (Abarca *et al.*, 2001). The distribution of the black Aspergilli in vineyards is represented by three subgroups: the *A. niger* "aggregate" which is the principal group, the *A.*

carbonarius whose incidence is 2-3 times less than *A. niger* "aggregate", and the *Aspergillus* uniseriate spp. as the least represented group (Battilani *et al.*, 2006b; Visconti *et al.*, 2008).

In recent years research has been accelerated in relation to mycofloral dynamics on the field. Based on a recent geostatistical study, Greece was an area which had a higher isolation percentage of *A. carbonarius* when compared with other countries of the Mediterranean basin (France, Israel, Italy, Portugal and Spain), as well as high contamination in its vineyards by black Aspergilli at harvesting time, exceeding 50% of berries (Battilani *et al.*, 2006b). It is also of major importance that, although black Aspergilli have a significantly low percentage of OTA-producing strains, not exceeding 10-15%, more than 90% of the isolated *A. carbonarius* strains from many studies investigating grape mycoflora are positive for ochratoxin A production (Serra *et al.*, 2003; Battilani & Pietri, 2004a; Battilani *et al*, 2006a; Belli *et al.*, 2006d; Gomez *et al.*, 2006). Moreover, the OTA producing capacity of these isolates, when examined in vitro, could reach sometimes levels up to 5000 ng mL^{-1} or g^{-1} of culture medium (Magnoli *et al.*, 2004; Belli *et al.*, 2005a; Valero *et al.*, 2006a).

The findings of Cozzi *et al.* (2006) are particularly interesting as they found higher levels of contamination by black Aspergilli and OTA in grapes originating from bunches damaged by *Lobesia botrana* larvae as compared to bunches without *L. botrana* attacks (Cozzi *et al.*, 2006). In this direction Belli *et al.* (2007a) published results that confirmed the theory that damaged berries were more susceptible to black Aspergilli colonisation. Other researchers noticed that rain prior to harvest was a common cause of berry damage, favouring *Aspergillus* infection (Serra *et al.*, 2003; Leong et al. 2006a; Cozzi et al. 2007). Berry damage caused by insects, birds or other fungal infection is one of the primary factors affecting disease development and OTA accumulation in berries (Visconti *et al.*, 2008; Martinez *et al.*, 2009).

2.3.2 Geographical and Climatological Attributes

There are studies which have attempted to examine the way that geological and climatic factors influence the presence of *A. carbonarius* and its ochratoxin A production potential. Important findings correlate strongly the incidence of *A. carbonarius* with latitude and longitude of the field, distance from the sea, and the meteorological conditions. Cozzi *et al.* (2007) found that meteorological conditions and closeness to sea played a major role in OTA presence, while Pateraki *et al.* (2007b) observed higher frequencies of isolation and OTA levels from sultanas dried near the sea level from those at the highest altitude levels (1000m). Batillani *et al.* (2006b) used the combination of day-degree and rainfall parameters to draw thermo-wetness maps which were correlated with the incidence of A. *carbonarius* in the

Mediterranean basin. In the same study the incidence was significantly correlated with latitude and longitude with a positive West–East and North–South gradient (Battilani *et al*, 2006b). This has also been confirmed by several other researchers that revealed data of OTA contamination in wines from different countries of Europe. These researchers have noted that the OTA content of European wine from the south of Europe is higher than in wine from northern Europe (Jørgensen, 2005; Burdaspal & Legarda, 2007; Melletis *et al.*, 2007; Clouvel *et al.*, 2008; Valero *et al.*, 2008).

2.3.3 Cropping Systems

Another important issue, not clearly defined yet and possibly playing a key role in OTA contamination of vineyards, is the cropping system followed. Cozzi *et al.* (2007) monitored the role of the cropping system in a 2-year survey carried out on four different systems, namely spur-pruned cordon, bower system, head (or small tree) system and espalier. In both years, the espalier cropping system produced the most contaminated grapes in terms of *A. carbonarius* infection and OTA accumulation. This could be explained by the closeness of bunches to the soil, which is the most important source of *A. carbonarius inoculum*, compared to spur pruned cordon and bower system. The higher humidity occurring in the espalier cropping system compared to the head system can explain the different contamination level, despite the similar distance of bunches from the soil (Cozzi *et al.* 2007). Studies have been also contacted to reveal the effect of organic *versus* conventional culturing. OTA content was determined in 44 organically and conventionally produced wines originating from different geographical regions, but unfortunately there was not any statistically significant difference between the two types of production (Chiodini *et al.*, 2006). In contrast Ponsone *et al.* (2007), when analyzed grapes cultured organically and conventional in Argentina, found that the percentage of samples colonized by *Aspergillus* section *Nigri* and the percentage of *A. carbonarius* were significantly influenced by the cropping system, and were less important in the organic cultured fields at harvesting (Ponsone *et al.*, 2007).

2.4 Ecophysiological attributes of mycotoxigenic fungi with specific reference to *Aspergillus carbonarius*

2.4.1 Water activity (a_w) and temperature

In contrast to bacterial growth, were temperature is the most important determinant of growth, water activity is the most important environmental factor affecting the ability of fungi

to germinate, grow and establish themselves on food. Temperature is also very important, this being highlighted in its well documented interactions with a_w in determining fungal growth (Pitt & Hocking, 1997; Adams & Moss, 1999; Dantigny *et al.*, 2005a). Temperatures differ among viticultural regions, among seasons, during stages of maturation, while a_w within the berry decreases during maturation upon accumulation of berry sugars. As a result, mentioning one factor without the other might be useless, and the studies of the ecology of *Aspergillus carbonarius* have to examine together these factors. Fungal growth results from the complex interaction of these factors together with incubation time. Thus an understanding of each factor involved is essential to understand the overall process and to predict fungal spoilage and OTA production (Pardo *et al.*, 2005a; Magan & Aldred, 2005).

Fungal growth is generally characterized by minimal, optimal and maximal a_w or temperature values for both germination and hyphal/mycelial extension. Many researchers assessed the influence of ecophysiological determinants based on the growth and OTA production on classic mycological media such as CYA, MEA and YES (Esteban *et al.*, 2004 & 2006; Palacios-Cabrera *et al.*, 2005; Romero *et al.*, 2007; Kapetanakou *et al.*, 2009). In the last decade experiments were mainly conducted on a synthetic grape juice medium (SGM or SNM), simulating grape composition at véraison, in order that the results can be comparable between the different studies. There is a significant number of studies on ecophysiology of *A. carbonarius* (Belli *et al.*, 2004a, 2004c; 2005a; Mitchell *et al.*, 2004; Leong *et al.*, 2006a; Valero *et al.*, 2006a, 2006b, 2007a, 2008; Pateraki *et al.*, 2007a; Tassou *et al.*, 2007a, 2007b, 2009). Most studies have shown that the optimum a_w for growth is at around 0.98 and a temperature of 30 to 35ºC. As regards the marginal condition for growth, this could be defined by a minimum a_w of 0.87 and a minimum temperature of 10-15ºC. The upper limits of growth are defined by a temperature between 38 and 40°C, and a_w area of freely available water. OTA production was generally found to be optimum at 20ºC and 0.93-0.98 a_w, with maximum accumulation at 10-20 days incubation. Generally, OTA production is greater at suboptimal conditions for growth. Accumulation tends to deplete when temperature reaches the upper marginal conditions of growth but this is not happening for the lower marginal temperature and a_w. Nevertheless, some results seem to be contradictory, mainly due to isolate and regional variations. Giving some examples, Belli *et al.* (2004a; 2004b; 2004c; 2005a) repeatedly referred to differences between growth rates and OTA production of several strains of *A. carbonarius* when tested under the same media and environmental conditions. Cabañes *et al.* (2002) analysed the OTA produced after 7, 14 and 21 days at 25ºC, by two *A. niger* aggregate and five *A. carbonarius* isolates, resulting in a different maximum OTA

accumulation that appeared to depend on the strain. Mitchel *et al.* (2004) presented differences in growth rates and OTA production between strains from several European countries, and sometimes between strains of the same origin. Testing isolates of different countries of origin may partly explain the different adaptation of the isolates to different ecophysiological conditions (Bellí *et al*, 2004b; Mitchell *et al.*, 2004).

2.4.2 Antagonism between different species

Another aspect of interest is the antagonism between different species colonising grapes. Studies conducted by Valero *et al.* (2007a, b) showed that *Aspergillus* section *Nigri* is very often dominant when grown together with other grape-associated filamentous fungi. When they simulated *in vitro* sun-drying of grapes, they revealed that when competing fungi were added to *A. carbonarius* inoculum, the OTA content was reduced. Competing mycoflora acted as an additional control factor against OTA accumulation at 30°C, but at 20°C, where OTA production is optimal, this did not happen (Valero *et al*., 2006b; 2007c). In another study the application of *Candida guillermondi* as an antagonistic biocontrol agent has also shown some success on reducing *Aspergillus* rots in Israel (Zahavi *et al.*, 2000).

2.4.3 Atmosphere composition

The surrounding atmosphere may also affect growth and OTA production by *A. carbonarius*. However, recent studies by Pateraki *et al.* (2007a) showed that modified atmospheres do not play a critical role. They found that for five strains of *A. carbonarius* using controlled atmospheres (25 and 50% CO_2) at different a_w levels on SGM and 25ºC it was difficult to control germination, growth and OTA production effectively, especially after 10 days exposure. It was also noted that a_w was a more significant factor than the modified atmosphere (Pateraki *et al.*, 2007a).

Another study evaluated growth of *A. carbonarius* and *A. niger* under modified atmosphere packaging (1% O_2 - 1% O_2/15% CO_2, 5% O_2). These studies also concluded that modified atmospheres is unlikely to be suitable as a sole method for OTA minimization and grape preservation, since of the atmospheres tested, only 1% O_2 combined with 15% CO_2 consistently reduced fungal growth and OTA synthesis by *A. carbonarius* and *A. niger* (Valero *et al.*, 2008).

Lazar *et al.* (2008) evaluated the antifungal activity of nitric oxide (NO) against the growth of the postharvest horticulture pathogens *Aspergillus niger*, *Monilinia fructicola* and

Penicillium italicum and found that short-term exposure to a low concentration of the gas was able to inhibit the subsequent growth of the fungi.

Finally, very interesting findings were reported from a study conducted by Dantigny and his colleagues, on the effect of ethanol vapours on growth of several food spoilage moulds, including *A. niger*. Ethanol appeared to be an effective additional barrier to inhibit fungal growth in food products and could represent an interesting alternative to the use of preservatives (Dantingy *et al.*, 2005b).

2.4.4 Fungicides

Other important factors, at the pre-harvest stage, are the culturing techniques and whether organic or conventional cultivation systems are employed. There are some studies showing the effect of fungicides used for conventional types of cultivation but more work is needed in this direction. Data to characterize the behaviour of *A. carbonarius* in relation to fungicide treatments are scarce. Belli *et al.* (2006b) tested the efficiency against this mould at 20 and 30ºC for a range of 26 fungicides designed to control other species infecting vines, noting that not all the fungicides that reduced growth reduced the OTA synthesis and fungicides that contained copper or strobilurins reduced both growth and the toxin production, contrary to sulphur-based fungicides. Studies by Mitchell (2007) suggested that there was an interaction between environmental factors such as water activity, growth and OTA production in the presence of fungicides. In some cases this resulted in a significant stimulation of OTA production under water stress.

In a recent study by Gustav *et al.* (2008), sulphur dioxide (SO_2) generator pads used to reduce the number of *A. carbonarius* isolates from grapes on SGM, but this only occurred in combination with storage at 0ºC. Moreover, exposure of *A. carbonarius* to a final level of 0.4 ppm of SO_2 resulted in fewer fungal colonies than in the control, but the surviving spores developed into fungal colonies, although they failed to sporulate (Gustav *et al.*, 2008).

Medina *et al.*, (2007a) examined the efficacy of natamycin to control *A. carbonarius* growth and OTA production under various combinations of a_w and temperature. Natamycin at concentrations of 50-100ng ml^{-1} appeared to be very effective on controlling growth and OTA production by strains of *A. carbonarius* over a range of environmental conditions on grape-based media. Medina *et al.* (2007b) also tested the efficacy of Carbendazim, a systemic fungicide that is widely used against fungal infections in grapes, found that it positively influenced OTA production by *A. carbonarius* in the field and concluded that this could increase OTA content in grape juices and wines. Similar conclusions were reached by

Tjamos *et al.* (2004) demonstrating that chemical applications with the fungicides Carbendazim or Chorus were ineffective in controlling the fungus in Corinth raisin cultivars. Nevertheless, the fungicide Switch, especially under low to intermediate *Aspergillus* infection of vineyards, could both significantly reduce the occurrence of OTA-producing *Aspergillus* spp. (Tjamos *et al.*, 2004). Valero *et al.* (2007b) evaluated the effect of preharvest grape pesticides, Chorus and Switch, in *Aspergillus* section *Nigri* infection in dehydrating grapes, the final ochratoxin A (OTA) content with Switch was slightly more efficient than Chorus in inhibiting mould colonization and in preventing OTA production both in inoculated and non-inoculated grapes (Valero *et al.*, 2007b). Finally, Belli *et al.* (2007b) also ended at the same conclusions when studied the effect of the two above mentioned fungicides and their mixture on the field for inhibition of growth and OTA production by *A. niger* aggregate and *A. carbonarius*.

2.5 Ochratoxin A

After the emergence of ochratoxin A (OTA) and its connection with *Aspergillus ochraceus* by Van der Merwe *et al.* (1965), more *Aspergillus* species were shown to produce ochratoxins in subsequent studies: *A. melleus, A. sulphureus, A. alliaceus, A. sclerotiorum, A. albertensis, A. auricomus, Neopetromyces muricatus*, and *A. lanosus*. All these species were originally grouped in *Aspergillus* section *Circumdati*, but *A. alliaceus, A. lanosus* and *A. albertensis* are now placed in section *Flavi*. *Aspergillus ochraceus*, that was polyphyletic as traditionally defined, recently was split into several species, some of which include OTA-producing isolates: *Aspergillus cretensis, A. flocculosus, A. pseudoelegans, A. roseoglobulosus, A. westerdijkiae*, as well as *A. ochraceus sensu strictu*. Ochratoxin A production has also been found in *A. glaucus* (section *Aspergillus*). Later, ochratoxin production was found in *A. niger* var. *niger* and subsequently in other species of *Aspergillus* section *Nigri*: *A. awamori* and *A. carbonarius* and recently, in two newly described species isolated from coffee, *A. lacticoffeatus* and *A. sclerotioniger* (Bayman & Baker, 2006).

OTA has a pentaketide skeleton, and contains a chlorinated isocoumarin moiety linked through a carboxyl group to L-phenylalanine via an amide bond. The chemical structure of OTA is shown in Figure 2.1. OTA is one of a family of mycotoxins including Ochratoxins B and C which are the dechlorinated analogue and the ethyl ester respectively. The isocoumarin carboxylic acid (ochratoxin *α*) and its dechlorinated analogue (ochratoxin *β*) are also

detectable in cultures of OTA-producing strains of *Aspergillus* and *Penicillium* and 4-hydroxy OTA has also been found in cultures of *Aspergillus ochraceus*. (JEFCA, 2001).

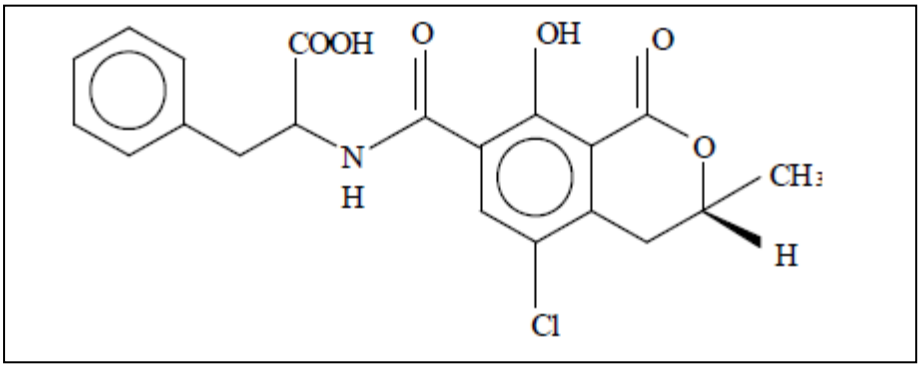

Figure 2.1 Stereo-chemical structure of ochratoxin A

Many researchers verified adverse health effects, mainly on kidney and liver and subsequently its teratogenic potential (Purchase & Theron, 1968; Brown *et al.*, 1976; Peraica *et al.*, 1999). OTA was also associated with an endemic nephropathy, called "Balkan Endemic Nephropathy" in the Balkan countries linked with the frequent consumption of contaminated porcine ham (Stoev, 1998). Nevertheless, there are still doubts about the acute linkage of OTA with this incident (Mally *et al.*, 2007). Until now, many surveys are still suggesting that the presence of OTA in food impacts human health (Creppy, 2002; Heussner *et al.*, 2006; Clark & Snedeker, 2006).

The toxicological status of OTA has been reviewed several times and detailed monographs have been published by the International Agency for Research on Cancer (IARC, 1993), by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2001) and European Food Safety Authority (EFSA, 2006). From the available evidence it is clear that OTA is potently nephrotoxic and carcinogenic. It is also teratogenic and immunotoxic, affecting both humoral and cell-mediated immunity. For these reasons IARC classified OTA as a possible carcinogen of group 2B (IARC, 1993). Ochratoxin A is efficiently absorbed by the body and it has a long residence time in body tissues, thus it is detectable in the blood of mice 18 days after receiving a single dose. Being a derivative of L-phenylalanine, it is a potent inhibitor of the incorporation of this amino acid into proteins by inhibiting phenylalanine t-RNA synthetase. It also inhibits the biosynthesis of tyrosine by inhibiting phenylalanine hydroxylase (Moss, 2008; EFSA, 2006).

2.6 Partitioning of Ochratoxin A during Vinification and Clarification of Wines

2.6.1 Presence of OTA in Greek wines

The widespread of human exposure to OTA is well documented by a number of surveys reporting the occurrence of the mycotoxin in a variety of food products. An assessment of dietary intake of OTA by the population of the European Community has been performed showing that the main contributors to OTA exposure are cereals and their products, followed by wine, coffee and beer. Moreover, grape products such as dried vine fruits and grape juice contribute to a significant extend to the children OTA exposure (European Commission, 2002).

OTA was detected in wine for the first time in 1996 by Zimmerli and Dick (1996). This study referred to OTA determination of 118 European table wines whose incidence of contamination was 70%, but with low range of OTA concentration in the samples (<0.005-0.4 μg kg^{-1}). Thereafter, several surveys have been contacted, mainly in Europe, showing that the problem is more severe for Southern Europe. A great number of surveys show a gradient in contamination correlated to the colour, and therefore to the production process of wine. Both OTA incidence and concentration were increased in the order white < rosé < red (Visconti *et al.*, 1999; Otteneder & Majerus, 2000; Soleas *et al.*, 2001; Belli *et al.*, 2004d; Blesa *et al.*, 2004; Ng *et al.*, 2004; Rosa *et al.*, 2004; Anli *et al.*, 2005; Mateo *et al.*, 2007). Several authors reviewing surveys for OTA presence in wines originated from all over the world, ended up to the same results. Otteneder & Majerus (2000), except from the 450 wines that analysed, they reviewed a total of 400 wines presenting the same gradient in incidence and concentration. Jorgensen (2005) reviewing SCOOP-2 report's 1470 wine samples and Visconti *et al.* (2008) reviewing 3512 wines, both reported a gradient of red>rosé>white type wines. Nevertheless, there are some studies that did not reveal the same conclusion (Shepard *et al.*, 2003; Stefanaki *et al.*, 2003). Another, common base in all these and many more surveys was the higher level of incidence and concentration of OTA in wines originated from Southern European regions of production in contrast with those of northern regions (Otteneder & Majerus, 2000; Pietri *et al.*, 2001; Anli *et al.*, 2005; Jorgensen, 2005; Visconti *et al.*, 2008). Only Soleas *et al.* (2001) did not find any evidence to support the South – North gradient. Finally, many studies agreed that special wines, more known as dessert wines, have often greater levels of OTA (Pietri *et al.*, 2001; Belli *et al.*, 2004d; Mateo *et al.*, 2007). Moreover, Burdaspal & Legarda (2007) and Valero *et al.* (2008b) found an incidence in dessert wines greater than 90%, whereas the

former study revealed a maximum concentration of 4.63 μg l^{-1} and the latter higher occurrence in wines that originated from must fortified before fermentation and those made from grapes dried by means of sun exposure. Belli *et al.* (2004d) found a concentration of 15.25 μg l^{-1} in a muscatel. Only a survey contacted by Blesa *et al.* (2004) showed that dessert wines were less contaminated than dry types of wine. As regards surveys for Greek wines, Markaki *et al.* (2001) analysed 31 samples of red wine and all contained OTA, with 9 of them exceeding 0.1 μg l^{-1}, but always lower from the maximum permitted EU level of 2.0 μg kg^{-1}. Soufleros *et al.* (2003) analysed 28 dry wines (14 red, 13 white, one rosé) and 7 sweet wines (3 red, 4 white) finding the same gradient of concentration as above (sweet wines > dry wines / red > white), while Stefanaki *et al.* (2003) analysed 268 wines but without finding any differences between red, rose, white types. Nevertheless, the Southern > Northern and sweet > dry gradients was again present. Generally, in Greece, more than 66% of samples showed detectable OTA levels and both red and sweet wines showed the highest levels (Markaki *et al.*, 2001; Soufleros *et al.*, 2003; Stefanaki *et al.*, 2003). Finally 100% of samples analyzed in Turkey had detectable levels of the toxin (Anli *et al.*, 2005). All the studies cited are primarily aimed at quantifying OTA, while little information is available about the origin of the toxin. According to Battilani & Pietri (2002) and Soufleros *et al.* (2003) the gradient between the colour types of wine may be connected with the longer mash standing in red vinification, while suggested that OTA found in sweet wines of southern European origin is probably formed after the harvest of the grapes, and mainly due to the technique of sun drying, prior to the alcoholic fermentation.

2.6.2 Fate of OTA during vinification

Several factors in winemaking procedures such as conditions of storage of the harvested grapes, type of maceration, and conditions of fermentation can play a crucial role for the presence of OTA in wines. In the production of wine, grapes undergo multiple stages during which solids and liquids are separated. In white vinification, grapes are crushed, then pressed to remove the skin and seeds. The juice may be treated with pectinase to enhance precipitation of grape solids before fermentation commences. In red vinification, grapes are crushed then fermented in the presence of skins and seeds to extract colour and tannins. This mixture is later pressed to remove skins and seeds. Both white and red wines undergo successive clarification stages to remove precipitated yeasts and other solids. Malolactic fermentation, in which malic acid is converted into lactic by lactic acid bacteria, may also occur after fermentation.

Lasram *et al.* (2008) studied the evolution of OTA in red and rosé vinification and resulted that the maceration of pomace had a significant effect on the increase of OTA content in red wine whereas the alcoholic fermentation had a reducing effect. After crushing, OTA content was distributed between the must and the pomace. Their data pointed out that the skin contained a large part of OTA produced by *Aspergillus carbonarius*, something that was also pointed out from other authors (Battilani & Pietri, 2002) after investigating the occurrence of the toxin in the skin and the pulp. However, the spontaneous malolactic fermentation showed no significant effect on the OTA content in wine. They also revealed that storage of red wine in tanks followed by draining caused a significant decrease of OTA of about 55% (Lasram *et al.*, 2008). Grazioli *et al.* (2006), studying the effect of processing stages of winemaking, ended up to the same conclusion, that no OTA is produced during winemaking, but each operation can modify OTA content. The OTA naturally present in grapes to a certain degree is released to the juice during crushing. Maceration increases the OTA content, while alcoholic and malolactic fermentation cause a reduction in OTA in the wine. In a similar study, but with artificially contaminated grapes, the evolution of ochratoxin A (OTA) content was assessed from must to wine during the making of Port Wine. The levels of OTA observed during the vinification dropped by up to 92% (Ratola *et al.*, 2005). Another study by Cecchini *et al.* (2006) pointed out the dependence of the yeast strain involved in the fermentation on the fate of OTA content in vinification. The absence of degradation products suggested an adsorption mechanism from the yeast lees that was relevant to the different yeast strains used. Moreover, OTA concentration in methanolic yeast lees from red must fermentation was higher than in white.

2.6.3 Removal /decontamination of OTA from wines

OTA detoxification strategies are classified depending on the type of treatment – physical, chemical or microbiological – and their objective is to reduce or eliminate the OTA toxic effects by destroying, modifying or absorbing this mycotoxin (FAO/WHO/UNEP, 1999). The ideal detoxification method would be easy to use and economical, and would not generate toxic compounds or alter other food quality parameters. Thus, firstly, the effect of processing stages on the toxin reduction should be studied and in the case that this would not be possible, other additional treatments (physical, chemical or microbiological) should be considered.

Fernandes *et al.* (2003, 2005) reported the reduction of OTA whenever solids and liquids were separated, thus these authors proposed that the partitioning of OTA to certain substrates was the primary means of removal during vinification.

Bejaoui *et al.* (2004a) succeeded a significant decrease of OTA levels in YPG medium and SGM by using six oenological *Saccharomyces* strains reaching a maximum decrease of 45%. When both heat and acid pretreated yeasts were used, OTA removal was enhanced, indicating that adsorption, not catabolism, is the mechanism to reduce OTA concentrations. Adsorption was also improved when the yeast concentration was increased and when the pH of the medium was lower. Approximately 90% of OTA was bound rapidly within 5 min and up to 72 h of incubation with heat-treated cells of either *S. cerevisiae* or *S. bayanus*. In a similar study investigating the performance of 20 strains of *Saccharomyces sensu stricto* to remove ochratoxin A (OTA) during vinification, the results were indicated that OTA-removal from grape must was probably carried out by the yeast cell wall, just like the aforementioned study resulted (Caridi *et al.*, 2006). In another study, 40 isolates representing the black apergilli species *Aspergillus carbonarius*, *A. niger* aggregate and *A. japonicus*, isolated on French grapes, were assessed for OTA degradation capacities in czapek yeast extract broth (CYB) and in a synthetic grape juice medium (SGM) contaminated with OTA. It was clearly observed that in both media these fungi had the ability to degrade OTA to OTa (ochratoxin a) (Bejaoui *et al.*, 2004b). Varga *et al.* (2000) also tested *A. fumigatus* and black *Aspergillus* strains to detoxify ochratoxin A in culture media and ended up to the same conclusion. Nevertheless, the use of black aspergilli to eliminate OTA that is a metabolite of the same sections of aspergilli it is not much promising, with the exception, probably, of non OTA producer strains of *A. niger* which is one of the few fungi which has received GRAS (generally recognized as safe) status from the US Food and Drug Administration (due to its low toxicity) and could therefore be of interest in further uses for the biological elimination of OTA in grape juices and musts. Finally, Mateo *et al.* (2010) tested ten *Oenococcus oeni* strains to remove OTA from culture medium with significant differences depending on the strain, incubation period, and initial OTA level in the medium. Mycotoxin reductions were higher than 60%.

Beyond the physical and biological methods discussed above, the most promising decontamination can be suggested that of chemical treatments during winemaking process. A variety of fining agents, including activated carbon, silica gel, potassium caseinate, egg albumin, and gelatine, have been evaluated in relation to their abilities to remove OTA in fortified wines (Amézqueta *et al.*, 2009). Castellari *et al.* (2001) pointed out that potassium

caseinate and activated carbon were found to be the best fining agents that could be used to remove OTA in wine. Potassium caseinate removed up to 82% of OTA, whereas activated carbon showed the highest specific adsorption capacity. The same results were revealed by Olivares-Marin *et al.* (2009) and Var *et al.*, (2008), both teams proving the efficiency of activated carbon on detoxifying wines. The later authors also tested the efficiency of bentonite, but caused only small decreases in the OTA levels.

2.7 Legislation Governing Maximum Levels of Ochratoxin A for Grape and Grape Products

Since the discovery of aflatoxins in the early 1960's, regulations have been established in many countries to protect the consumer from the harmful effects of these mycotoxins. The recognition that aflatoxins and other mycotoxins can cause major illness in humans and animals has led to limits being set for aflatoxins and some other mycotoxins in different countries around the world, often however on an *ad hoc* basis. The European Union aims to harmonise legislation between the countries of the Union. Various factors play a role in the decision-making process required for setting these limits. These include scientific factors such as the availability of toxicological data, survey data, knowledge about the occurrence and distribution of mycotoxins in commodities, and analytical methodology. Economic and political factors such as commercial interests and sufficiency of food supply have their impact as well (EMAN).

For setting up a statutory maximum level, a summary toxicological assessment of the substance with regard to its impact on human health and on the environment is reached by cooperation between the following organisations:

International Programme on Chemical Safety (IPCS, www.who.int/pcs/)

International Agency on Research on Cancer (IARC, www.iarc.fr)

Joint FAO/WHO Committee on Food Additives and Contaminants (JECFA, www.who.int/pcs/jecfa/jecfa.htm)

Within the EU, this assessment is the responsibility of the Scientific Committee on Food (SCF, http://europa.eu.int/comm/food/fs/sc/scf/index_en.html). Throughout these discussions, and taking into account international standards or norms (e.g. Codex Alimentarius) in order to ensure that international trade is not impeded without justification, adoption of maximum levels is taken leading to a Directive or Regulation.

On the basis of Council Regulation (EEC) No. 315/93 maximum levels for a number of mycotoxins in certain foodstuffs have been set by Commission Regulation (EC) No. 1881/2006. Within the latter the Commission Regulation (EC) No 123/2005 as regards ochratoxin A was embodied, whereas Commission Directive 401/2006 regulates the method of sampling and analysis. The maximum levels are detailed in the Table 2.1.

Table 2.1 Maximum Levels for Ochratoxin A as regulated by Commission Regulation (EC) No 1881/2006.

Product	**OTA (*µ*g/kg) Maximum Level**
Unprocessed cereals	5.0
All products derived from unprocessed cereals, including processed cereals products and cereals intended for direct human consumption	3.0
Dried vine fruit (currants, raisins and sultanas)	10.0
Roasted coffee beans and ground roasted coffee, excluding soluble coffee	5.0
Soluble coffee (instant coffee)	10.0
Wine (including sparkling wine, excluding liqueur wine and wine with an alcoholic strength of not less than 15 % vol) and fruit wine	2.0
Aromatised wine, aromatised wine-based drinks and aromatised wine-product cocktails	2.0
Grape juice, concentrated grape juice as reconstituted, grape nectar, grape must as reconstituted, intended for direct human consumption	2.0
Processed cereal-based foods and baby foods for infants and young children	0.50
Dietary foods for special medical purposes intended specifically for infants	0.50

2.8 Predictive Mycology

2.8.1 Introduction

For over 20 years, predictive microbiology has focused on bacterial food-borne pathogens, and some spoilage bacteria. Few studies have concerned to model fungal development. Predictive modelling is a versatile tool that should not be limited to bacteria, but should be extended to moulds. Mathematical modelling of fungal growth was reviewed earlier (Gibson *et al.*, 1994; Gibson & Hocking, 1997; Dantigny, 2004; Davidson, 2007), but it still

remains a subject of research. Because of the inherent differences between fungal and bacterial growth, such tools should take into account the specificities of mould growth (Gibson & Hocking, 1997; Dantigny *et al.*, 2005a). The term "predictive mycology" was therefore coined to differentiate the modelling of fungal growth and mycotoxin production from that of bacteria (Dantigny *et al.*, 2005a).

2.8.2 Mould Specificities and Growth Measurement

Fungal growth involves germination, hyphal extension and eventually forming mycelium. Spores are widely disseminated in the environment, and they are principally responsible for spoilage. Under favourable conditions, spores will swell. Thereafter, when the length of the germ tube is between one half and twice of the spore diameter, the spore is considered to have germinated. Germination can be considered as the main step to be focused on, because a product is spoiled as soon as visible hyphae can be observed. However, few studies have concerned germination kinetics (Marin *et al.*, 1996; 1998a; Sautour *et al.*, 2001a; Dantigny *et al.*, 2002; Pardo *et al.*, 2005b; 2005c) and the lack in literature is greater regarding to *A. carbonarius* (Dantigny *et al.*, 2007). This limitation can be explained in part by the acquiring sufficient and reproducible data. In fact, this kind of study requires microscopic observation for evaluating the length of the germ tube. Moreover, measurements should be carried out without opening the Petri-dishes (Dantigny, 2004; Dantigny *et al.*, 2006). Although, Marin *et al.* (2008a) showed that for some species of airborne fungi the ergosterol content can be used as mould growth indicator for primary modelling. But it must be noted that ergosterol content can vary with substrate, growth conditions, and the age of mycelium and due to interspecies differences (Gibson & Hocking, 1997). More work was dedicated to the measurement of hyphal extension rate, which is usually reported as radial growth rate (mm d^{-1}) (Gibson *et al.*, 1994; Baranyi *et al.*, 1996). It is probably the simplest and most direct measure, but does not necessarily represent the true nature of fungal growth. Fungal hyphae can penetrate the three dimensional matrix of foods, and since that point the radial growth does not represent the actual growth of the fungus (Gibson & Hocking, 1997). Another attempt modelling fungal growth has been presented from Panagou *et al.* (2005) using the gradient plate technique first introduced by Szybalski and Bryson on 1952, but furthermore refined by Wimpenny and Waters (1984; 1987). Panagou inoculated *Monascus ruber* van Tieghem on the surface of square Petri-dishes with gradients of NaCl at right angles with gradients of pH, which were incubated at different temperatures. Visible fungal was expressed to optical density units, recorded by image analysis and graphically presented in form of three dimensional grids.

Finally, the data were applied to several models in order to generate plots of growth / no growth interface (Panagou *et al.*, 2005). Other researchers are measuring the increase of a fungal metabolite, usually an enzyme, in order to make an assessment for growth. Numerous studies trying to estimate the influence of several environmental factors on fungal growth by the increment of enzymes such as phosphomonoesterase, protease, b-glucosidase, endocellulase, laccase and many other (Marín *et al.*, 1998b; Keshri & Magan, 2000; Fragoeiro & Magan, 2005). Again there is lack in literature referring to *A. carbonarius* and moreover this technique does not supply appropriate data sets for growth modelling. A prerequisite for a useful and reliable model must be a database containing large amounts of relevant data, preferably in the form of growth or survival curves.

2.8.3 Primary Modelling

According to Whiting and Buchanan (1993), models that describe the response of microorganisms to a single set of conditions *over time* are termed as "primary" models. A primary model for microbial growth aims to describe the kinetics of the process with as few parameters as possible, while still being able to accurately define the distinct stages of growth (McKellar & Lu, 2004). When coming to fungi, the concept of primary modelling seem to be less demanding in mathematical terms since the stages of growth are depleted with lag to germination and a linear growth state. Since now two approaches are referred to fungal growth modelling, the first being the germination of fungal spores and the second the hyphal radial extension. The germination of spores of *Fusarium moniliforme* and *F. verticillioides* as a function of time was first studied at different a_w and temperatures (Marín *et al.*, 1996). The percentage of germination *vs.* time was modelled with the modified Gompertz equation at different conditions. There are two different ways of looking at spore germination:

(1) the percentage of germination at a certain time, where the percentage of germination *vs.* time can be modelled with the modified by Zwietering *et al.* (1990) Gompertz equation (Marin *et al.*, 2006; 1998a; Pardo *et al.*, 2005b; 2005c; 2005d; Dantigny *et al.*, 2007):

$P = A \cdot \exp \cdot (-\exp[\mu_m \cdot e(1) \cdot (\lambda_{ge} - t)/A + 1])$

where A (%) was the asymptotic P value at $t \rightarrow +\infty$, μ_m (h^{-1}) was the slope term of the tangent line through the inflection point (t_G) as defined further, λ_{ge} (h) was the t-axis intercept of the tangent through the inflection point and t was the time (h), and:

(2) the time to obtain a certain germination percentage, or germination time. The germination time can be considered as the probability of a single spore germinating.

Accordingly, the logistic function is used (Dantigny *et al.*, 2002; 2005a; 2007; Samapundo *et al.*, 2007a):

$$P = \frac{P_{max}}{(1 - e^{(k(\tau - t))})}$$

where P_{max} (%) was the asymptotic P value at $t \rightarrow +\infty$, k (h^{-1}) was the slope term of the tangent line through the inflection point, τ (h) was the inflection point where P equals half of the P_{max} and t was the time (h). By using any of these equations, it is possible to estimate accurately the time necessary to reach a certain percentage of germination.

Shortly after the completion of germination, the mycelium is visible to the naked eye (when the colony reaches approximately 3 mm in diameter). Therefore fungal growth can be easily estimated from macroscopic measurements of the radius of the colony. The modified Gompertz model has been again used for empirically modelling mould growth on the basis of its proven flexibility to different asymmetrical growth data (Char *et al.*, 2005; Marin *et al.*, 2008a).

Although, more common is the primary model developed originally for bacterial growth by Baranyi *et al.* (1993), which has been adapted to fit colony diameter growth curves of 4 species from *Aspergillus* section *Nigri* (Gibson *et al.*, 1994):

$$y(t) = y_0 + gA(t) - \frac{1}{m} In\left(1 + \frac{e^{mgA(t)} - 1}{e^{m(y_{max} - y_0)}}\right)$$

where:

$$A(t) = t + \frac{1}{v} In\left(\frac{e^{-vt} + q_0}{1 + q_0}\right)$$

and

$$q_0 = \frac{1}{e^{v\lambda} - 1}$$

Since then is appearing in many studies referring to fungal growth modelling: *Aspergillus* section *Flavi* (Baranyi *et al.*, 1996), *Penicillium roqueforti* (Valík *et al.*, 1999), *P. brevicompactum* (Membré & Kubaczka, 2000), *P. verrucosum* and *P. proliferatum* (Samapundo *et al.*, 2005; 2007b), *A. flavus* (Marin *et al.*, 2009), 14 fungal species representing general common food contaminants found in intermediate moisture foods, including *A. carbonarius* (Marin *et al.*, 2008a;Tassou *et al.*, 2007a). When plotting diameters (or radiuses) of a mould colony growing in an agar Petri plate against time, a lag phase is

observed, followed by a linear phase, but in most of the cases no decrease in growth rate is observed before the edge of the Petri plate is reached. Under constant conditions a fungal colony would then probably grow indefinitely, if the agar plate was unlimited; thus, in the absence of the edge of the plate, the Baranyi's model parameter *m* (Richards' curve, curvature after the exponential phase) should be fixed in a way to eliminate the upper asymptote (*m*=1). The use of these models enables the estimation of important growth parameters such as the maximum colony growth rate (g_{max}, mm d^{-1}) and lag phase duration (λ, d) (Samapundo *et al.*, 2005; 2007a; Marin *et al.*, 2008a).

Nevertheless, the most common way assessing fungal growth rates is a simple linear model with breakpoint:

$r = m \cdot (t - \lambda)$,

where *r* was the colony radius (mm), *m* the radial growth rate (mm d^{-1}), and λ is the lag time (d). The linear section of the graph (with growth rate of *m*) is extrapolated to a zero increase in diameter, and the intercept on the time axis is defined as the lag prior to growth (λ). In most cases, the fits are excellent, as is being revealed from numerous studies for fungi: *Aspergillus ochraceus* (Lee & Magan, 1999; 2000), *Mucor racemosus* (Dantigny *et al.*, 2002), *P. chrysogenum* (Sautour *et al.*, 2003) *P. verrucosum* (Pardo *et al.*, 2005d), *Botrytis cinerea* (Lahlali *et al.*, 2007), several food spoilage moulds (Cuppers *et al.*, 1997; Sautour *et al.*, 2001b; Dantigny *et al.*, 2005b; Marin *et al.*, 2008a) and *Aspergillus* section *Nigri* including *A. carbonarius* or the latter one alone (Mitchel *et al.*, 2004; Parra *et al.*, 2004; Parra & Magan, 2005; Belli *et al.*, 2004a; 2005a; Leong *et al.*, 2006a; Medina *et al.*, 2007a; 2007b; Pateraki *et al.*, 2007a; Romero *et al.*, 2007; Samapundo *et al.*, 2007a; Valero *et al.*, 2007a; Marin *et al.*, 2008a). The main disadvantage of the latter model is that when the experimental design includes suboptimal conditions, the estimation of lag phase by extrapolation cannot be reliable (Marin *et al.*, 2008a).

Finally, it is reported for the inactivation of spores a classical first order equation:

$dN/dt = -kN$,

where N is the number of surviving spores after treatment (cfu/ml), *t* is the time (min) and *k* is an inactivation rate (min^{-1}). In a same way, the known Bigelow model is used, with the D and z values (Dantigny *et al.*, 2005a).

2.8.4 Secondary Modelling

Models that describe the effect of environmental conditions, e.g., physical, chemical, and biotic features, on the values of the parameters of a primary model are termed "secondary"

models (Whiting & Buchanan, 1993). The concept of predictive microbiology is that detailed knowledge of the growth responses of microorganism to environmental conditions enables objective evaluation of the effect of processing, distribution and storage operations on the microbiological safety and quality of foods. If the microbial ecology of a processing operation or a product during distribution or storage is understood, survival and/or growth of an organism of concern may be predicted on the basis of a mathematical relationship between microbial growth rate and environmental conditions (McMeekin *et al.*, 1993).

Two major types of models are recognized. Empirical models are derived from an essentially pragmatic perspective. They simply describe the data in a convenient mathematical relationship and consequently often give little or no insight into the underlying process (McMeekin *et al.*, 1993). Because empirical models are descriptions of the experimental conditions, they should not be used to make predictions outside the limits of the original experiments (Gibson & Hocking, 1997).

Mechanistic, or deterministic, models are built up from theoretical bases and, if they are correctly formulated, may allow the interpretation of the modelled response in terms of known physical, chemical, and biological phenomena and processes (McMeekin *et al.*, 1993). An advantage of mechanistic approaches is that they tend to provide a better foundation for subsequent development and expansion of models. Conversely, as none of the models in use in predictive microbiology can be considered to be mechanistic, they can only be used to make predictions by interpolation. In general, even with good quality data the mechanistic models do not provide better fit to data and are usually harder to work with than semi-mechanistic or empirical models currently used (Ross & Dalgaard, 2004). Semi-mechanistic models are termed empirical models including an aspect of mechanistic modelling and *vice versa*. Mechanistic or semi-mechanistic models usually include parameters from differential equations of applicable known theories (growth kinetics, substrate utilization and depletion rates, substrate or end-product diffusion rates etc.) and attempt to describe what is actually occurring during growth. Such models can give more insight into the behaviour of a biological system than empirical models can (Gibson & Hocking, 1997).

In the following pages a brief report of secondary models is presented, always relatively with mycelial growth and toxin production studies in the late literature.

Secondary Models:

Based on the Arrhenius Equation

This group of models relates growth rate with several environmental factors by extended types of Arrhenius thermodynamic model originally proposed to describe the effect of

temperature on rates of chemical reactions. After taking the natural logarithm, and with the proper re-parameterization this equation leads to a range of secondary models, based on adherence to the reaction kinetics described by the Arrhenius model, but including terms to account for the observed deviations. In this group belong the mechanistic modifications of Hinshelwood, Schoolfield and Ross all reviewed previously, but few of these types of models have been routinely applied in predictive microbiology, possibly because the models are highly nonlinear and initial parameter estimates are difficult to determine (Ross & Dalgaard, 2004; Zwietering, 1991). Moreover, they had never been applied to mycelial growth rates.

More close to predictive mycology is the empirical modification of the Arrhenius equation that Davey (1989) has proposed to model the effects of temperature and water activity, which is linear and thus allows for explicit solution of the optimum parameter values. The "linear Arrhenius" or "Davey" models have been used to model growth of moulds on solid microbiological media under the influence of several environmental factors. Panagou *et al.* (2003) applied an extension of the Davey equation to model temperature, a_w and pH effect on growth rates of *Monascus ruber*, and Samapundo *et al.* (2007d) to model b_w and temperature on growth of *Aspergillus flavus* and *A. parasiticus* on corn.

Square-Root-Type or Bêlerádek-Type

In many cases the classical Arrhenius equation is inappropriate to describe the effect of suboptimal temperature on growth rates of microorganisms because the (apparent) activation energy (E) itself is temperature dependant. To overcome this problem Ratkowsky *et al.* (1982) suggested a simple empirical model. When this model was fitted to experimental growth rates the data were square-root transformed to stabilize their variance and this simple model and its numerous expansions are named square-root-type, Ratkowsky-type, or Bêlerádek-type models. For the reason that the simple square-root-type model was intended to be applied only to the low temperature region, namely from the minimum temperature at which growth can occur to that just below the optimum temperature, Ratkowsky *et al.* (1983) introduced a four parameter extension to enable the whole biokinetic temperature range to be modelled. The Ratkowsky-type model has several applications to bacterial growth and some can be found in predictive mycology, either by its own or by combining it with other models. Cuppers *et al.* (1997) successfully modelled the influence of temperature with Ratkowsky model for *P. roqueforti*, *Trichoderma harzianum*, *Paecilomyces variotii*, *A. niger* and *Emericella nidulans*. Parra & Magan (2005) used an extension of this model for the temperature dependence, combined with Gibson model for a_w dependence on *A. niger* growth.

Polynomial models

The effect of many different environmental parameters (e.g., temperature, NaCl/a_w, pH, nitrite, CO_2, organic acids, and natural antimicrobials) has been described by these linear models. Polynomial models were extensively used during the 1990s and they remain widely applied although square-root-type and cardinal parameters models are becoming increasingly popular. Polynomial models are attractive, first, because they are relatively easy to fit to experimental data by multiple linear regression, which is available in most statistical packages. Second, polynomial models allow the environmental parameters and their interactions to be taken into account. Thus, application of polynomial models is a simple way to summarize information from a data set (Ross & Dalgaard, 2004).

A first approach on modelling fungal growth with this type of equations is presented by Gibson *et al.* (1994), where a_w is transformed to b_w in order to perform a better fitting of the growth data to the quadratic function proposed and since known as Gibson transformation for a_w:

$$b_w = \sqrt{(1 - a_w)}$$

and the proposed 2nd order Polynomial being:

$$\ln\ g = C_0 + C_1 b_w + C_2 b_w^2$$

used to fit growth data of *A. flavus, A. oryzae, A. parasiticus* and *A. nomius* against water activity. The same exactly mathematical method was adopted from Barayni *et al.* (1996) to investigate the relatedness of *A. flavus, A. oryzae, A. parasiticus* and *A. nomius*. Cuppers *et al.* (1997) used the same model after having substitute b_w with NaCl concentration in order to assess its effect on growth of *P. roqueforti*, *Trichoderma harzianum*, *Paecilomyces variotii*, *A. niger* and *Emericella nidulans*. A two-variant quadratic function, again with the transformation of Gibson for a_w was used from Valik *et al.* (1999) to study the effect of b_w and pH on growth rates of *P. roqueforti*, while Panagou *et al.* (2003) included a third variable, temperature in the same model for *Monascus ruber*. Guynot *et al.* (2005) also used a 2nd order polynomial with 3 variables, $C_6H_7KO_2$ concentration, a_w and pH for predicting growth rates of *Eurotium amstelodami*, *E. herbariorum*, *E. rubrum*, *A. flavus* and *A. niger*. Many other studies have applied polynomial equations for modelling environmental factors on microbial growth and lag phase. Pardo *et al.* (2005b) used the same mathematical means to estimate influence of a_w and T on lag phase duration of *A. ochraceus*, while Lahlali *et al.* (2007) and Samapundo *et al.* (2007d) on growth rate of *B. cinerea* and *A. flavus* and *A. parasiticus* respectively. The latter author also used the method for a_w and salt concentration (Samapundo *et al.*, 2007e) on *Fusarium* and *Aspergillus* spp. and on b_w on *Fusarium proliferatum* and *F.*

verticilliodes (Samapundo *et al.*, 2005). In the last paper an attempt made to use a 3rd order polynomial for b_w and temperature influence.

Nevertheless, higher order polynomial models, e.g., cubic or quadratic models have been criticized for being too flexible and for attempting to model, rather than eliminate, experimental error. Because of the very flexible nature of higher order polynomial models they should not be used as secondary model within predictive microbiology unless very high quality experimental data are available and support the application of these models. Moreover, polynomial models have properties that limit their usefulness as secondary predictive models. Polynomials include many coefficients that have no biological interpretation. The high number of coefficients and their lack of biological interpretation make it difficult to compare polynomial models with other secondary predictive models. The important information included in, e.g., the T_{min} parameter of a square-root-type model is not provided by polynomial model (Ross & Dalgaard, 2004).

The Gamma Concept

The concept of dimensionless growth factors, now known as the gamma (g) concept, was introduced in predictive microbiology by Zwietering *et al.* (1992). The gamma (g) concept relies firstly on the observation that many factors affecting microbial growth rate act independently, and that the effect of each measurable factor on growth rate can be represented by a discrete term that is multiplied by terms for the effect of all other growth rate affecting factors

Secondly, the effect on growth rate of any factor can be expressed as a fraction of the maximum growth rate (i.e., the rate when that environmental factor is at the optimum level). Under the gamma concept approach, the cumulative effect of many factors poised at suboptimal levels can be estimated from the product of the relative inhibition of growth rate due to each factor, as indicated by the complete equations for temperature, pH and a_w (Zwietering *et al.*, 1996). The relative inhibitory effect of a specific environmental variable is described by a growth factor "gamma" (γ), a dimensionless measure that has a value between 0 and 1. The relative inhibitory effect can be determined from the "distance" between the optimal level of the factor, and the minimum (or maximum) level that completely inhibits growth by recourse to a predictive model. In the gamma model approach, the reference growth rate is μ_{max}, so that reference levels of temperature, water activity, etc., are those that are the optimum for growth rate, usually represented as T_{opt}, $a_{w\ opt}$, pH_{opt}, etc. The combined effect of several environmental factors is then determined by multiplication of their respective γ factors (Ross & Dalgaard, 2004). Panagou *et al.* (2003) presented an expellant example of

gamma factor concept when applied to model a_w, T and pH effect on growth rate of *Monascus ruber*.

Cardinal Parameter Models

The basic idea behind cardinal parameters models (CPMs) is to use model parameters that have a biological or graphical interpretation. When models are fitted to experimental data by nonlinear regression, this has the obvious advantage that appropriate starting values are easy to determine. General CPMs rely on the assumption that the inhibitory effect of environmental factors is multiplicative, an assumption that was formalized in the gamma (g) concept discussed above. Thus, general CPMs consist of a discrete term for each environmental factor, with each term expressed as the growth rate at the optima of the factor; i.e., each term has a numerical value between 0 and 1 and at optimal growth conditions all terms have a value of 1 and thus μ_{max} is equal to μ_{opt}. Within predictive mycology various CPMs were developed. A first approach of CPMs to food spoilage fungi was introduced by Cuppers *et al.*(1997) using a model originally built up for microbial growth from Rosso *et al*. (1995).

Expressions of CPM related with fungal growth are the temperature effect on *Penicillium roqueforti*, *Trichoderma harzianum*, *Paecilomyces variotii*, *Aspergillus niger* and *Emericella nidulans* studied by Cuppers *et al.* (1997). Rosso and Robinson (2001) and Sautour *et al.* (2001b) used the same method to model the effect of a_w on *Aspergillus flavus*, *A. nomius*, *A. oryzae*, *A. parasiticus*, *A. candidus*, *A. sydowii*, *Eurotium amstelodami*, *E. chevalieri*, and *Xeromyces bisporus* the first two authors and *P. chrysogenum*, *A. flavus*, *C. cladosporioides* and *Alternaria alternata* the latter group of authors. An expansion of this model is presented from Panagou *et al.* (2003) in order to include the effect of temperature beside that of a_w and pH for the fungus *Monascus ruber*. Finally, Marin *et al.* (2009) used the model of Rosso and Robinson (2001) to assess a_w influence on *A. flavus* growth.

Artificial Neural Networks

Artificial neural networks (ANNs) are algorithms that can be used to perform complex statistical modelling between a set of predictor variables and response variables. Their particular advantage is that they have the potential to approximate underlying relationships of any complexity between those variables. The use of ANN in predictive growth modelling remains relatively little developed, and direct comparison of the performance of different ANN techniques is still lacking (Ross & Dalgaard, 2004). Although, an interesting ANN performance for the effect of pH, a_w and temperature on radial growth rate of *Monascus ruber* is presented by Panagou *et al.* (2007).

Probability Models

In probability models in predictive microbiology the data are usually that the response (e.g., growth, detectable toxin production) is observed under the experimental conditions, or that it is not. Responses such as detectable toxin production can be coded as either 0 (response not observed) or 1 (response observed) or, if repeated observations have been made, as probability (between 0 and 1). The probability is related to potential predictor variables by some mathematical function using regression techniques. Because regression techniques do not exist for dichotomous data, the regression equation is usually related to the log odds, or *logit*, of the outcome of interest (Ross & Dalgaard, 2004). Studies from the field of mycology are presented from Sautour *et al.* where the probability (%) of conidial germination of *P. chrysogenum* after 25 days of incubation was modelled against a_w, temperature and pH by a polynomial equation (Sautour *et al.*, 2001a), and T_{90}(%) was estimated, taking into account the influence of the same environmental factors on the fungus (Sautour *et al.*, 2001c) with a 2^{nd} order polynomial.

Growth/No Growth Interface Models

Models defining combinations of environmental conditions that *just* prevent growth have become known as 'G/NG interface," "growth boundary," or more simply "growth limits" models. Various approaches have been suggested to define the G/NG boundary with most common empirical or deterministic approaches and logistic regression techniques.

A global logistic model incorporating a dummy variable for the growth medium was used for the estimation of the growth–no growth interface of yeast *Issatchenkia occidentalis* as a function of NaCl, citric and sorbic acid concentrations. From this work, the growth/no growth interface for a selected probability can be deduced by substituting logit (*p*) by the corresponding polynomial equation and plotting the resulting equation as a function of two or three variables, while maintaining the rest of them at predetermined levels (Lopez *et al.*, 2007). Marin *et al.* (2008b) built a probability model to predict the growth of *Aspergillus carbonarius* as a function of moisture content and storage temperature of pistachios nuts. Marin *et al.* (2009) modelled probability growth data by using linear logistic regression analysis to determine the growth/no-growth boundaries of *A. flavus* under different water activity levels. Battey *et al.* (2001) and Panagou *et al.* (2003) used the same methodology by expanding the polynomial to include more factors. The first team modelled the probability of growth for *A. niger* and *P. spinulosum* against time, pH, titratable acidity, °Brix, sodium benzoate and potassium sorbate concentrations in ready-to-drink beverages, while the latter

team modelled probability of growth of *Monascus ruber* against NaCl concentration, temperature and pH.

Toxin modelling

If literature of growth/no-growth interface referring to toxigenic fungi is limited, studies relating probability modelling of toxin production are minimal. A first integrated approach has been carried out by Pitt (1993) whose first thing to consider was the difficulty of toxin modelling due the parallel production and degradation of aflatoxin and the poorly understood fungal secondary metabolism. Pitt assumed the rate of toxin formation proportional to the rate of production of new cell mass and the rate of toxin degradation proportional to the production of the concentration of dead cell mass and aflatoxin. An Arrhenius-type function was selected for the effect of temperature, a linear function for a_w and a parabolic for pH. By this methodology the author tried to conclude to a model that will take into account all environmental factors and predict the relative growth and toxin formation (Pitt, 1993). But the numerous assumptions that followed during model construction minimize its usefulness, and thus this work had no much progress later. Marin *et al.* (1999) investigated the potential of modelling fumonisin B_1 production under the influence of temperature and a_w by *Fusarium moniliforme* and *F. proliferatum* on maize grain. Although that detailed two-dimensional profiles to assess fumonisin B_1 production under the environmental factors essayed was successfully developed, the differences between productions at the different temperatures were not high enough to provide general models. Since the effect of a_w on fumonisin accumulations was much more marked, its impact was modelled through a 3^{rd} degree polynomial for some of the temperature sets. However, in this study the crucial aspects of initial inoculum, time, and fluctuation of environmental conditions were not taking into account, and thus the prediction is meaningless outside the limited environmental conditions tested.

Nowadays, the trend is, instead of trying to model the concentration during time, to model the probability of presence of the toxin at an actual time. In this direction, Marin *et al.* (2008b) built a probability model to predict the growth and OTA production boundaries of *Aspergillus carbonarius* strains under different incubation temperatures and moisture content pistachios. Tassou *et al.* (2009) also modelled the probabilities of growth and OTA production of *Aspergillus carbonarius* isolates on media of different a_w and incubation temperatures and demonstrated graphically the Growth/No Growth and OTA production boundaries.

2.9 Strategies on Prevention and Control of *A. carbonarius*

The presence of OTA in grapes, grape products and wine is dependent on opportune climate conditions which can facilitate germination and mycelial colonisation to occur. The most important factors governing these components are water activity, temperature and their interaction with the nutrient status of the food matrix. For grapes, pre-harvest contamination is a critical factor in determining the contamination in wine, especially of red wine where the skins are left in the initial phases of production. For processing of grapes, pH is also important.

For the production of vine fruits the grapes are generally sun-dried for 7–14 days and turned regularly to ensure an even drying. This technique is also applied in some specialty dessert wines in Greece and other countries of Mediterranean basin. During this process the sugar is concentrated as the moisture content decreases resulting in an almost selective medium for xerotolerant moulds such as *Aspergillus* section *Nigri* species. If a rain episode occurs during the process, then, this can result to a higher risk of OTA contamination due to uneven drying (Magan & Aldred, 2005).

Softening of the berry skin and swelling after véraison cause the skin to split in some berries, allowing infection by *A. carbonarius* and subsequent sporulation. Appropriate vineyard management during the period between véraison and harvest is vital for reducing berry splitting, and thus, minimising *Aspergillus* rots and the risk for OTA formation. Fungicides, such as Switch® (cyprodinil+fludioxonil, Syngenta, Basel, Switzerland), have been reported to be effective in reducing *Aspergillus* rots when applied shortly before véraison, with follow-up sprays before harvest conferring additional reductions. *Aspergillus* rots may be controlled by minimising berry damage through careful management of irrigation and canopy. Management of fungi such as *B. cinerea* and *Erysiphe necator* (powdery mildew), which are capable of damaging berries, is another strategy for reducing the incidence of *Aspergillus* bunch rots (Leong *et al.*, 2006d; Belli *et al.*, 2007). In the same way grape berry damage by *Lobesia botrana* has been shown to considerably increase the contamination level of black aspergilli and consequent OTA accumulation in grapes. It is, therefore, important to ensure adequate insect control in combination with fungicide treatment to realize effective pest management (Cozzi *et al.*, 2006; Visconti *et al.*, 2008).

Another promising innovation is proposed by Battilani *et al.* (2006b) with the use of Geostatistical analysis tools to identify areas with a high risk of grape infection by black aspergilli. This study examined geostatistical data and relate key climatic information on a

regional basis to levels of contamination of wine grapes with *A.* Section *Nigri* and *A. carbonarius*, responsible for ochratoxin A contamination in wine and vine fruits. This approach can be found very useful in developing accurate risk maps and a web-based knowledge system for risk from OTA and this way indicate the distribution in the field and allow prediction of occurrence (Battilani *et al.*, 2006b; C.A.S.T., 2003).

Battilani & Pietri (2004a) and Martínez-Rodríguez & Carrascoza (2009) discuss the application of HACCP system during the winemaking process to control the formation and concentration of OTA in wine. OTA concentrations in the final product should not exceed the limits established in the legislation. If the contrary happens, the traceability system and the existing records will allow the failure and the batch that has become out of control to be located, and thus to find the CCP that has become out of control, modifying this if necessary.

In conclusion, fungicidal and insecticidal treatments can reduce OTA contamination. Developing of risk maps, based on critical control points, can help to prevent and control OTA accumulation in grapes. Availability of rapid methods for OTA analysis is also important for preventive and corrective intervention at critical control points. During vinification of (red) grapes only a small amount of the initial present OTA remains dissolved in the wine, while most of it is absorbed by solid winery by-products (grape pomace and lees). Carbon reduces OTA concentrations in wines, but negatively affects quality. Good Agriculture Practices (balanced soil tillage, irrigation, nitrogen fertilization, and pruning) and Good Manufacturing Practices (reduced harvest to vinification time, segregation of rot bunches) help considerably to reduce OTA contamination risk (Visconti *et al.*, 2008).

Chapter 3: Mycoflora Dynamics in Organic/non-Organic Vine Grape Production in Greece

3.1 Introduction

Several surveys in European and other traditional wine-producing countries, such as Australia and South American countries have confirmed the frequent presence of *A. carbonarius* on grapes (Magnoli *et al.*, 2003; Battilani *et al.*, 2004b; Belli *et al.*, 2004b; 2006d; Guzev *et al.*, 2006; Leong *et al.*, 2006b; Tjamos *et al.*, 2006; Melki Ben Fredj *et al.*, 2007; Visconti *et al.*, 2008). The presence and incidence of the responsible mycoflora is affected by several factors of the vine environment, i.e., the status of the grape berries, the number of damaged berries, the meteorological conditions, the localization of the vineyard, the cropping system and the chemical treatments (Battilani *et al.*, 2006b; Blesa *et al.*, 2006; Belli *et al.*, 2005b; 2007a; 2007b; Leong *et al.*, 2006b; Clouvel *et al.*, 2008). Studying these factors *in vivo* and understudying the ways they are affecting the presence of black aspergilli on the vineyard is important, because it will allow early detection of *A. carbonarius* which is considered the major cause of OTA contamination of grapes.

The incidence of the black aspergilli responsible for OTA presence in grapes increases as berries mature, starting from the early stages of berry development, but become very well established from véraison until grape ripening (Battilani & Pietri, 2002; Belli *et al.*, 2005b; Bejaoui *et al.*, 2006; Serra *et al.*, 2003; 2006b). The occurrence of black aspergilli has been correlated mainly with temperature, and to a lesser extent to relative humidity and pre-harvest rainfall (Belli *et al.*, 2005b; Battilani *et al.*, 2006b; Leong *et al.*, 2007), while an increased incidence is reported from the warmer regions of southern Europe (Battilani *et al.*, 2004b; Belli *et al.*, 2004b; 2006d; Guzev *et al.*, 2006; Tjamos *et al.*, 2006; Melki Ben Fredj *et al.*, 2007). Battilani *et al.* (2004b) reported differences in susceptibility to infection and OTA production among cultivars commonly grown in Italy, while the same was also noted by Bejaoui *et al.* (2006) for French cultivars and Tjamos *et al.* (2004; 2006) for Greek ones. The common viticulture chemical treatments and their efficacy on *A. carbonarius* control have been studied by several authors (Tjamos *et al.*, 2004; Belli *et al.*, 2004; Valero *et al.*, 2007b), while organic *versus* conventional culturing have been also investigated (Chiodini *et al.*, 2006; Ponsone *et al.*, 2007).

The present chapter aims to correlate the incidence of *A. carbonarius* in Greek cultivars intended for winemaking with the above mentioned factors, and particularly with the cropping system applied, in a way that will reveal useful information regarding the control of the species responsible for OTA production in the fungal mycoflora of grapes.

3.2 Materials and methods

3.2.1 Data collection

3.2.1.1 Study areas

The vineyards were located with the cooperation of 2 Greek wine producers, Arkas S.A. (Domain Spiropoulos) located in Tripoli, Arcadia, and Achaia Clauss Co. located in Patra, Achaea. Both wineries' vineyards lie in the greater range of Peloponnesus as shown in Figure 3.1. They are located in the prefectures of Achaea, Arcadia and Corinthia. In total 21 different vineyards, in 10 locations laid at heights starting from an altitude of 20 m and a distance of 5 km from the sea and reaching about 750 m altitude and 50 km from sea. They were chosen from both conventionally and organically cultured cultivars. For every sampled vineyard recordings were made of the longitude, latitude, altitude, and distance from the sea, the weather conditions and the culturing technique followed by the producer (Tables 3.1, 3.2). The laboratory work was carried out at the Institute of Technology of Agricultural Products at Lycovrisi, Attiki (National Agricultural Research Foundation).

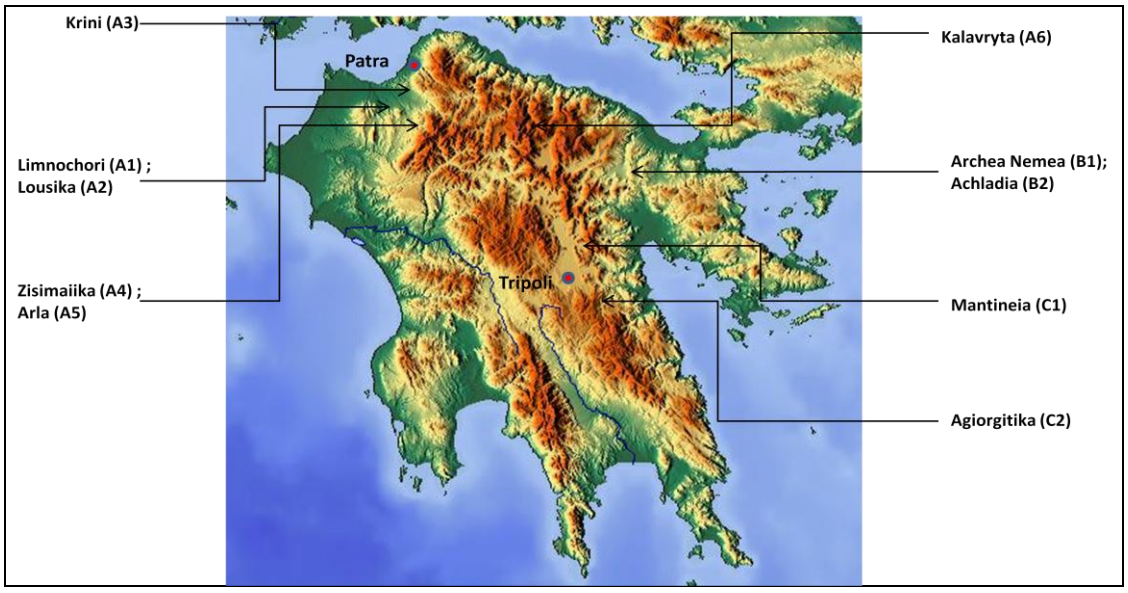

Figure 3.1 Locations and coding of studied vineyards in the 3 Peloponnese prefectures: A = Achaea, B = Corinthia, C = Arcadia.

Table 3.1 Geographic localisation of the studied vineyards

Prefecture Location name (code)	Altitude (m)	Latitude	Longitude	Distance from sea (km)	Climate type*
Achaea					
Limnochori (A1)	20	38°8'34"N	21°28'49"E	5 km	LM
Loussika (A2)	60	38°6′34″N	21°35′28″E	5 km	LM
Krini (A3)	150	38°11'46"N	21°46'12"E	5 km	LM
Zissimeika (A4)	150	38°3'3"N	21°36'32"E	15 km	LM
Arla (A5)	200	38°3'22"N	21°35'55"E	15 km	LM
Kalavrita (A6)	750	38°3'1"N	21°55'17"E	40 km	M
Corinthia					
Achladia (B1)	350	37°50'23"N	22°18'56"E	45 km	M
Archaia Nemea (B2)	400	37°48'58"N	22°42'26"E	20 km	M
Arcadia					
Mantinia (C1)	650	37°40'24"N	22°21'53"E	50 km	M
Agiorgitika (C2)	650	37°29'40"N	22°29'13"E	25 km	M

* Climate type coding:
LM (Landlocked Mediterranean) = Climate with moderate rainfall, mild winter and dry summer.
M (Mountainous) = Climate with icy winter, cool summer and rainfall all the year. The temperature has great fluctuation within a day and between different seasons.

Table 3.2 Monthly Mean Rainfall and Temperature for the 2 types of climate studied.

	July	August	September
	Landlocked Mediterranean		
Rainfall (mm)	10	10	10
Temperature (°C)	27	26	23
	Mountainous		
Rainfall (mm)	20	20	30
Temperature (°C)	25	24	20

3.2.1.2 Study cultivars

The wine producing grape cultivars cultured in the 21 vineyards and recorded for the mycological study were Mavrodaphni, Cabernet Sauvignon, Agiorgitico, Moschophilero and

Syrah from red varieties, and Roditis, Sauvignon Blanc, Chardonnay and Muscat Blanc from white. Mavrodaphni, Agiorgitico, Moschophilero, Roditis and Muscat Blanc cultivars represent the most common Greek varieties cultured in the Peloponnesian regions and give some of the most famous V.Q.P.R.D. Greek wines such as "Moschatos Patron", "Mavrodaphni Patron", "Moschophilero Mantineias" and "Agiorgitico Nemeas". On the other hand, the remaining famous international varieties and Greek Roditis are frequently used for the production of regional wines of high quality.

3.2.1.3 Vineyards management

All vineyards of Arkas S.A. were treated according to the E.U. legislation for organic farming and only the following commercial chemicals were applied:

Fungicides

YPER 50WP = 50% w/w copper hydroxide, as Cu in water-dispersible powder (VECTOR AGRO S.A.)

Kumulus 80WG = 80% w/w sulphur in water-dispersible granules (BASF Agro)

SULPHUR-B.F.L. 96 DP = 96% w/w sulphur dry powder (Phosphoric Fertilisers Industry S.A.)

Fertilizers

Genitron Spyrou S.A. Organic Fertilizer

Geotron Spyrou S.A. Nitrate Organic Fertilizer

Vineyards of Achaia Clauss Co. were cultured with the conventional wine-making grape culturing, with herbicide as well as fungicide treatment and fertilizer applications. Varieties, culturing, and sampling time for the vineyards studied are presented in Table 3.3.

Table 3.3 Varieties, culturing technique and sampling for the vineyards studied.

Vineyard[1]	**Cropping**	**Samplings**[3]			
Variety studied (Colour)	**System**[2]	2004	2005	2006	2007
A1					
Roditis (w)	C			R	
A2					
Roditis (w)	C		R	V-R	
A3					
Roditis (w)	C	R	R	V-R	R
Mavrodaphni (r)	C	R	R	R	V-R
Cabernet Sauvignon (r)	C	R	R		
Muscat Blanc (w)	O		R		
A4					
Roditis (w)	C		R		
A5					
Roditis (w)	C	R	R		
A6					
Roditis (w)	C		R		
Cabernet Sauvignon (r)	C			R	
B1					
Agiorgitico (r)	O		R	R	
B2					
Agiorgitico (r)	C			R	
C1					
Sauvignon Blanc (w)	O	R		R	
Chardonnay (w)	O	R		R	
Moschophilero (r)	O	R	R	V-R	R
Cabernet Sauvignon (r)	O	R	R	V-R	R
Agiorgitico (r)	O	R		V-R	
Syrah (r)	O			V-R	V-R
C2					
Moschophilero (r)	O				V-R

[1] **w** = white cultivar, **r** = red cultivar
[2] **C** = Conventional, **O** = Organic
[3] **R** = Ripening, **V** = Véraison

3.2.1.4 Field Samplings

The study started during the harvesting period of 2005 (September, October), continued for two developmental stages of the berries, early véraison and ripening (harvest time), in 2006 and 2007. Moreover, mycological data was also obtained for some vineyards in 2004. For the present mycological study, five plants were marked along two major diagonal transects of each vineyard. Three bunches were collected from each marked plant at véraison and at harvest. Bunches collected were at a height of 0.5-1.0 m from the ground. They were

kept in paper bags and stored in portable refrigerators during transfer to the laboratory and analysed within 24h.

3.2.2 Mycological study

3.2.2.1 Fungal isolation

From each bunch, three berries were randomly selected. Three or four pieces of skin were aseptically (in the Laminar flow bench) removed using surface sterilised tweezers from every selected grape of the bunch and placed on agar plates containing Dichloran Rose Bengal Chloramphenicol agar (DRBC, Merck). The pieces of skin were placed on the substrate with the outer skin side downwards (Pitt & Hocking, 1997). Additionally, a whole bunch was crushed with the help of a stomacher crusher and decimal serial dilutions were prepared in order to compare the populations with frequency of isolation by direct plating. Plates were incubated at 25^{o}C for 7 days. From the third day of incubation, the plates were monitored in the stereomicroscope for the presence of *Aspergillus* and *Penicillium* species. Colonies were enumerated and for every *Aspergillus* isolate, a suspension of spores was maintained in 10% glycerol solution maintained at –80^{o}C (Serra *et al.*, 2003).

3.2.2.2 *Aspergillus* spp. identification

The Aspergilli isolates were further cultured on Malt Extract Agar (MEA, Merck) and Czapek Yeast Extract Agar (CYA, prepared at laboratory according to Samson *et al.*, 2000) for identification. Their identification was based on their morphological and microscopic characteristics and in accordance with the guidelines of Samson *et al.* (2000) and Pitt & Hocking (1997). Several isolates were also compared with selected reference strains of *A. niger, A. japonicus* and *A. carbonarius*, kindly provided by the Applied Mycology Group of Cranfield University (Prof. N. Magan, Cranfield University, UK).

On the eighth day of cultivation the MEA plates were checked for identification of *Aspergillus* section *Nigri* and particularly for *A. carbonarius strains.* The macroscopic identification was made by the use of a stereoscope and by eye observation of the characteristics of isolates, while isolates were also observed with a high powered compound microscope. For every colony believed to belong to the black *Aspergilli* a small quantity of conidia was placed with a microbiology cringle to a vial with sterilized and double-distillate water and 10% glycerol or 0.1% agar-agar solution, and maintained at –80^{o}C.

3.2.2.3 Ochratoxigenic ability of Aspergilli isolates

Qualitative: *Aspergillus* isolates were grown on Coconut cream medium (40-50% coconut cream, 1.5% agar) at 25^{o} C for 7 days. The plates were then viewed inverted under long wave UV light and checked for the presence of fluorescence under the colony indicating OTA production (Dyer & McCammon, 1994; Heenan *et al.*, 1998).

Quantitative: Cultures of aspergilli were grown on CYA plates at 25^{o} C for 7 days. Three agar plugs were removed upon the radial of every colony and after weighted were placed into a vial with 0.5 ml methanol for the infusion of OTA. After 60 min, the extract was filtered (Millex®-HV 0.45μm, 13mm), diluted 100 fold (0.1 ml to 10ml) and kept in vials at 4°C until the injection to the HPLC (Bragulat *et al*. 2001).

HPLC analysis: The samples were analysed using an HPLC device (Hellamco Hewlett Packard Series 1100) equipped with a fluorescence detector (330nm excitation wavelength; 460nm emission wavelength). Chromatographic separations were performed with a C18 column (Waters spherisorb ODS-2 5 um, 250 mm×4.6 mm). The mobile phase was pumped at 1.0 ml min^{-1} and consisted of an isocratic solution of acetonitrile (ACCN): H_2O: acetic acid at 51:47:2. OTA was quantified on the basis of the HPLC fluorometric response compared with that of an OTA standard. The detection limit for OA was <0.01 μg g^{-1} of medium substrate.

3.2.2.3 Storage of fungal isolates

As soon as the mycelium had colonised 50% of the Petri plate to obtain mature conidia, a small quantity of them was also stored in the same way as above. This method was also used for isolation of species and strains of *Aspergillus* genus from several musts and grapes varieties of Peloponnese region. Moreover, surface isolations from machinery and raw material have been made from the wineries we visited. Spores of the isolated strains or isolates are kept in deep freezer (-80°C) and recovered every 6 month.

3.3 Results

3.3.1 Total mycoflora

All isolated and identified colonies found, belonged to the following genera: *Aspergillus*, *Penicillium*, *Alternaria*, and *Botrytis* spp., that were almost always present, and *Cladosporium*, *Epicocum*, *Fusarium*, *Rhizopus*, *and Trichoderma* spp., that were rarely identified in the sampled grapes (Data not shown).

The results of the mycological study for each of the years studied and separate vineyards are presented in Tables 3.4 – 3.7, while in Figure 3.2 the occurrence and the population composition of *Aspergillus* section *Nigri* spp. at harvest time for all studied vineyards are shown.

Table 3.4 Mycoflora dynamics for 2004 at ripening stage of vine grape

Cultivar / Vineyard code	Colour / Culture	% *Aspergillus* section *Nigri*	% *Penicillia* spp.	% Other Fungi
MD/A3	r/C	50	38	12
CS/A3	r/C	51	12	37
RD/A3	w/C	94	2	4
RD/A5	w/C	74	18	8
MP/C1	r/O	25	13	62
CS/C1	r/O	0	50	50
AG/C1	r/O	0	57	43
SB/C1	w/O	20	20	60
CR/C1	w/O	20	40	40
Total		37.1	27.8	35.1

[1]MD = Mavrodaphni, CS = Cabernet Sauvignon, AG = Agiorgitico, RD = Roditis, MP = Moschophilero, SB = Sauvignon Blanc, CR = Chardonnay
[2]For the localisation of each vineyard according to its code it is recommended to advise Figure 3.1 and Tables 3.1 & 3.2
[3]r = red, w = white, O = Organically cultured, C = Conventionally cultured

Table 3.5 Mycoflora dynamics for 2005 at ripening stage of vine grape

Cultivar / Vineyard code	Colour / Culture	% *Aspergillus* section *Nigri*	% *Penicillia* spp.	% Other Fungi
MD/A3	r/C	11	30	57
CS/A3	r/C	4	69	25
RD/A2	w/C	87	8	4
RD/A3	w/C	65	28	5
RD/A4	w/C	60	21	18
RD/A5	w/C	75	6	18
RD/A6	w/C	11	56	31
MP/C1	r/O	10	23	65
CS/C1	r/O	4	36	59
AG/C1	r/O	26	6	66
MB/A3	w/O	71	1	27
Total		27.4	33.3	39.3

[1]MD = Mavrodaphni, CS = Cabernet Sauvignon, AG = Agiorgitico, RD = Roditis, MP = Moschophilero, MB = Muscat Blanc
[2]For the localisation of each vineyard according to its code it is recommended to advise Figure 3.1 and Tables 3.1 & 3.2
[3]r = red, w = white, O = Organically cultured, C = Conventionally cultured

Table 3.6 Mycoflora dynamics for 2006 at véraison and ripening stage of vine grape

Cultivar[1] / Vineyard code[2]	Colour / Culture[3]	Sampling period	% *Aspergillus* section *Nigri*	% *Penicillia* spp.	% Other Fungi
MD/A3	r/C	ripening	98	0.7	0.4
CS/A6	r/C	ripening	31	48	20
AG/B2	r/C	ripening	57	26	16
RD/A1	w/C	ripening	86	2	10
RD/A2	w/C	véraison	62	4	33
		ripening	85	4	10
RD/A3	w/C	véraison	24	19	55
		ripening	90	0	9
AG/C1	r/O	véraison	0.1	80	19
		ripening	0.2	72	27
AG/B1	r/O	ripening	13	66	19
MP/C1	r/O	véraison	0.1	80	19
		ripening	1	67	31
CS/C1	r/O	véraison	0.4	87	11
		ripening	1	60	38
SR/C1	r/O	véraison	0.3	73	26
		ripening	1	55	42
SB/C1	w/O	ripening	2	68	29
CR/C1	w/O	ripening	0.5	44	54
Total		véraison	1.6	78.8	19.6
Total		ripening	23.9	48.3	27.8

[1]MD = Mavrodaphni, CS = Cabernet Sauvignon, AG = Agiorgitico, RD = Roditis, MP = Moschophilero, SB = Sauvignon Blanc, CR = Chardonnay

[2]For the localisation of each vineyard according to its code it is recommended to advise Figure 3.1 and Tables 3.1 & 3.2

[3]r = red, w = white, O = Organically cultured, C = Conventionally cultured

Table 3.7 Mycoflora dynamics for 2007 at véraison and ripening stage of vine grape

Cultivar / Vineyard code	Colour / Culture	Sampling period	% *Aspergillus* section *Nigri*	% *Penicillia* spp.	% Other Fungi
MD/A3	r/C	véraison	19	0	80
		ripening	94	0.4	5
RD/A3	w/C	ripening	60	2	37
MP/C2	r/O	véraison	0.8	0	99
		ripening	7	67	25
MP/C1	r/O	ripening	0	2	97
AG/B1	r/O	véraison	0	78	21
		ripening	2	90	7
CS/C1	r/O	ripening	0.7	1	98
SR/C1	r/O	ripening	0	0	100
Total		véraison	6.6	17.9	75.3
Total		ripening	32.0	22.3	45.6

[1]MD = Mavrodaphni, CS = Cabernet Sauvignon, AG = Agiorgitico, RD = Roditis, MP = Moschophilero, SR = Syrah

[2]For the localisation of each vineyard according to its code it is recommended to advise Figure 3.1 and Tables 3.1 & 3.2

[3]r = red, w = white, O = Organically cultured, C = Conventionally cultured

These results indicated that the occurrence of *Aspergillus* section *Nigri* species was relatively high every year in the total mycofloral populations and represented >23% and for 2004 reached a mean occurrence of 41%. In the composition of *Aspergillus* section *Nigri* population, *A. carbonarius* represented 22 – 35%, with 2004 the only exception where it was about 10%. Nevertheless, mycofloral analysis for that year was not part of the thesis per se and detailed identification was not done. From the isolates identified as *A. carbonatius*, >80% were able to produce OTA. From the OTA-producers the highest detected production was 23.11 μg g^{-1} while the majority of them produced <10 μg g^{-1} of growth medium.

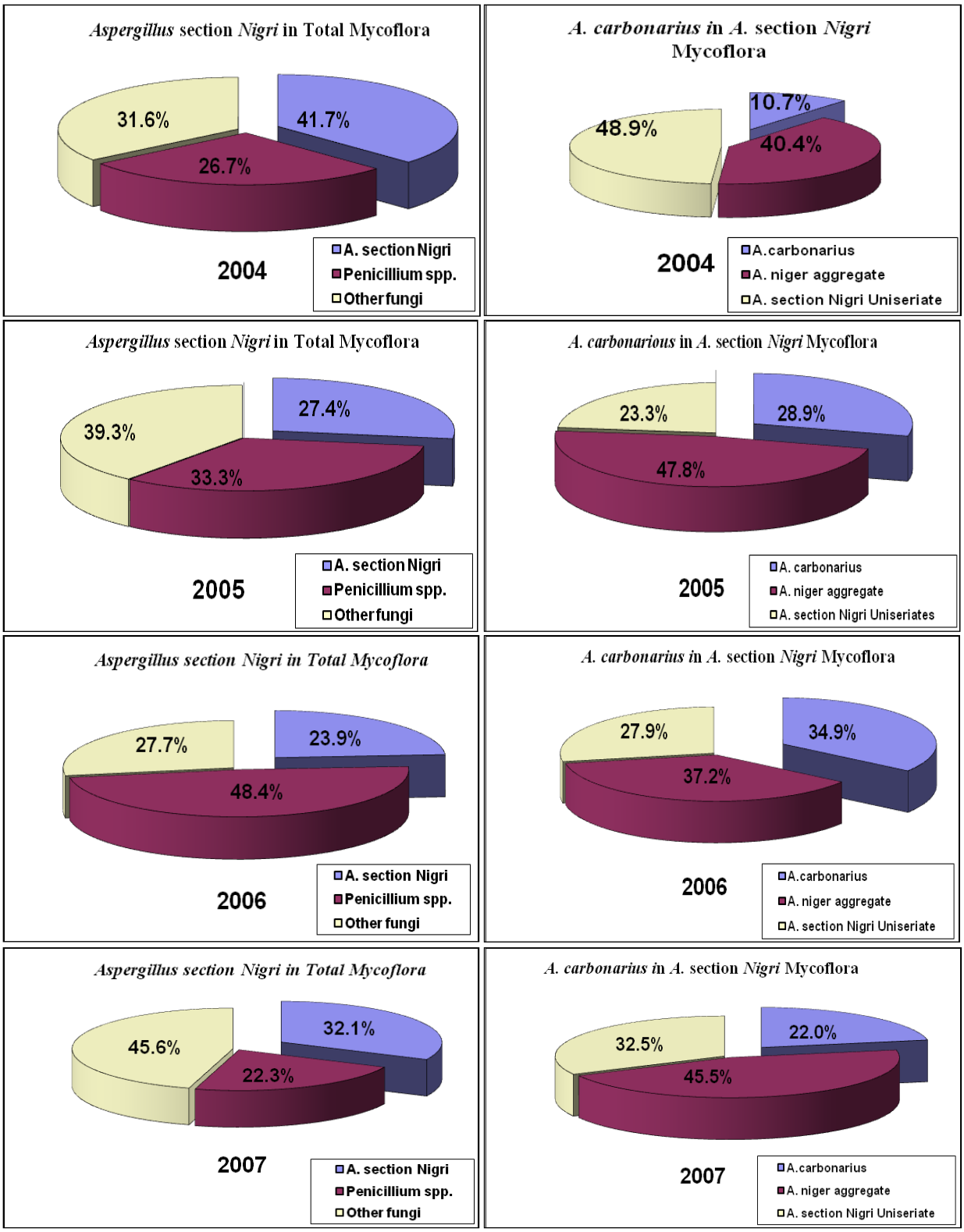

Figure 3.2 Occurrence and population composition of *Aspergillus* spp. at harvest time on berries of wine grape cultivars in Peloponnese for the 4 studied years (2004-2007).

3.3.2 Ripening *vs* véraison

Considering berry status, six different varieties (2 white and 4 red) cultured from 9 different vineyards were monitored for their mycofloral dynamics during 2006-07. Figure 3.3 shows that for the total mycoflora there was a strong correlation between the two berry statuses. This was also evident when the results are considered in relation to cropping system, organic or conventional followed (Figure 3.4).

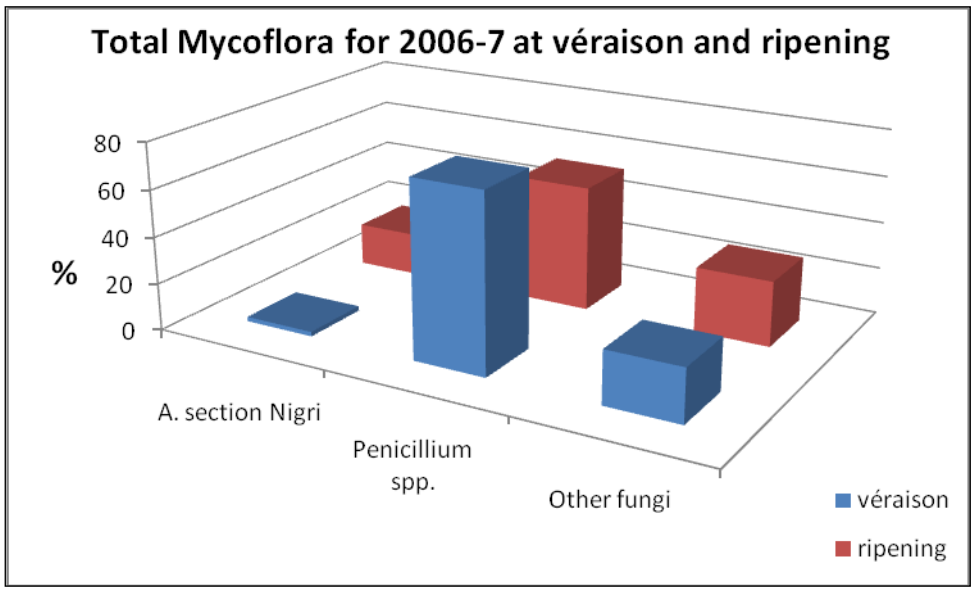

Figure 3.3 Total mycoflora of wine making vineyards between the véraison and ripening stage of grape.

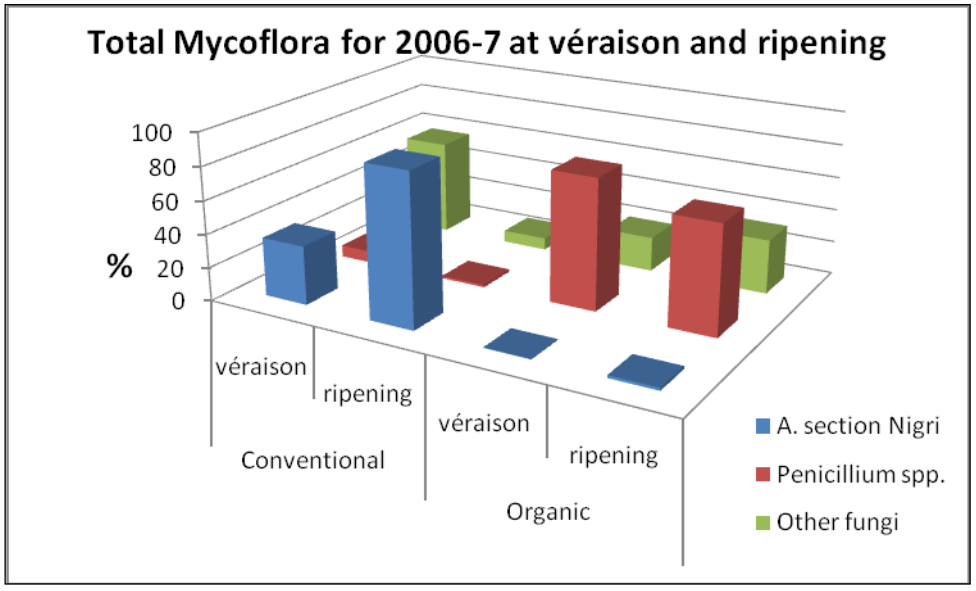

Figure 3.4 Total mycoflora of organically and conventionally cultured vineyards between the véraison and ripening stage of grape.

It is also worthwhile noting that the "other fungi" percentage increased during the ripening of grapes for the organic vineyards in contrast to the conventional, where the use of chemical treatments appeared to decrease them. Although *A.* section *Nigri* did not show the same patterns. A similar trend was found when examining every variety separately (Figure 3.5), especially for the two conventionally cultured Roditis and Mavrodaphni cultivars for which the increase from véraison to ripening was more marked.

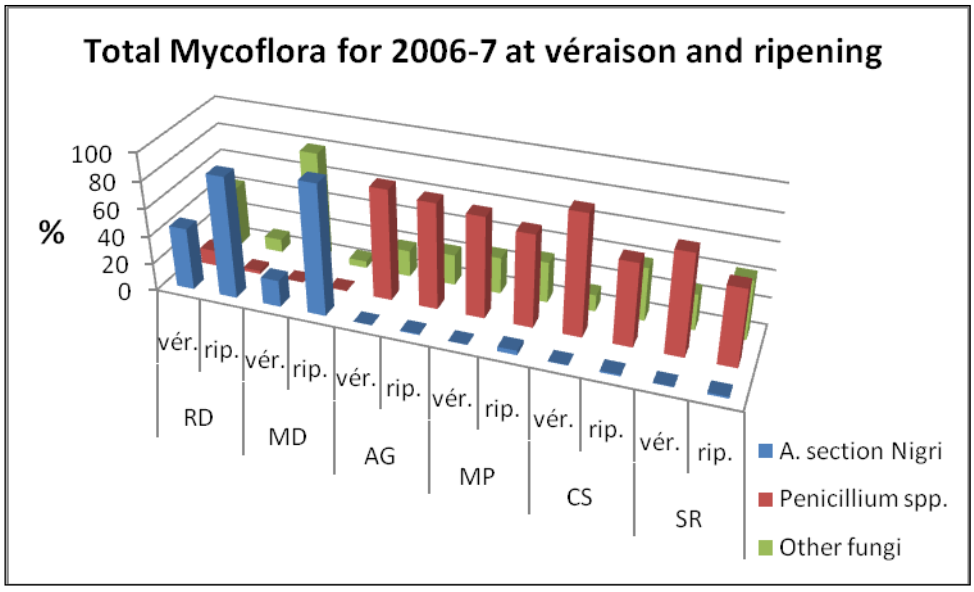

Figure 3.5 Mycoflora dynamics for 6 varieties of wine making vineyards. Roditis (RD) and Mavrodaphni (MD) were of conventional culturing, while Agiorgitico (AG), Moschophilero (MP), Cabernet Sauvignon (CS) and Syrah (SR), of organic.

3.3.3 Organic *vs* conventional culturing technique

When data are distributed between conventionally and organically treated vineyards results showed a strong correlation with the cropping type. Results are demonstrated in Figures 3.6 – 3.8.

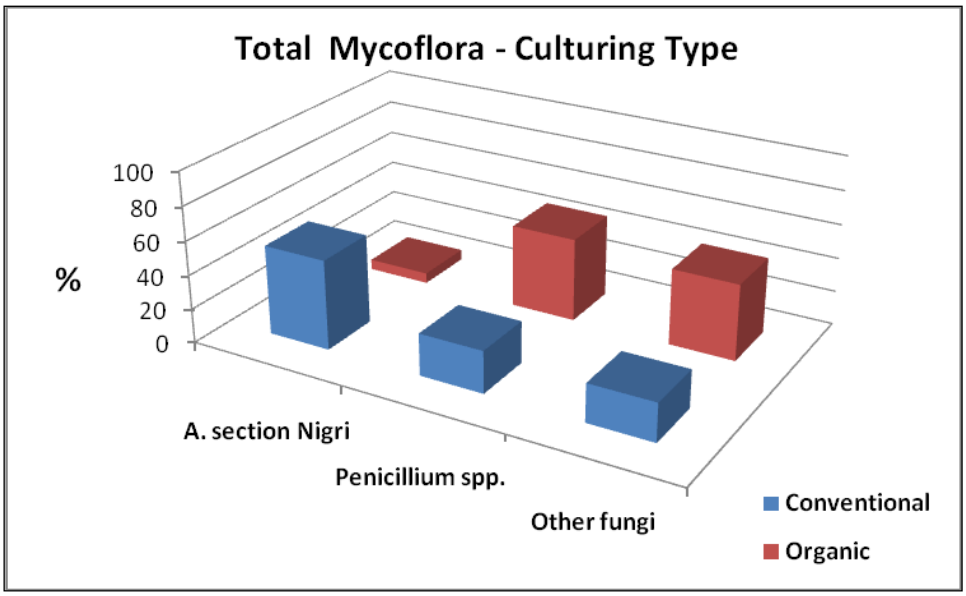

Figure 3.6 Total mycoflora of wine making vineyards between the véraison and ripening stage of grape in relation to culturing type.

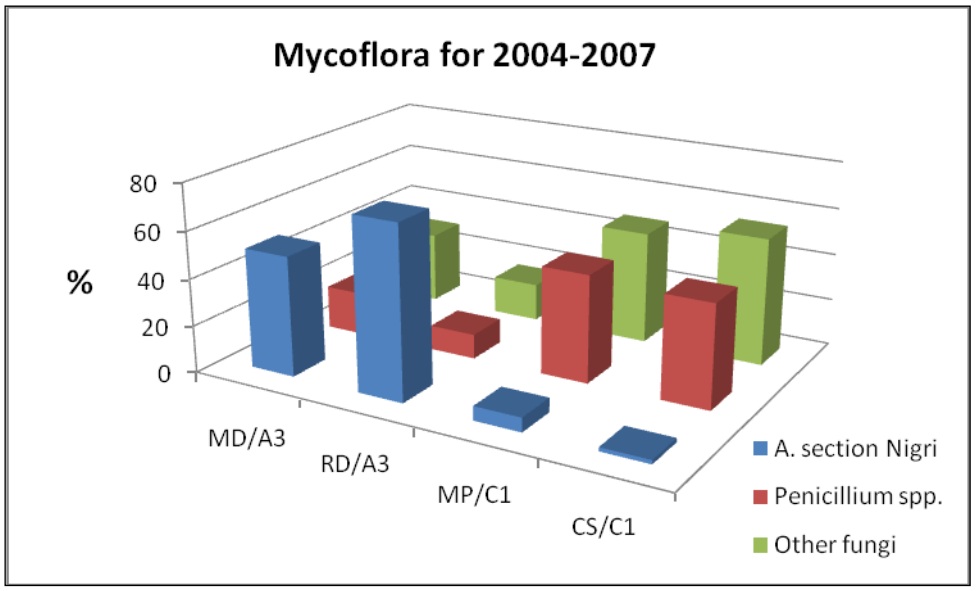

Figure 3.7 Total mycoflora at harvesting time for conventionally cultured Mavrodaphni (MD) and Roditis (RD) cultivars and for the organically cultured Moschophilero (MP) and Cabernet Sauvignon (CS).

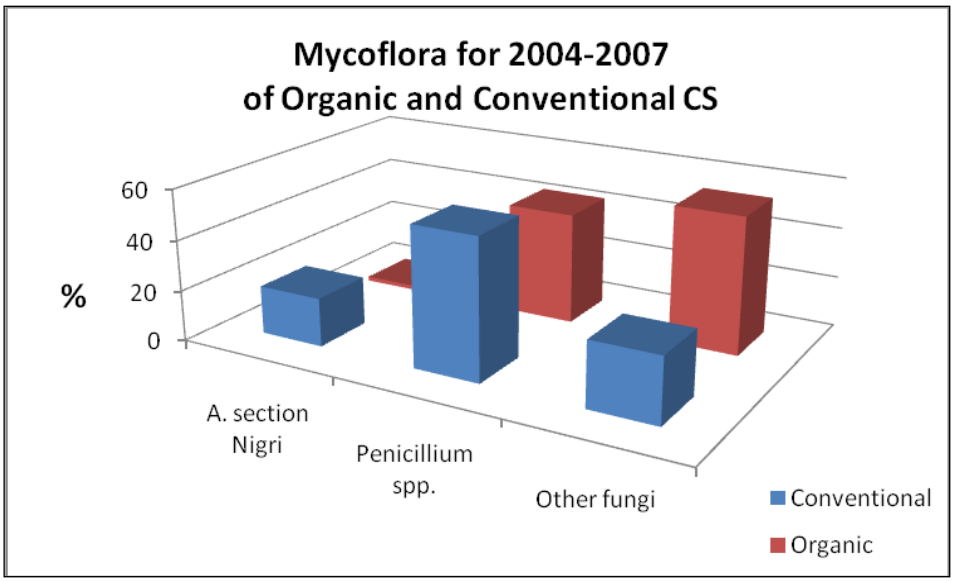

Figure 3.8 Total mycoflora at harvesting time for conventionally and organically cultured cv. Cabernet Sauvignon.

The percentage of samples colonized by *Aspergillus* section *Nigri* was significantly influenced by the cropping system followed by the two wine producers. *A.* section *Nigri* from conventional vineyards studied were generally the dominant fraction in contrast with the organically where the section was dominant only in one case of a Muscat Blanc variety grown in the lowest altitude from all the organically grown varieties.

It is worthwhile noting that Cabernet Sauvignon (Figure 3.8; Table 3.5), although showing the same trend in relation to cropping system, it was the least contaminated variety among the conventionally cultured examined.

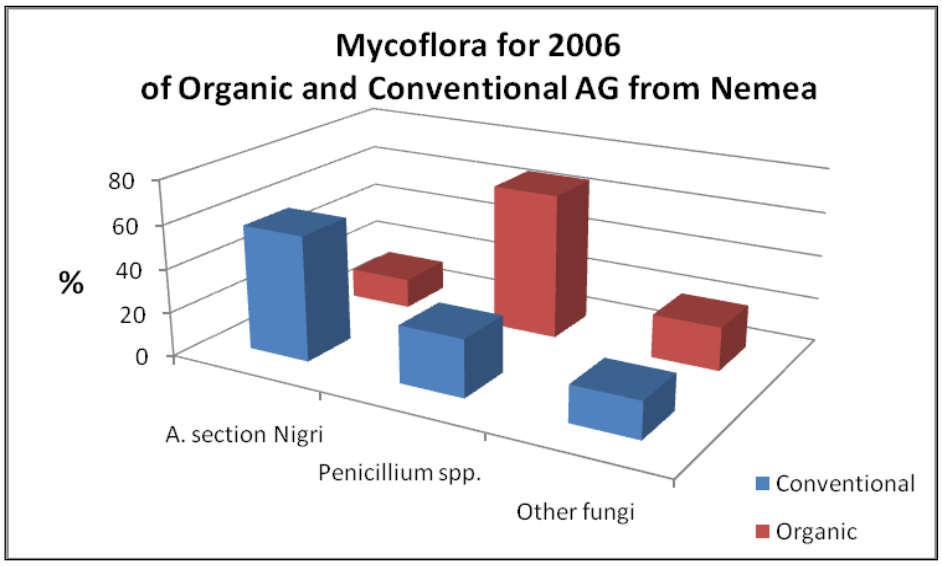

Figure 3.9 Total mycoflora at harvesting time for conventionally and organically cultured Agiorgitico cultivar for 2006.

In Figure 3.9 the population distribution for an Agiorgitico variety cultured conventionally and organically in lateral regions in Nemea are shown (B1, Achladia Nemeas, organic and B2, Archaia Nemea, conventional; Table 3.1). Agiorgitico is presented from the overall results as a rather resistant variety to *Aspergillus* section *Nigri* spp., but when the cropping system followed was a conventional one, even if the location remained the same as that of organic cultured ones, the results was far worse from the latter.

3.3.4 Geo-climatic factors

Some interesting findings were found when the results were correlated to the altitude and distance from sea. As has been shown in Figure 3.9 the occurrence of black *Aspergilli* increases for the Roditis cultivars as culturing altitude decreases. A similar trend is shown in Figure 3.10, but this was correlated to smaller distances from sea (5 & 15km) since when the distance was greater, the altitude also increased.

As regards the climatic conditions for the period of study (véraison and ripening) the discrimination of Mountainous (M) and Landlocked Mediterranean (LM) climate types is the same with the discrimination made for altitude (Tables 3.1, 3.2), and therefore results are not shown again. As shown in Figure 3.10 and 3.11, the vineyards of LM climate are far more susceptible to black Aspergilli infection from those located at higher altitudes and thus in a Mountainous climate.

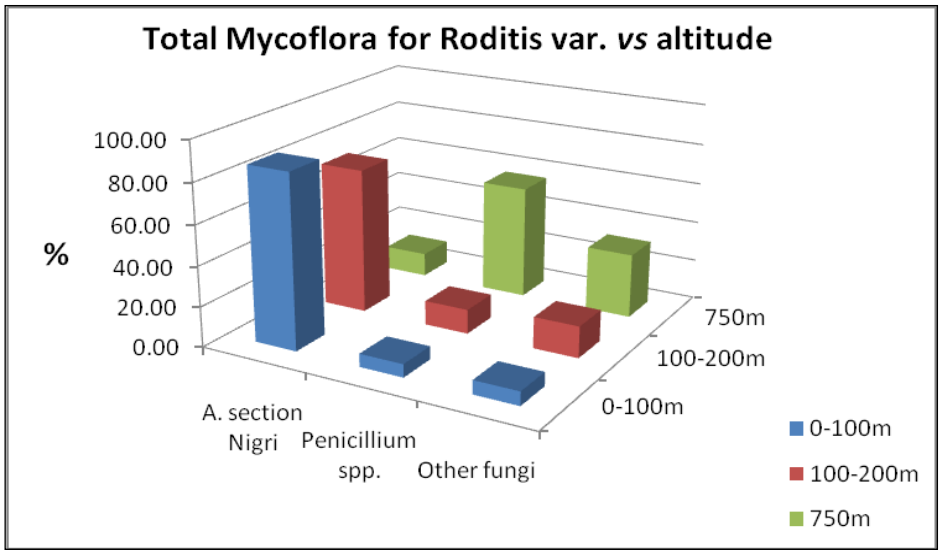

Figure 3.10 Total mycoflora at harvesting time for Roditis variety at different altitudes of Achaea region.

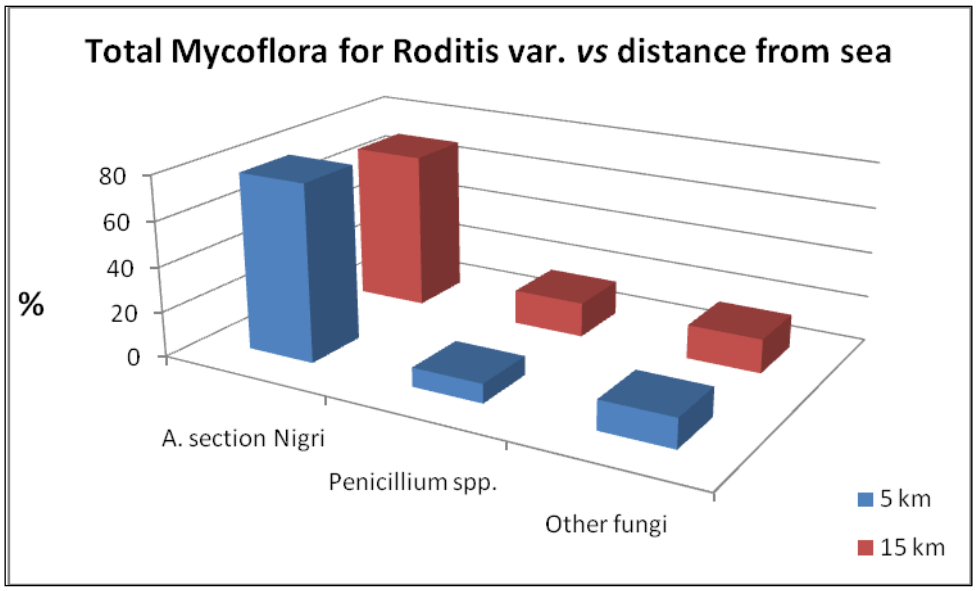

Figure 3.11 Total mycoflora at harvesting time for Roditis variety at different distances from sea of Achaea region (Altitude 50-200m).

3.3.5 Grape Varieties

As regards the cultivars of vines the results shown in Figure 3.12 draw a clear discrimination between two groups. Those commonly cultivated at high altitudes and which result in wines of high acidity and low sugar content (Cabernet Sauvignon, Agiorgitico, Sauvignon Blanc and Syrah), had low susceptibility to contamination by *A.* section *Nigri*. In contrast, the varieties of Mavrodafni, Roditis and Muscat Blanc, usually cultured in lower altitudes, and with high sugar content, were found to be more susceptible to black *Aspergilli*. From these varieties the Roditis and Muscat Blanc have characteristic thin berry-skin, which make them more susceptible to fungal or insect infection. The colour of the sample did not play a central role in occurrence of black *Aspergilli*, since both white and red varieties found to be highly contaminated [e.g. MD (r) & MB (w)].

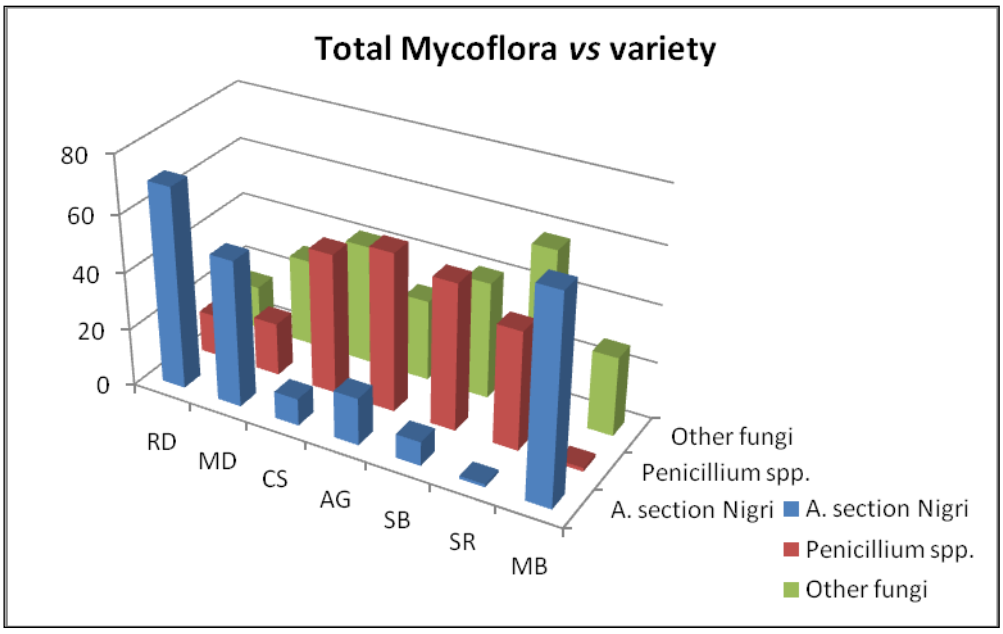

Figure 3.12 Total mycoflora of the studied varieties.

3.4 Discussion

The grape varieties included in this study are representative of a wide range of characteristics. Regarding geographical distribution, Cabernet Sauvignon is probably the most common grape variety in the world and it is widely grown in Greece, giving high quality red dry wines. Sauvignon Blanc, Muscat Blanc and Syrah are less common varieties in Greece. Nevertheless, Muscat Blanc is a variety that has been adapted to the regional vineyard characteristics of Achaea and is designated with the V.Q.P.R.D. certification. Equally important varieties for Greek wine-making production and also of V.Q.P.R.D. certification are the varieties of Mavrodaphni, intended usually for the production of sweet wines, and Agiorgitico that gives red dry wines of high quality. Roditis is a variety that in Peloponnese is used for the production of local table wines with high consumption in Greece. The position of these varieties (Roditis, Mavrodaphni, Agiorgitico, Muscat Blanc and Cabernet Sauvignon) on the Greek wine making industry and market, and the fact that they produce important quality wines, make the severity of the reported infection from *Aspergillus* section *Nigri* more important.

Aspergillus section *Nigri* and in particular *A. carbonarius* play a central role in OTA contamination of grape products (Cabañes *et al.*, 2002; Bellí *et al.*, 2005b; Jørgensen, 2005; Battilani *et al.*, 2006a). In this study they were thus the main fungi considered. The occurrence of *A.* section *Nigri* in the total mycoflora of all vineyards tested was always high

(23.9 – 41.7%) throughout the 4 years of study, although there have been recorded higher incidences in more limited surveys in Greece (Battilani *et al.*, 2006b; Tjamos *et al.*, 2006). However, some samplings of Roditis and Mavrodaphni varieties from Achaea showed incidences >90% for some years. From the *A.* section *Nigri* isolates, *A. carbonarius* represented 20-35%, with the only exception being 2004, where they represented only 10% of the total black Aspergilli isolates. OTA-producers within the *A. carbonarius* fraction were always >80%. These results are in accordance with other studies from Italy (Battilani *et al.*, 2004b), Spain (Bau *et al.*, 2005; Gomez *et al.*, 2006), France (Bejaoui *et al.*, 2006) and Tunisia (Lasram *et al.*, 2007) of the Mediterranean basin.

The study between the two stages of grape maturation, véraison and ripening, revealed interesting information about the dynamics of the potential OTA producer fungi. Firstly, it was clearly shown that the final occurrence of *A.* section *Nigri* in the total mycoflora is proportional to the infection at véraison. When high incidences were present at véraison, then, at the following samplings just before harvest, the incidence increased dramatically. The same was observed from several other studies regarding the two stages of maturation in grapes intended for wine-making (Serra *et al.*, 2003; 2005; 2006b; Bejaoui *e al.*, 2006; Tjamos *et al.*, 2006; Lasram *et al.*, 2007). Nevertheless, different results have been found. For example, Battilani *et al.* (2006a) and Belli *et al.* (2004b) reported the same incidence of *A.* section *Nigri* and *A. carbonarius* between the two maturation stages of grape berries. The factor of "sour rot" or "black mold" that is dominant in Greece and elsewhere should also be taken into account as an amplifier of spore contamination of the berries between véraison and harvest (Guzev *et al.*, 2006; Tjamos *et al.*, 2004). In a similar way, the main characteristic of the most contaminated samples of the present study was the increased proportion of damaged berries, which apart from "sour rot" incidence, may be correlated also with the thin berry skin and high sugar proportion of these samples.

When the results are differentiated as organic and conventional cultured vineyards, the picture changes dramatically, showing a mean incidence of infected berries for conventionally cultured vineyards >52% and in some cases >70% (i.e. Roditis or Mavrodaphni samplings). In contrast, organically cultured vineyards present a rather low mean incidence of infection (6%) at harvesting time, with some samples revealing an absence of black *Aspergilli* at harvest time. The quality of the samples, in terms of damaged berries, was far better for organically cultivated vineyards, while even for intact samples of conventional cultured plants the incidence remained high. Similar results have been reported from other studies regarding the cropping system followed before harvest of wine-making vineyards. Ponsone *et al.* (2007)

monitored two cultivars in Argentina under organic and non-organic cultivation system, with main differences between the two techniques being the use of herbicides for the latter. The two cultivars under organic cropping had reduced incidences of *A.* section *Nigri* spp. at harvest time compared with the non-organic. This was revealed even in the case where population of black Aspergilli was greater at véraison for organic type vineyards. Always, at harvesting time organically cultured samples had at least half the incidence from conventionally grown ones. Data of Tjamos *et al.* (2006) showed that the organically grown varieties Athiri and Cabernet Sauvignon were less contaminated among the sampled wine grape cultivars, while the conventionally grown Cabernet Sauvignon was the most contaminated one.

Valero *et al.* (2007b), examined fungicide applications of either Chorus (cyprodinil) or Switch (37.5% cyprodinil + 25% fludioxonil) 21 days before harvest. When the fungicides were applied twice pre-harvest with one extra application at véraison, the inhibition of *A.* section *Nigri* was reduced. The effectiveness of these two fungicides was also shown by others (Tjamos *et al.*, 2004; Belli *et al.*, 2006b; 2007b). However, since there is awareness of the traditional SO_4Cu or copper hydroxide effectiveness for fungal inhibition and accounting the general trend for minimising chemical treatments in agriculture, the present study did not focused on the effect of commercially available fungicides. Moreover, from the results of the present study, in conventionally cultured vineyards there was a decrease in other contaminant fungi following the maturation of grapes, while the opposite happens with "*Aspergillus* section *Nigri*". In contrast, organically cultured varieties presented an increase of other contaminant fungi from véraison to ripening. These results suggest that when the inhibition of mycoflora by fungicides is not fully successful, the reduction of mycoflora from the chemical treatment allows rapid *A. carbonarius* colonisation to occur when the environmental conditions are favourable. As regards the varieties studied, the results are in accordance with Tjamos *et al.* (2006) findings that revealed the Agiorgitiko cultivar cultivated in mountainous regions of Greece showed a low level of *A. carbonarius* even if the total *Aspergillus* spp. infection level was high and the Muscat Blanc cultivar from the Aegean Islands found to be among the most contaminated ones. Moreover, in surveys investigating the presence of OTA in Greek wines, the most contaminated samples refer to sweet wines, which are usually produced in Greece by Muscat Blanc and Mavrodaphni cultivars (Stefanaki *et al.* 2003; Soufleros *et al.* 2003).

There is clearly an effect of the localisation of vineyard to the population of fungi. This comes out firstly from the decreased infection from *A.* section Nigri of Roditis varieties

grown at higher altitudes in contrast with those located in lower and near to the sea. Secondly, from the fact that Muscat Blanc grown in Achaea region, although being of organic cropping, had similar incidence of black *Aspergilli* with other varieties of the same origin. In contrast, varieties grown in the mountainous Arcadia always had lower incidences from the Mediterranean climate of Achaea. A similar trend was also reported by Tjamos *et al.* (2006) for Greek cultivars, where more contamination was found in those originating from Mediterranean climates near the sea, like the Aegean Islands, in contrast with those grown in mountainous climates of Central Greece.

Chapter 4: Ecology and Control of *A. carbonarius* in Grapes

4.1 Introduction

Mycotoxins are toxic metabolites produced by certain fungi that can infect and proliferate on various agricultural commodities in the field and/or during storage. The occurrence of these toxins on grains, nuts and other commodities susceptible to mould infestation is influenced by several environmental factors. Temperature, humidity, latitude, stage of maturation and mechanical damage of the fruit or the kernel are some factors that can affect the growth and toxin production of the fungi (Pitt and Hocking 1997; Magan and Olsen 2004). Mycotoxins may exhibit various toxicological manifestations; some are teratogenic, mutagenic and /or carcinogenic in susceptible animal species and are associated with various diseases in domestic animals, livestock, and humans in many parts of the world (JEFCA 2001). Black aspergilli are the main ochratoxin A (OTA) producing species most frequently encountered in warm and tropical climates in a variety of foods (Abarca *et al.* 1994). In particular, ochratoxin A (OTA) is a secondary metabolite of several species of filamentous fungi (*Aspergillus* section *Circumdati* and *A.* section *Nigri*) with nephrotoxic, immunosuppressive, teratogenic and carcinogenic effects to animals and humans (O'Brien and Dietrich 2005; Murphy *et al.* 2006; Richard 2007). Moreover, there are several reports from many countries in the world describing the high frequencies of OTA contamination in a large number of food groups (Creppy 2002; Counil *et al.* 2005), leading international organizations and authorities to thoroughly investigate and report risk assessments of the problem (JEFCA 2001; Berg 2003; Heussner 2006). Many authors report OTA contamination of cereal and vegetable products, even meat products like sausages contaminated via poor quality animal feed (Samson *et al.* 2000; Magan and Olsen 2004). Grapes, raisins, grape juice and wine can be considered as high-risk products because of colonisation by *Aspergillus carbonarius* (Chulze *et al.* 2006; Leong *et al.* 2006a). Several research studies have determined the mycoflora responsible for the presence of OTA in these products and have shown that *Aspergillus* Section *Nigri* and in particular *A. carbonarius* have a central role in OTA contamination of these commodities (Abarca *et al.* 2001; Cabañes *et al.* 2002; Esteban *et al.* 2004; Bellí *et al.* 2005; 2007; Jørgensen 2005; Battilani *et al.* 2006a; Pateraki *et al.* 2007; Visconti *et al.* 2008). This has led the EU to develop legislative limits of 2ng ml^{-1} for wine and grape juice and 10ng ml^{-1} for vine dried fruits (EC Regulation 1886/2006). The frequent presence of OTA in these products above the acceptable limits has been previously

demonstrated (McDonald *et al.* 1999; Stefanaki et *al.* 2001; Soufleros et *al.* 2003; Belli et *al.* 2004b; Magnoli et *al.* 2004; Mankotia 2004).

Moreover, strains of *A. carbonarius* have been isolated and identified in several Mediterranean wine producing countries such as Spain, Italy, Israel, Portugal and Greece (Serra *et al.* 2003; Battilani *et al.* 2004; Bellí *et al.* 2004a; Mitchell *et al.* 2004; Tjamos *et al.* 2004, 2006) and other parts of the world (Chulze *et al.* 2006; Leong *et al.* 2006; Romero *et al.* 2007). Based on a recent geostatistical study (Battilani *et al.* 2006b), Greece was an area which had a high isolation percentage of *A. carbonarius* when compared with other countries (France, Israel, Italy, Portugal and Spain), as well as high contamination in its vineyards by black aspergilli at harvest time, exceeding 50% of berries.

Fungal growth is influenced by several environmental (abiotic) parameters, but generally temperature and water activity (a_w) are regarded as the principal controlling factors determining the potential for growth (Scott 1957; Magan and Lacey 1984, 1988; Panagou *et al.* 2003; Plaza *et al.* 2003; Dantigny *et al.* 2005). Alike, these two factors are of paramount importance in influencing growth and OTA formation by mycotoxigenic spoilage fungi on wine grapes. The differences of temperature among the viticultural regions, the daily mean temperature, the reduction of water availability within the grapes due to sugar content increases and production techniques influences growth and OTA production by *A. carbonarius* (Pitt and Hocking 1997; Belli *et al.* 2006; Serra *et al.* 2006). Although some research is available on the ecophysiology of isolates of *A. carbonarius*, some results seem to be contradictory mainly due to isolate and regional variations (Bragulat *et al.* 2001; Mitchel *et al.* 2004; Belli *et al.* 2005a). Very little information is available on the tolerances of environmental factors and OTA production by isolates of *A. carbonarius* from Greece and how this compares with other regions in the Mediterranean region.

In this instance one of the objectives of this study was to examine the effect of temperature and a_w on *in vitro* growth and OTA production of two *A. carbonarius* isolates from wine grapes in southern Greece. The effects on (a) lag phase prior to growth, (b) growth rates and (c) temporal and maximum OTA production at marginal and optimum conditions identified. Surface response curves were developed for prediction of growth and OTA production which could be useful to predict times when growth and OTA may be produced.

Mathematical modelling has proved to be a valuable tool to predict bacterial growth as a function of environmental factors such as temperature, pH and water activity (Davey 1994; Zwietering *et al.* 1994; Rosso *et al.* 1995; McMeeking *et al.* 2002). However, the modelling of filamentous fungi has not received the same level of attention, possibly due to inherent

difficulties in quantifying fungal growth and produce reliable and reproducible data (Gibson *et al.* 1994; Gibson and Hocking 1997; Dantigny *et al.* 2005). Recently, the need for improved understanding of the factors controlling fungal growth in foods has attracted the attention of several researchers who have developed probabilistic, mechanistic, semi-mechanistic, empirical and thermal death models for a variety of toxigenic and spoilage fungi (Gibson *et al.* 1994; Cuppers *et al.* 1997; Valík *et al.* 1999; Membré and Kubaczka 2000; Patriarca *et al.* 2001; Rosso and Robinson 2001; Sautour *et al.* 2001, 2002; Valík and Piecková 2001).

In the case of *A. carbonarius* there are several published studies reporting the effect of temperature and a_w on fungal growth using modelling approaches (Bellí *et al.* 2004b, 2005; Mitchell *et al.* 2004; Magan and Aldred 2005; Pardo *et al.* 2005). However, these studies are focused almost exclusively on secondary polynomial model development producing response surface contour plots. In addition, these models were used for fitting the experimental data and defining optimum conditions for germination and growth, whereas no attempt was made to validate them with independently derived data and assess their prediction capability.

Within the aims of the present study was to develop and evaluate comparative empirical models, including models with biological meaningless parameters and cardinal models, in an attempt to describe the effect of temperature and water activity on the growth rate of an ochratoxigenic *A. carbonarius* isolate on a synthetic grape nutrient medium, and to validate the developed models with independent data from the literature.

Moreover, a special category of predictive models, and namely probabilistic models have been used to determine microbial responses in relation to time and provide estimates for parameters such as lag phase duration and growth rate. The latter determine the probability of microbial growth or toxin production, i.e. whether growth or toxin production might occur or not, under a specific range of environmental factors (Whiting 1995). So far, several modelling approaches have been developed for *A. carbonarius* to quantify the effect of temperature and a_w on fungal growth and OTA production (Bellí *et al.* 2004b, 2005; Magan and Aldred 2005; Pardo *et al.* 2005; Marín *et al.* 2006; Tassou *et al.* 2007; Romero *et al.* 2007). However, these studies have been focused almost exclusively on the development of kinetic models providing information in the form of growth rate and lag phase duration or produce response surface and contour plots, whereas there is little information available on the development of a probabilistic approach to quantify fungal growth and OTA production boundaries for a specific range of environmental conditions. Recently, Marín *et al.* (2008b) has developed a probabilistic model to determine the growth/no growth boundaries and OTA production by *A.*

carbonarius in pistachio nuts. To the best of our knowledge, no similar probabilistic model has been developed so far for *A. carbonarius* on grapes and grape products.

One the novelty of the present thesis is the development of probabilistic models to predict the growth/no growth and OTA production boundaries of two ochratoxigenic isolates of *A. carbonarius* from Greek wine grapes on a synthetic grape juice medium, and (ii) to validate the performance of the developed models with independent data from the literature.

4.2 Materials and methods

4.2.1 Fungal isolates

All studies in this chapter were carried out with two OTA-producing strains of *A. carbonarius* (ATHUM Culture Collection of Fungi, National and Kapodistrian University of Athens; ATHUM 5659 & ATHUM 5660), isolated from wine grapes (cultivar Roditis) during the harvesting period of 2005 in the Peloponnesus region, South Greece (Tassou *et al.*, 2007). They were identified with macroscopic and microscopic observation (Pitt and Hocking 1997; Samson *et al.* 2000) and by comparison with type strains from CABI (CABI Bioscience, Farnham, Surrey, U.K.). Their OTA production ability was confirmed on CYA medium using the method developed by Bragulat *et al.* (2001) (25°C/7days), being 4.9 and 8.9 μg g^{-1} respectively.

4.2.2 Medium and water activity modification

A synthetic grape-juice medium (SGM) representative of grape composition between véraison and harvest was used in all growth experiments. The medium consisted of the following ingredients: D(+) glucose, 70 g; D(-) fructose, 30 g; L(-) tartaric acid, 7 g; L(-) malic acid, 10 g; $(NH_4)_2HPO_4$, 0.67 g; KH_2PO_4, 0.67 g; $MgSO_4 \cdot 7H_2O$, 1.5 g; NaCl, 0.15 g; $CuCl_2$, 0.0015 g; $FeSO_4 \cdot 7H_2O$, 0.021 g; $ZnSO_4 \cdot 7H_2O$, 0.0075 g; (+) catechin hydrate, 0.05 g; agar, 25 g; and distilled water, *ca* 1000 ml (Mitchel *et al.* 2004). The medium was adjusted to pH 3.5 with 2M KOH and had an a_w of 0.98 which represented the unmodified control treatment. The a_w of the unmodified medium was 0.98, measured by a Novasina Thermoconstander RTD 33 (Novasina AG, Zürich, Switzerland) water activity meter at 20°C, and it was used as the control treatment. The a_w of the SGM was adjusted by the addition of amounts of the non-ionic solute glycerol (Merck, Darmstadt, Germany). The amounts required were calculated using a standard curve of known glycerol concentrations and a_w levels (Bellí *et al.* 2004a) and corresponded to the preferred levels for every experiment. The

medium was autoclaved and poured into sterile 9 cm Petri plates. Additional non-inoculated plates of each treatment were made to ensure that a_w levels remained accurate during experimental periods. These were found to not be significant when measured with a HygroClip® water activity meter (Rotronic HygroLab set 3, Huntington, NY, USA).

4.2.3 Inoculation, incubation and growth measurement

The fungus was grown on SGM medium for 10 days at 25°C to obtain sporulating cultures. Spore suspensions were obtained by flooding the plates with 15 ml sterile phosphate buffer solution (pH 7.0) containing 0.1% of a wetting agent (Tween 80, Merck, Darmstadt, Germany) and gently scraping the surface of the medium with a sterile spatula. After filtering through sterile medical tissue (Aseptica, Athens, Greece), the final concentration of spores was assessed by a counting chamber and adjusted to 10^6 spores ml^{-1} (Neubauer Brand, Wertheim, Germany). All treatments were inoculated centrally with a 5 μl spore suspension of 10^6 spores ml^{-1}. This resulted in a 3–4 mm diameter droplet at the centre of the Petri plates containing 15-20 ml SGM medium. The experiment for the impact of the two environmental factors on growth and OTA production was carried out at a_w levels of 0.85, 0.90, 0.93, 0.96 and 0.98 in combination with temperatures of 10, 15, 20, 25, 30, 35 and 40°C. The experiment for the development of the predictive models was carried out at 0.85, 0.90, 0.93, 0.96 and 0.98 a_w, and at the same temperatures with the later. The effect of temperature and a_w on fungal growth and OTA production was investigated by means of a full factorial design. Four replicated plates for each treatment were used and the whole experiment was repeated twice (n = 8). For every treatment of a_w/temperature the Petri plates were enclosed in polyethylene bags and kept in constant temperature chambers. Growth measurements were carried out for up to 50 days. Mycelial extension was measured daily or periodically depending on treatment by measuring the diameter of the colonies in two directions at right angles to each other. The radial extension rates were obtained from these data.

4.2.4 Determination and quantification of OTA

OTA production was assessed by a modification of the Bragulat *et al.* (2001) method in all growth experiments performed. An agar plug of 3 mm radius was taken from the central area of each colony after 5, 10, 15, 25 and 35 days incubation. The sampling was stopped at this time point because previous studies suggested that degradation of OTA can occur over longer periods (Leong *et al.* 2006b). The samples were weighed and placed in dark glass vials. They were then vortexed with 0.5 ml of methanol and kept in the solvent for 1 hr. The

extracts were filtered (Millex® Syringe Driven Filter Unit, Millipore Co. Bedford, MA, USA) and stored at 4°C until HPLC analysis.

Analysis was performed with a HPLC system (Hewlett Packard Series 1100), equipped with an Agilent 1100 fluorescence detector (330 nm excitation wavelength; 460 nm emission wavelength). Chromatographic separations were performed with a C18 Waters Spherisorb ODS2 column (5 μm, 250×4·6 mm). The flow rate of the mobile phase used (acetonitrile: water: acetic acid; 51:47:2) was 1ml min^{-1}. The detection limit of the method was <0·01 μg OTA g^{-1} of SGM and extracts were diluted to 1:100 just before inoculation to HPLC system. The OTA standard used for the reference curve was supplied by Sigma. The OTA production results were analysed with a computer equipped with the HP ChemStation 1990-99 software (Hewlett Packard Company, CA, USA).

4.3 Results

4.3.1 Impact of water activity and temperature on growth and ochratoxin A production of two *A. carbonarius* isolates from wine grapes in Greece

4.3.1.1 Statistical treatment of the data and primary modelling

The data collected by measuring the extension of the mycelium were fitted to Baranyi equation in order to determine maximum specific growth rates (mm d^{-1}) and lag phases prior to growth (days) by using DMFit v. 2·0 (Institute of Food Research, Norwich Research Park Norwich, U.K.), an Excel add-in program to fit curves where a linear phase is preceded and followed by a stationary phase. DMFit is based on a reparameterized version of the model of Baranyi and the main difference between this model and other sigmoid curves like Gompertz, Logistic, etc. is that the mid-phase is really very close to linear, unlike those classical sigmoid curves which have a pronounced curvature there (Baranyi *et al.* 1993; Barayni and Roberts 1994).

The linear growth phases were only used for statistical analyses. To exclude the stationary phase the model of Baranyi (Baranyi *et al.* 2003; Baranyi and Roberts 1994) was modified by setting a 0 value for the "Richard curvature" parameter (this defines the curvature at the end of the linear phase). The derived maximum specific radial growth rates were then examined for the effect of a_w × temperature × isolate by ANOVA using Statistica software (Statsoft, Tulsa, OK, USA).

Polynomial multiple linear regression analysis and the resulting response surface models were obtained in order to predict growth rates and OTA production for *A. carbonarius* isolates with the examined environmental factors also using the Statistica software.

4.3.1.2 Impact of temperature and a_w on lag phase prior to growth of the two *A. carbonarius* isolates

No significant differences were found between the two isolates with respect to lag phase duration. Thus, as an example, data for one isolate is shown in Figure 4.1. This shows the influence of temperature and a_w on lag phase (in days) at 15–35°C. The results at 10 and 40°C are not included as no growth occurred at all a_w treatments over the experimental period. The shortest lag phases (0.54-0.58 days) were at 30-35°C and 0.96 a_w. At lower temperatures (15 and 20°C) the shortest lag phases were 2.32 and 1.45 days respectively at the highest a_w level used (0.98). It was also interesting that the lag phase duration between different a_w levels had a greater variance at a set temperature treatment, than between different temperatures and the same a_w level.

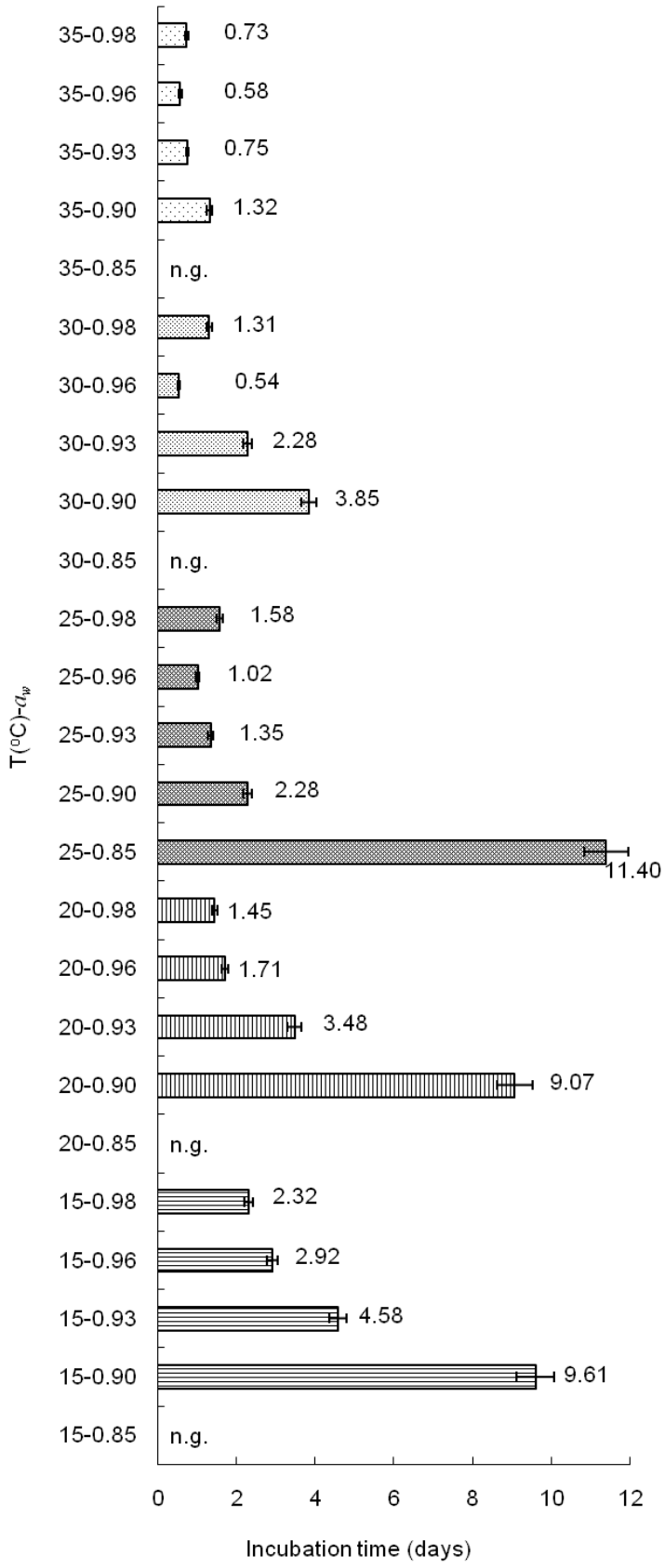

Figure 4.1 *A. carbonarius* lag phase duration (days) in relationship with the environmental factors studied, temperature (°C) and water activity (a_w) level. (Data are mean values from the two strains ± standard error).

4.3.1.3 Impact of temperature and a_w on the mycelium growth of the two *A. carbonarius* isolates

Table 4.1 shows the maximum specific growth rates (estimated by the Excel add-in program DMFit, IFR, Norwich, UK) of both isolates of *A. carbonarius* at different temperatures (15-35°C) and in the a_w range of 0.850–0.980. Data for the temperatures of 10 and 40°C are not shown as no growth was detected at these conditions. All DMFit growth curves had values of $R^2 > 0.99$ (data not shown).

Table 4.1 Maximum specific growth rates (mm d^{-1}) of the two *Aspergillus carbonarius* isolates at different a_w and temperature levels on SGM.

Isolate	a_w	Growth rate (mm d^{-1})*				
		15°C	**20°C**	**25°C**	**30°C**	**35°C**
A. carbonarius ATHUM 5659	0·850	n.g.**	n.g.	0·37±0·01	n.g.	n.g.
	0·900	0·44±0·01	0·88±0.01	3·06±0·04	3·43±0·05	3·75±0·08
	0·930	1·49±0·01	2·02±0·04	4·87±0·10	5·93±0·12	6·38±0·15
	0·960	2·76±0·09	4·01±0·09	6·43±0·21	7·07±0·37	7·33±0·46
	0·980	2·36±0·06	3.18±0·01	5·05±0·21	6·49±0·30	6·81±0·16
A. carbonarius ATHUM 5660	0·850	n.g.	n.g.	0·39±0·01	n.g.	n.g.
	0·900	0·43±0·01	0·64±0·01	3·07±0·04	3·64±0·04	3·89±0·06
	0·930	1·39±0·02	2·06±0·04	4·69±0·05	5·77±0·15	6·65±0·17
	0·960	2·92±0·07	3·91±0·08	6·64±0·14	7·00±0·41	7·35±0·29
	0·980	2·52±0·05	3·23±0·08	4·41±0·18	6·61±0·28	7·16±0·11

* Data are mean values of duplicate experiments (three replications each) ± standard error
** No growth was observed after 55 days of incubation

Temperatures of 30 and 35°C were optimum for growth of the *A. carbonarius* isolates. The highest growth rates were at 35°C and at 0.96 a_w, although the shortest lag period prior to growth was observed at 30°C and the same a_w. Although 25°C was not optimum for growth, mycelial extension occurred at 0.85 a_w only at these combination of conditions. At all other temperatures no growth has occurred over the 55 day incubation period. Growth was significantly reduced at 15 and 20°C when compared with that at ≥25°C. Generally, 0.96 a_w favoured the growth of *A. carbonarius* isolates regardless of the temperature.

Analysis of variance (Table 4.2) showed that the single factors of a_w and temperature and their interaction were highly significant (P<0.000001) on growth rate. This was supported by

the test of the SS Whole Model vs. SS Residual which resulted in an R^2 of > 0.99. There was no significant difference between isolate and its interaction with the two environmental factors. Thus, subsequent data treatment included data sets for both isolates together.

Table 4.2 Analysis of variance of the effects of isolate, a_w, temperature, and their interaction, on growth of *A. carbonarius*.

Factors	**SS**	**d*f***	**MS**	**F**	***p***
Intercept	1165.284	1	1165.284	44880.20	0.000001*
Isolate	0.008	1	0.008	0.30	0.587359^{ns}
a_w	740.968	6	123.495	4756.32	0.000001*
Temperature	408.787	4	102.197	3936.04	0.000001*
Isolate × a_w	0.327	6	0.055	2.10	0.057184^{ns}
Isolate × Temperature	0.021	4	0.005	0.21	0.934760^{ns}
Temperature × a_w	238.148	24	9.923	382.17	0.000001*
Isolate × Temperature × a_w	1.006	24	0.042	1.61	0.046928^{ns}

*: statistically significant at P<0.000001; ns: not significant

Figure 4.2 shows the surface response contour plot of growth rates for both isolates after quadratic smoothing of the data. The function describing the suggested model of growth rate vs. a_w and temperature and its coefficients were estimated by multiple linear regression resulting in a second order polynomial with R^2 > 0.99 and the function:

$$\boldsymbol{\mu}\ (h^{-1}) = -7.683+2.406\times\boldsymbol{a_w}+3.362\times\boldsymbol{T}-0.317\times\boldsymbol{a_w}^2+0.104\times\boldsymbol{a_w}\times\boldsymbol{T}-0.416\times\boldsymbol{T}^2.$$

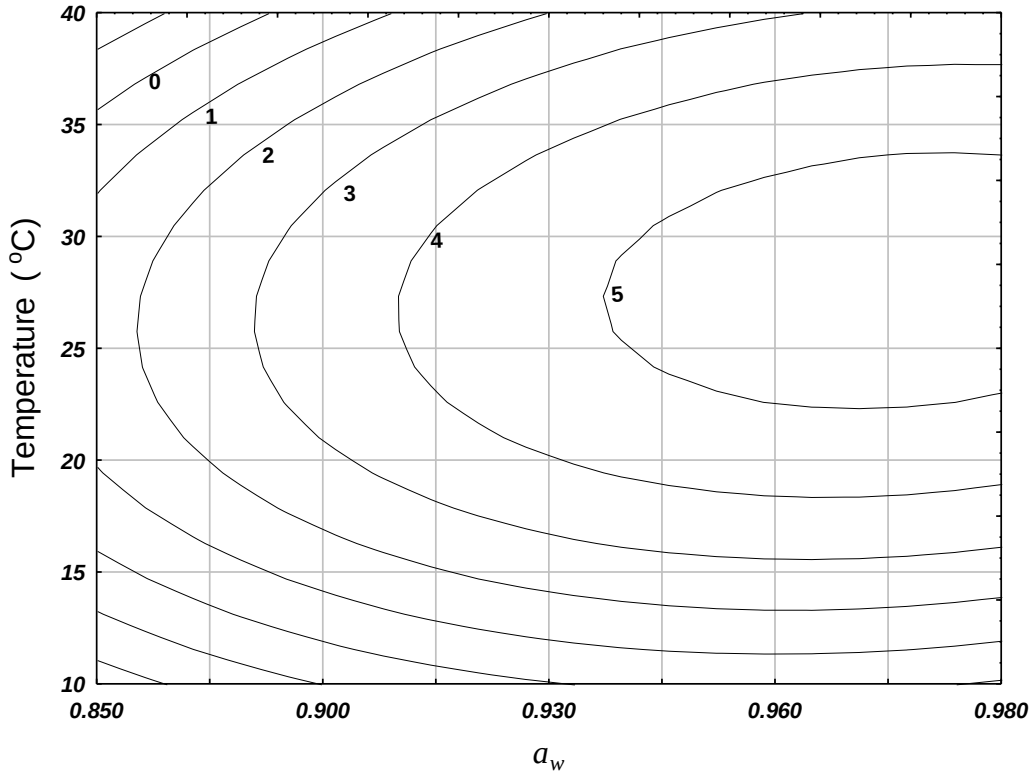

Figure 4.2 Surface response (quadratic) contour plot showing the effect of a_w and temperature on the maximum specific growth rate (h^{-1}) of *A. carbonarius* isolates, on the SGM.

4.3.1.4 Impact of temperature and a_w on OTA production by the *A. carbonarius* isolates

Figure 4.3 shows the contour plot of OTA production against a_w and temperature after distance weighted least squares smoothing of the data for both two isolates. Maximum OTA was produced at 20°C and 0.96 a_w. In general, optimum conditions for OTA production were 0.93–0.96 a_w at 20°C, then at 0.93 a_w and 15°C. Significantly less production occurred at all a_w treatments at ≥25°C.

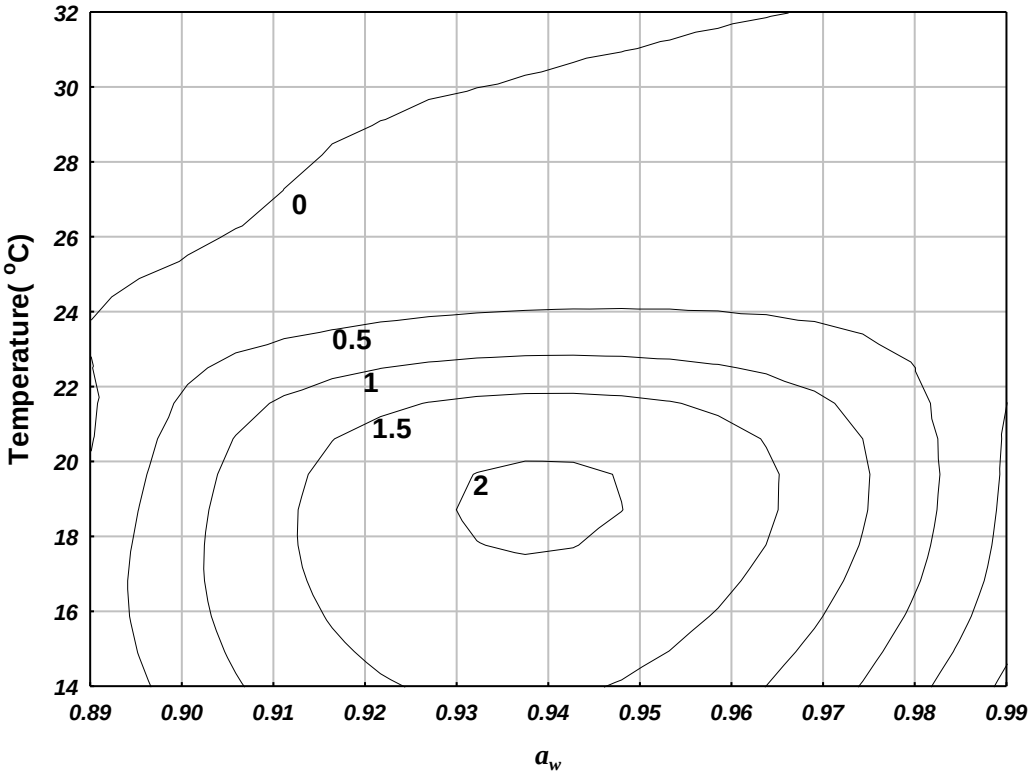

Figure 4.3 DWLS surface response contour plot for OTA production (μg g^{-1}) by the two *A. carbonarius strains*, after 10 days of growth on the SGM, in relation to a_w and temperature.

The temporal changes in OTA production at 20^{o}C and three a_w levels are shown in Figure 4.4. The highest amounts of OTA were produced after 15-25 days. The maximum amounts of OTA production detected were 3.14 and 2.67μg g^{-1} respectively for the two isolates (data not shown).

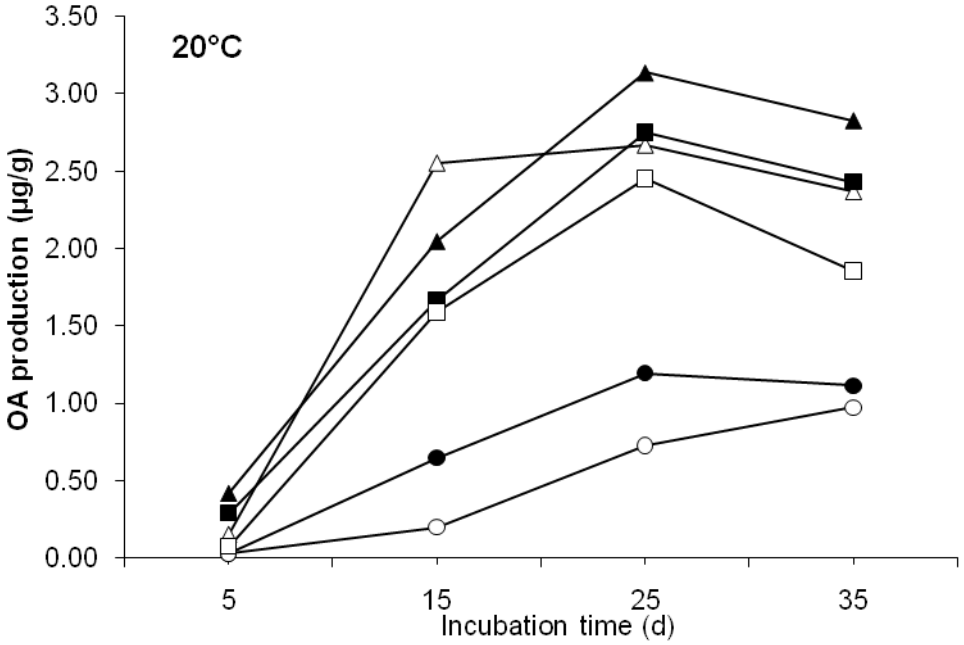

Figure 4.4 Ochratoxin A production by *Aspergillus carbonarius* ATHUM 5659 (solid symbols) and ATHUM 5660 (outlined symbols) over time at water activity 0·930(■, □), 0·960(▲, Δ), 0·980(●, ○), and temperature of 20°C.

4.3.2 Modelling the effect of temperature and water activity on the growth of an ochratoxigenic isolate of *Aspergillus carbonarius* from Greek wine grapes

4.3.2.1 Model development

A standard two-step approach was followed to develop a model for the influence of temperature and a_w on fungal growth. First, estimates of the maximum specific colony growth rates (μ_{max}) were obtained by applying Baranyi's primary model (Baranyi *et al.* 1993; Baranyi and Roberts 1994) as described before (Section 4.3.1.1). The average estimates of μ_{max} were then fitted to secondary models to describe the single and combined effects of temperature and water activity on fungal growth.

A quadratic response surface model was the first model used. The following transformation of water activity was applied, as introduced by Gibson *et al.* (1994):

$$b_w = \sqrt{1 - a_w} \quad (1)$$

Therefore, the quadratic expression of the natural logarithm of maximum colony growth rate had the following form:

$$ln\ \mu_{max} = a_0 + a_1 \cdot b_w + a_2 \cdot b_w^{\,2} + a_3 \cdot T + a_4 \cdot T^2 + a_5 \cdot T \cdot b_w \quad (2)$$

where $a_0 \ldots a_5$ are design parameters estimated by non-linear regression. The natural logarithm transformation was introduced to stabilize the variance of the fitted values for growth rate (Gibson and Hocking 1997).

The extended combined model proposed by Parra and Magan (2004), based on the Gibson-type a_w dependence (Eq. 1) and the Ratkowsky-type temperature dependence on growth rate, was the second modelling approach to study the effect of temperature and a_w on the growth of *A. carbonarius*. The model has the general form:

$$\ln \mu_{max} = a_0 + a_1 \cdot b_w + a_2 \cdot b_w^{\,2} + a_3 \cdot T \cdot [1 - \exp(a_4 \cdot T)] \quad (3)$$

where μ_{max} is the maximum specific growth rate (mm day^{-1}) and $a_0 \ldots a_4$ are regression coefficients.

The model of Miles *et al.* (1997) was the third approach followed to study the effect of the entire biokinetic range of temperatures and a_w levels on the growth of the fungus. The model is based on the following equation:

$$\sqrt{\mu_{max}} = b \cdot (T - T_{min}) \cdot \{1 - \exp[c \cdot (T - T_{max})]\} \cdot \sqrt{(a_w - a_{wmin}) \cdot \{1 - \exp[d \cdot (a_w - a_{wmax})]\}} \quad (4)$$

where b, c and d are coefficients to be fitted and T_{min}, T_{max}, $a_{w,min}$, $a_{w,max}$ are the minimum and maximum values of temperature and water activity, respectively, beyond which growth is not possible.

The linear Arrhenius-Davey equation (Davey 1989) was the forth model tested, based on the following equation:

$$\ln \mu_{\max} = a_0 + a_1 \cdot a_w + a_2 \cdot a_w^2 + a_3 / T + a_4 / T^2 \quad (5)$$

where T is absolute temperature (°K), a_w is water activity and $a_0 \ldots a_4$ are coefficients to be determined.

Finally, the Rosso equation (Rosso *et al.* 1995; Rosso and Robinson 2001) for the effect of temperature and water activity on fungal growth was selected:

$$\mu_{\max}(T, a_w) = CTPM_2(T, a_w) = \mu_{opt} \cdot \tau(T) \cdot \lambda(a_w) \quad (6)$$

where

$$\tau(T) = \left(\frac{(T - T_{\min})^2 \cdot (T - T_{\max})}{(T_{opt} - T_{\min}) \cdot \left[(T_{opt} - T_{\min})(T - T_{opt}) - (T_{opt} - T_{\max})(T_{opt} + T_{\min} - 2T) \right]} \right) \quad \text{and}$$

$$\lambda(a_w) = \left(\frac{(a_w - a_{w\min})^2 \cdot (a_w - a_{w\max})}{(a_{wopt} - a_{w\min}) \cdot \left[(a_{wopt} - a_{w\min})(a_w - a_{wopt}) - (a_{wopt} - a_{w\max})(a_{wopt} + a_{w\min} - 2a_w) \right]} \right)$$

The terms $T_{\min}$, $T_{\max}$, $a_{w,\min}$, $a_{w,\max}$ correspond to the values of temperature and water activity, respectively, below and above which no growth occurs. Additionally, T_{opt} and $a_{w,\text{opt}}$ are the values of temperature and water activity at which $\mu_{\max}$ is equal to its optimal value (μ_{opt}).

The in-house programme DMFit (Institute of Food Research, Norwich, UK) was used to fit the growth curves and estimate the maximum specific colony growth rate ($\mu_{\max}$). Non-linear regression was carried out using the quasi-Newton algorithm of the NLIN procedure of Statistica v6.0 (Statsoft Inc., Tulsa, OK, USA) to fit the secondary growth models. The indices used for statistical comparison of models were the regression coefficient (R^2) and the root mean square error (RMSE).

4.3.2.2 Model validation

The developed models were validated with independent data from the literature (Bellí *et al.* 2005) for eight strains of *A. carbonarius* grown at different a_w and temperature regimes on the same synthetic grape juice medium (SNM). The prediction capability of the models was evaluated using graphical plots as well as the bias (B_f) and accuracy (A_f) factors (Ross 1996).

4.3.2.3 Results

The growth curves based on colony diameter changes were typical of fungal growth presenting a straight line after a short lag period. The colony growth rate was calculated with the Baranyi primary model as the slope of the linear segment of each growth curve. Parameter estimation for Eqs 2 and 3 was based on the transformation of a_w to b_w. However, for easier comparison, all graphs are presented in a_w terms. No growth was observed at 10 and 40°C in the time frame of the experiments, regardless of a_w treatment. In addition, no growth took place at a_w values equal to 0.85 at all experimental temperatures. The response of the fungus to the environmental variables examined was quantified with five different models. Initially, the natural logarithm of the maximum specific colony growth rate ($\ln \mu_{max}$) was modelled vs. temperature and b_w with a quadratic response surface model (Equation 2; Figure 4.5; Table 4.3).

Table 4.3 Parameter estimation and performance statistics of the coefficients of the models for the μ_{max} of *A. carbonarius*.

Equation type	Parameter	Estimated value	R^2	RMSE
Polynomial	a_0	-2.815 ± 0.961	0.978	0.179
	a_1	0.167 ± 0.052		
	a_2	-0.003 ± 0.001		
	a_3	18.263 ± 5.973		
	a_4	-68.627 ± 12.122		
	a_5	0.319 ± 0.086		
Parra	a_0	-2.582 ± 1.023	0.939	0.285
	a_1	26.241 ± 8.898		
	a_2	-68.627 ± 19.348		
	a_3	0.071 ± 0.011		
	a_4	-0.143 ± 0.113*		
Miles	a_0	1.147 ± 0.625	0.967	0.619
	a_1	6.50 ± 3.57		
	a_2	126.96 ± 12.74		
	$a_{w,min}$	0.862 ± 0.023		
	$a_{w,max}$	0.995 ± 0.036		
	T_{min}	-0.15 ± 0.23*		
	T_{max}	43.88 ± 5.25		
Davey	a_0	-225.293 ± 84.625	0.939	0.287
	a_1	519.30 ± 176.67		
	a_2	-269.978 ± 93.998		
	a_3	-6800.57 ± 9906.90*		
	a_4	$-45.751 \pm 14.69 \cdot 10^{5*}$		
Rosso	μ_{opt} (days^{-1})	9.84 ± 0.82	0.977	0.569
	$a_{w,min}$	0.824 ± 0.045		
	$a_{w,max}$	0.999 ± 0.031		
	$a_{w,opt}$	0.962 ± 0.005		
	T_{min}	-4.17 ± 1.99*		
	T_{max}	43.25 ± 13.58		
	T_{opt}	34.62 ± 3.17		

* Not significant at $P<0.05$

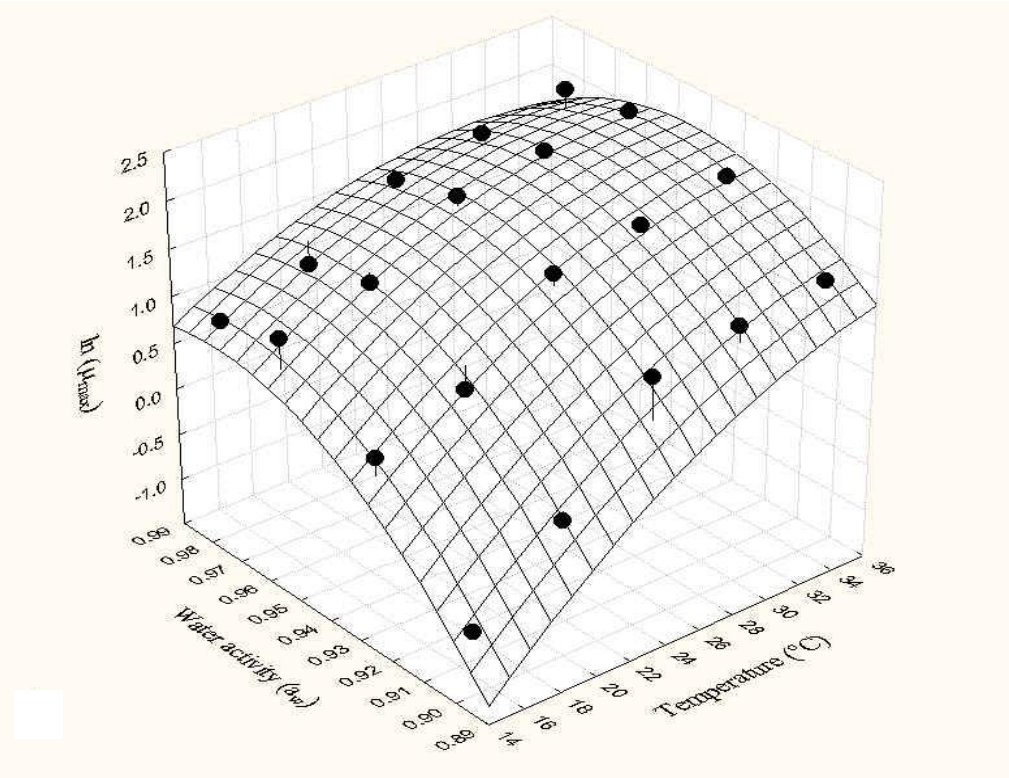

Figure 4.5 Quadratic response surface predicting the effect of temperature and a_w on the natural logarithm of maximum specific colony growth rate (ln μ_{max}) of *A. carbonarius* on a synthetic grape juice medium. Data points are mean values of three replications from two experiments.

From the generated response surface it is evident that the maximum specific colony growth rates form parabolic curves with relatively parallel positions, implying that the environmental factors act independently and pose additive effects. Curvature is observed in the optimal/super-optimal region for temperature, i.e. at 25-35°C. It is more intense at lower a_w values over the whole range of temperature, but as a_w moves to optimal/super-optimal values, curvature is greatly reduced. Growth rates presented a peak at 35°C for all a_w levels tested. The maximum specific colony growth rate was obtained at 35°C and 0.96 a_w.

The second model tested was the extended combined model proposed by Parra and Magan (Equation 3; Figure 4.6; Table 4.3). The strain was characterised by a sharp decrease in colony growth rate from $a_{w,opt}$ to $a_{w,max}$ and from a slower decrease from $a_{w,opt}$ to $a_{w,min}$. The a_w for optimal growth rate was *ca* 0.96 at all temperatures tested.

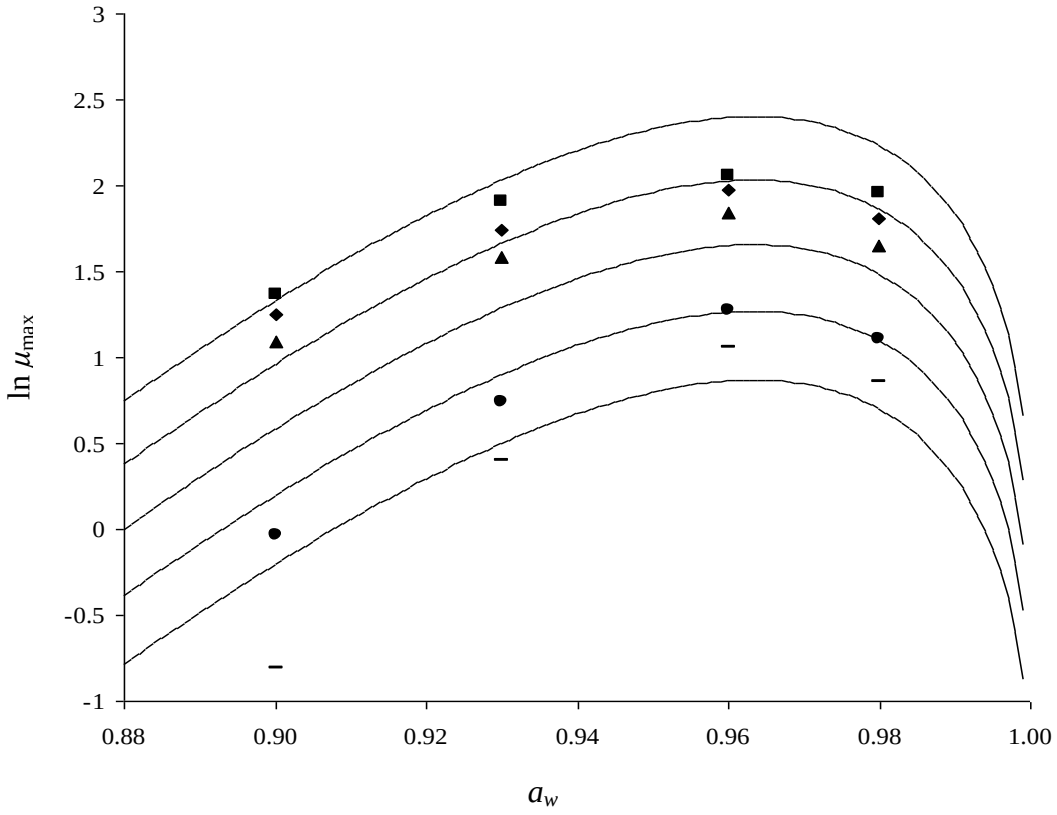

Figure 4.6 Fitted curves of the Parra model (Eq 3) describing the a_w dependence of ln μ_{max} of *A. carbonarius* growing on a synthetic grape juice medium at different temperature levels. Data points are mean values of three replications from two experiments.

The third model was an extended square root-type model proposed by Miles *et al.* (1997) in which the relative effect of temperature and a_w was modelled vs. the square root of the maximum specific colony growth rate ($\sqrt{\mu_{max}}$) (Equation 4; Figure 4.7; Table 4.3). This model has the advantage to provide parameter estimates (cardinal parameters) with biological meaning, such as minimal temperature for growth (T_{min}), maximum temperature for growth (T_{max}), minimal a_w for growth ($a_{w,min}$) and maximum a_w for growth ($a_{w,max}$) (Table 4.3). Based on this model the $a_{w,opt}$ for growth was found to be at *ca.* 0.97 for all incubation temperatures (Figure 4.8) whereas the minimal and maximum a_w for growth were 0.86 and 0.99, respectively. However, this model provided a rather unrealistic temperature estimate for minimal growth (T_{min}), -0.15, and a higher maximum growth temperature (T_{max}) for *A. carbonarious*, *ca* 43°C (Table 4.3).

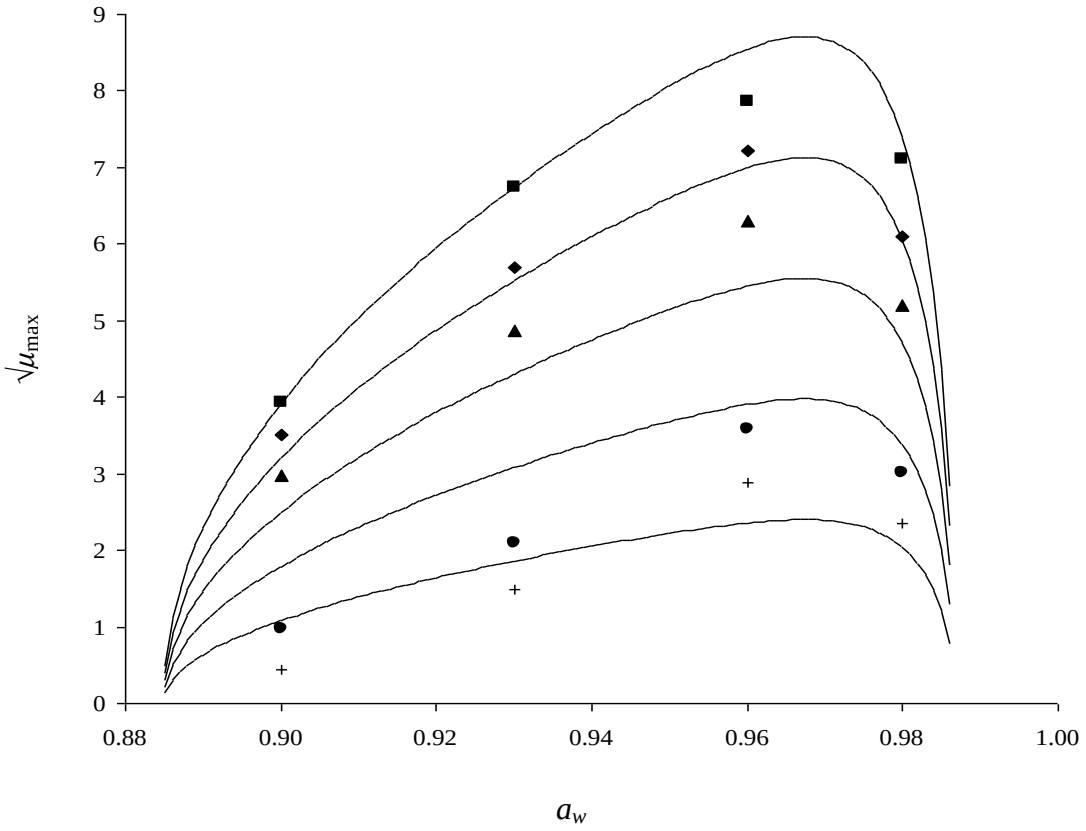

Figure 4.7 Fitted curves of the Miles model (Eq 4) describing the effect of a_w and temperature on the square root of maximum specific colony growth rate ($\sqrt{\mu_{max}}$) of *A. carbonarius* growing on a synthetic grape juice medium. Data points are mean values of three replications from two experiments.

Good fitting was also obtained by the modified (linear) version of the Arrhenius equation as proposed by Davey (1989) (Equation 5; Figure 4.8; Table 4.3). However, a weak point of this model is the lack of physiological interpretation for the significance of the values of the estimated regression parameters. The a_w for optimum growth was 0.96 regardless of incubation temperature (Figure 4.8). Besides, the parameters $1/T$ and $1/T^2$ were not statistically significant (Table 4.3) indicating that fungal growth was influenced primarily by water activity and to a much lesser extent by incubation temperature.

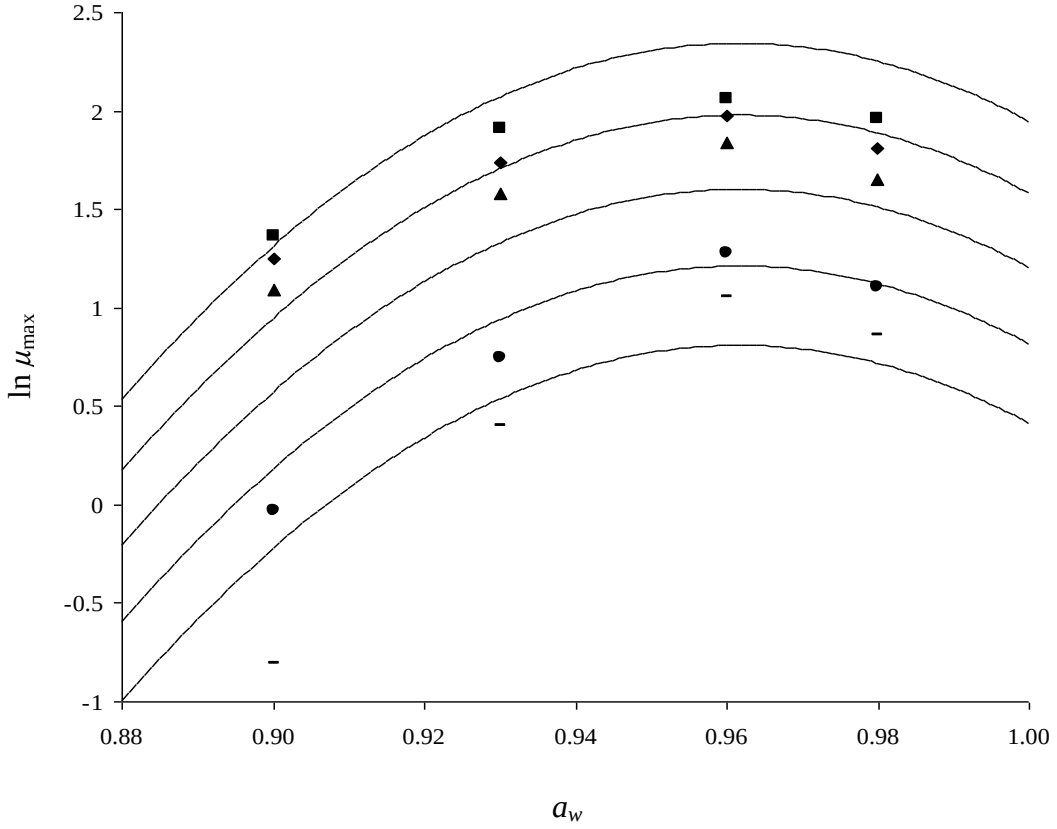

Figure 4.8 Fitted curves of the Davey model (Eq 5) describing the effect of a_w and temperature on ln μ_{max} of *A. carbonarius* growing on a synthetic grape juice medium. Data points are mean values of three replications from two experiments.

Finally, the complete model of Rosso gave a good quality fit for the data set of *A. carbonarius* providing cardinal values of environmental factors (minimal, maximum and optimal values) (Equation 6; Figure 4.9; Table 4.3). The estimates for $a_{w,min}$, $a_{w,max}$ and T_{max} were comparable with the values of Eq. 4 (Miles model) although the latter model gave a slightly higher estimate of $a_{w,min}$ for growth. Additionally, the Rosso-type model provided estimates for optimum values of growth rate, temperature and water activity (Table 4.3), but again an unrealistic estimate for T_{min} was predicted. As far as the statistical evaluation is concerned, all models exhibited good fit to experimental data in terms of R^2 and RMSE. The lowest RMSE values were observed in the polynomial, Parra and Davey models. This is associated with the *ln* transformation of maximum specific colony growth rates used in these models, in contrast to the untransformed values introduced to the Rosso model and the square root transformation of the square root-type models.

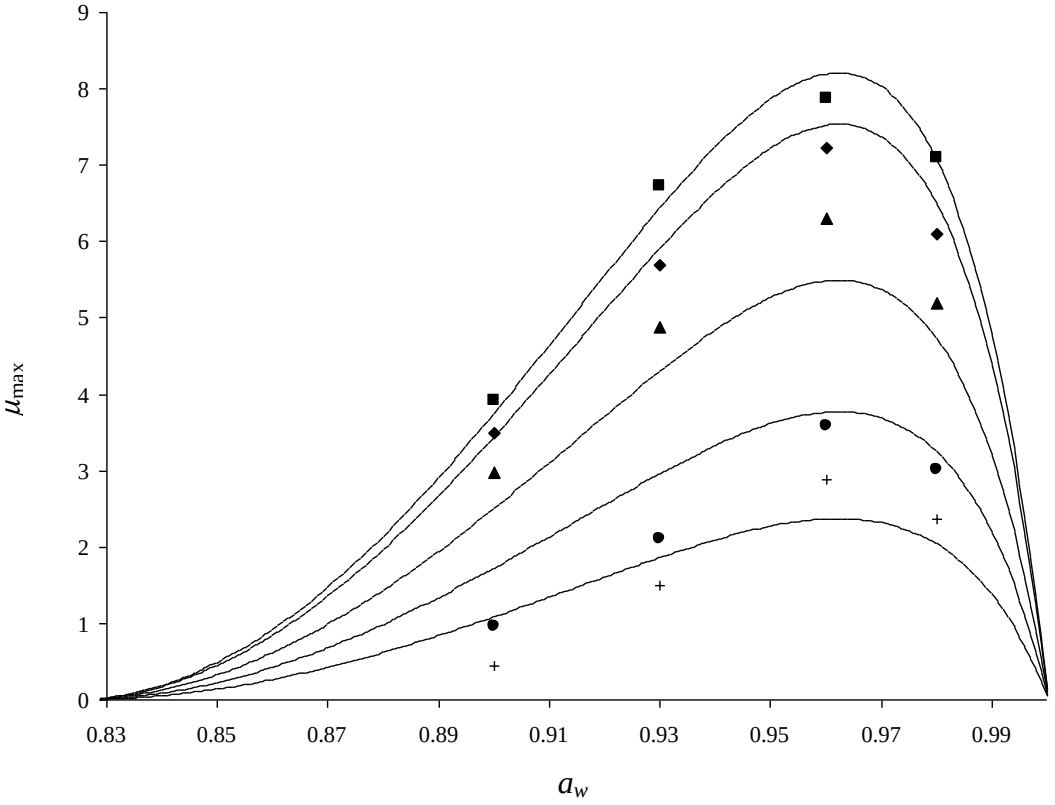

Figure 4.9 Fitted curves of the Rosso model (Eq 6) describing the effect of a_w and temperature on the maximum specific colony growth rate (μ_{max}) of *A. carbonarius* growing on a synthetic grape juice medium. Data points are mean values of three replications from two experiments.

The graphical comparisons of the observed growth rates of eight *A. carbonarius* strains from independent literature data vs. the predicted growth rates by the five developed models is presented in Figure 4.10. The performance of validation in terms of calculated bias (B_f) and accuracy (A_f) factors is shown in Table 4.4. The calculated B_f values were > 1, indicating that, in general, the models predicted higher maximum specific growth rates than observed. Moreover, the values of A_f indicated that all models predicted colony growth rates with approximately the same deviation from the observed growth rates.

Figure 4.10 (Next page) Predictions of the polynomial (a), Parra (b), Miles (c), Davey (d), and Rosso (e) models for the μ_{max} of eight strains of *A. carbonarius* from independently derived data (Bellí *et al.* 2005).

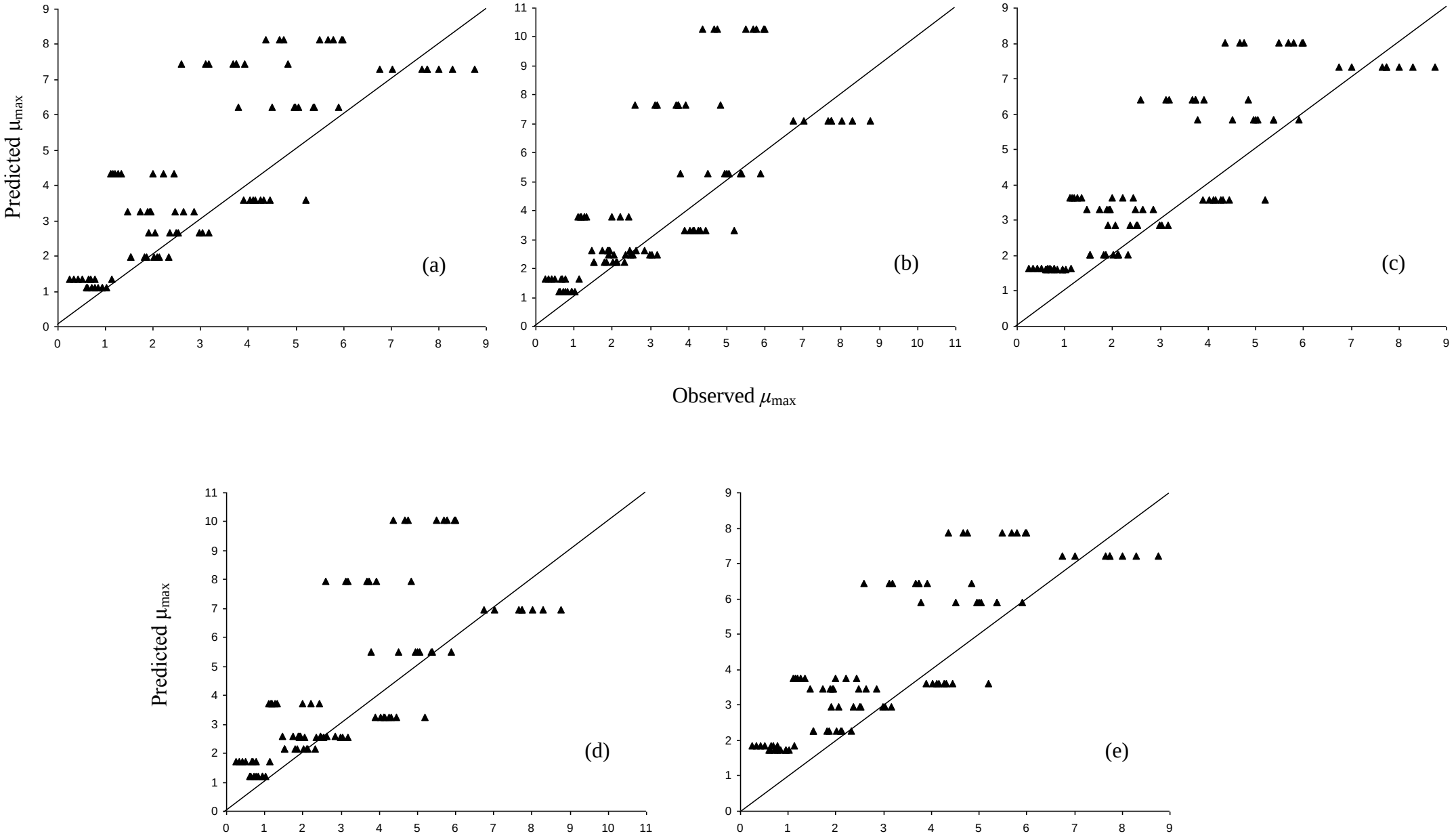

Fig. 4.10

Table 4.4 Validation indices (bias and accuracy factors) for the performance of the developed models on independently derived data from the literature (Bellí *et al.* 2005).

Equation type	Bias factor	Accuracy factor
Polynomial	1.45	1.54
Parra	1.44	1.57
Miles	1.49	1.57
Davey	1.45	1.58
Rosso	1.54	1.62

4.3.3 Effect of temperature and a_w on growth and ochratoxin A production boundaries of two *Aspergillus carbonarius* isolates on a simulated grape juice medium

4.3.3.1 Modelling of the growth/no growth interface

For each replicate response of the two fungal isolates, visible growth or no growth were scored as values of 1 or 0, respectively. Data were fitted to a logistic regression model based on the approach of Ratkowsky and Ross (1995) in order to determine the growth/no growth boundaries of the fungi under the assayed environmental factors. The model employed was a second-order logistic regression model in the form shown in the following equation:

$$\mathbf{log}\, it(P) = \mathbf{ln}\left[\frac{P}{1-P}\right] = a_0 + a_1 t + a_2 T + a_3 a_w + a_4 t^2 + a_5 T^2 + a_6 a_w^2 + a_7 tT + a_8 t a_w + a_9 T a_w \quad (1)$$

where, P is the probability of growth (in the range of 0-1), a_i are coefficients to be estimated, a_w is the water activity of the medium, t (days) is incubation time, and T (°C) is temperature. The equation was fitted using Minitab® version 14.1 (Minitab Inc., State College, PA, USA) logistic regression procedure. The automatic variable selection option with a stepwise selection method was used to choose the significant effects (P<0.05). The predicted growth/no growth interfaces for P=0.1, 0.5, and 0.9 were calculated using Microsoft Excel Solver. The following statistical indices were calculated to measure the goodness-of-fit of the developed models: the Hosmer-Lemeshow goodness-of-fit statistic, the maximum rescaled R^2 and the concordance rate (McKellar and Lu 2001; Koutsoumanis and Sofos 2005; Skandamis *et al.* 2007).

4.3.3.2 Modelling of OTA production

For the same set of a_w/temperature conditions a separate logistic regression model was developed to quantify the effect of both factors on OTA production. For this reason, OTA analysis results were assigned values of either 1 when OTA concentration was above the limit of detection (>l.d.), or 0 when OTA concentration was below the limit of detection (<l.d.). Logistic regression was used to calculate the probability of OTA production given a certain combination of storage conditions (a_w and temperature) and incubation time. A similar second order logistic regression model was developed as follows:

$$\mathbf{log}\, it(P) = \mathbf{ln}\left[\frac{P}{1-P}\right] = b_0 + b_1 t + b_2 T + b_3 a_w + b_4 t^2 + b_5 T^2 + b_6 a_w^2 + b_7 tT + b_8 t a_w + b_9 T a_w$$

(2)

where, P is the probability of OTA production (in the range of 0-1), and b_i are coefficients to be estimated. The equation was fitted using Minitab® version 14.1 (Minitab Inc., State College, PA, USA) logistic regression procedure. The automatic variable selection option with a stepwise selection method was used to choose the significant effects (P<0.05). The predicted OTA interfaces for P=0.1, 0.5, and 0.9 were calculated using Microsoft Excel Solver. The goodness-of-fit of the developed model was assessed by the same statistical indices as mentioned above.

4.3.3.3 Comparison of the developed models with independent data

The predictions at 50% probability level of models for the two isolates of *A. carbonarius* were compared with two literature data sets in which conditions for growth were similar to those used for the development of the logistic models. Specifically, the first validation data set was that of Bellí *et al.* (2005) in which the growth and OTA production of eight isolates of *A. carbonarius* were monitored on the same synthetic grape juice medium in relation to temperature (15-37°C) and water activity (0.90-0.99 a_w). The second data set was again of Bellí *et al.* (2004b) who studied the effect of water activity (0.90-0.995 a_w) and temperature (10-37°C) on the growth rate of ten isolates of *Aspergillus* section *Nigri* from which four were *A. carbonarius*, on the same synthetic grape juice medium. In both data sets, the value of 0.99 a_w was not included in the validation approach as it was outside the initial a_w range used in our work for the development of the probabilistic model. It has to be noted that in both publications the reported growth data are provided in the form of kinetic parameters (growth rates, mm day^{-1}) and not as incidence (probability) of growth/no growth. For the purpose of validation, values of 0 or 1 have been assigned by the authors of the present work to validation data for a selected time period of 20 days, where, according to our experience, growth or no growth should have been occurred.

4.3.3.4 Results

The parameter estimates and statistics with the significant effects (P < 0.05) of the logistic regression model for the growth of the two fungal isolates are shown in Tables 4.5 and 4.6. The degree of agreement between predictions and observations was 99.4%

concordant and 0.6% discordant for *A. carbonarius* ATHUM 5659 (Table 4.5) indicating successful data fitting. Overall, 5.5% of observed data fell on the "wrong" side of the predicted boundary at a probability level of 0.5, from which 4.1% were false positives (i.e. growth predicted when no growth was observed) and 1.5% false negatives (i.e. no growth predicted but growth observed). The goodness-of-fit was also evaluated by the Hosmer-Lemeshow statistic (χ^2 =11.98, *df* = 8, *P* = 0.152) and the maximum rescaled R^2 (0.845) which proved the good adjustment of the model to the observations. A similar pattern was observed for the other isolate of *A. carbonarius* ATHUM 5660 (Table 4.6). In this case, the concordance of the model was 99.5%, whereas 3.4% and 0.7% of the predictions were false positive and false negatives, respectively. The Hosmer-Lemeshow statistic and the maximum rescaled R^2 showed high agreement of predicted with observed probability of growth and hence adequate fit of the data.

Table 4.5 Estimated parameters and statistical indices of the logistic regression model for the growth/no growth interface of *Aspergillus carbonarius* ATHUM 5659.

Parameter	Estimated value	Standard Error	*P*
Intercept	-1163.41	243.25	0.000
t	3.76	1.22	0.002
T	7.45	1.40	0.000
a_w	2144.02	475.82	0.000
t^2	-0.035	0.013	0.008
T^2	-0.095	0.017	0.000
a_w^2	-1040.71	242.24	0.000
$t \cdot T$	-0.022	0.006	0.001
$t \cdot a_w$	-1.95	1.14	n.s.
$T \cdot a_w$	-2.30	0.78	0.003
Hosmer-Lemeshow	11.98 (df = 8, P = *0.152*)		
Maximum rescaled R^2	0.845		
Concordant rate (%)	99.4		
Discordant rate (%)	0.6		
False positive [a]	4.1%		
False negative [b]	1.4%		

[a] Growth was not observed when the model predicted growth at probability $P > 0.5$

[b] Growth was observed when the model predicted no growth at probability $P < 0.5$

n.s.: Not significant ($P > 0.05$)

Table 4.6 Estimated parameters and statistical indices of the logistic regression model for the growth/no growth interface of *Aspergillus carbonarius* ATHUM 5660.

Parameter	Estimated value	Standard Error	*P*
Intercept	-1083.51	260.13	0.000
t	4.85	1.42	0.001
T	9.39	1.74	0.000
a_w	1894.01	504.45	0.000
t^2	-0.039	0.014	0.006
T^2	-0.106	0.018	0.000
a_w^2	-874.51	254.41	0.001
$t \cdot T$	-0.022	0.006	0.001
$t \cdot a_w$	-2.91	1.33	0.030
$T \cdot a_w$	-3.72	0.98	0.000
Hosmer-Lemeshow	0.82 (df = 8, P = 0.999)		
Maximum rescaled R^2	0.869		
Concordant rate (%)	99.5		
Discordant rate (%)	0.5		
False positive [a]	3.4%		
False negative [b]	0.7%		

[a] Growth was not observed when the model predicted growth at probability $P > 0.5$

[b] Growth was observed when the model predicted no growth at probability $P < 0.5$

Plots of probability of growth for a_w and temperature at 5, 15 and 25 days of incubation are presented in Figures 4.11 and 4.12. It is characteristic that the probability plot shifted to lower temperatures for the same a_w level, especially between 5 and 15 days for both fungal isolates. In addition, the probability of growth for *A. carbonarius* ATHUM 5660 was higher at the lowest a_w assayed (0.85) at 15 and 25 days (Figure 4.12) indicating that this fungal isolate could be more xerophilic compared with *A. carbonarius* ATHUM 5659. The predicted growth interfaces with respect to time at probabilities of 0.1, 0.5 and 0.9, together with the observed growth/ no growth data from which the predictions were derived are depicted in Figures 4.13 and 4.14 for each isolate. These graphs are also representative of the low percentage of model disagreement with the experimental data. No growth was observed at 10°C and 40°C, regardless of a_w level. As

time increased, the predicted growth interface shifted to lower water activity values for both isolates. The advancement of the interface was clearer between 5 and 15 days, but from this time onwards little change was evident, with the exception of 40°C and 0.98 a_w where slow fungal growth was observed at 25 days.

Table 4.7 Estimated parameters and statistical indices of the logistic regression model for OTA presence of *Aspergillus carbonarius* ATHUM 5659.

Parameter	Estimated value	Standard Error	*P*
Intercept	-1619.59	247.17	0.001
t	2.73	1.46	0.042
T	-3.36	1.10	0.002
a_w	1709.83	528.52	0.001
t^2	-0.009	0.011	n.s.
T^2	-0.062	0.008	0.000
a_w^2	-933.25	284.14	0.001
$t \cdot T$	-0.011	0.004	0.033
$t \cdot a_w$	-2.12	1.50	n.s.
$T \cdot a_w$	6.94	1.42	0.000
Hosmer-Lemeshow	4.05 (df = 8, P = 0.852)		
Maximum rescaled R^2	0.856		
Concordant rate (%)	98.9		
Discordant rate (%)	1.1		
False positive [a]	1.4%		
False negative [b]	2.7%		

[a] OTA absence was observed when the model predicted presence at probability $P > 0.5$

[b] OTA presence was observed when the model predicted absence at probability $P < 0.5$

n.s.: Not significant ($P > 0.05$)

Table 4.8 Estimated parameters and statistical indices of the logistic regression model for OTA presence of *Aspergillus carbonarius* ATHUM 5660.

Parameter	Estimated value	Standard Error	*P*
Intercept	-1395.49	285.59	0.000
t	16.07	3.11	0.000
T	-0.247	0.098	0.008
a_w	2640.74	580.71	0.000
t^2	-0.051	0.011	0.001
T^2	-0.061	0.008	0.000
a_w^2	-1276.59	300.49	0.000
$t \cdot T$	-0.006	0.005	n.s.
$t \cdot a_w$	-15.18	3.01	0.000
$T \cdot a_w$	3.47	1.09	0.002
Hosmer-Lemeshow	12.14 (df = 8, P = 0.145)		
Maximum rescaled R^2	0.832		
Concordant rate (%)	99.1		
Discordant rate (%)	0.9		
False positive [a]	0.7%		
False negative [b]	2.0%		

[a] OTA absence was observed when the model predicted presence at probability $P > 0.5$

[b] OTA presence was observed when the model predicted absence at probability $P < 0.5$

n.s.: Not significant ($P > 0.05$)

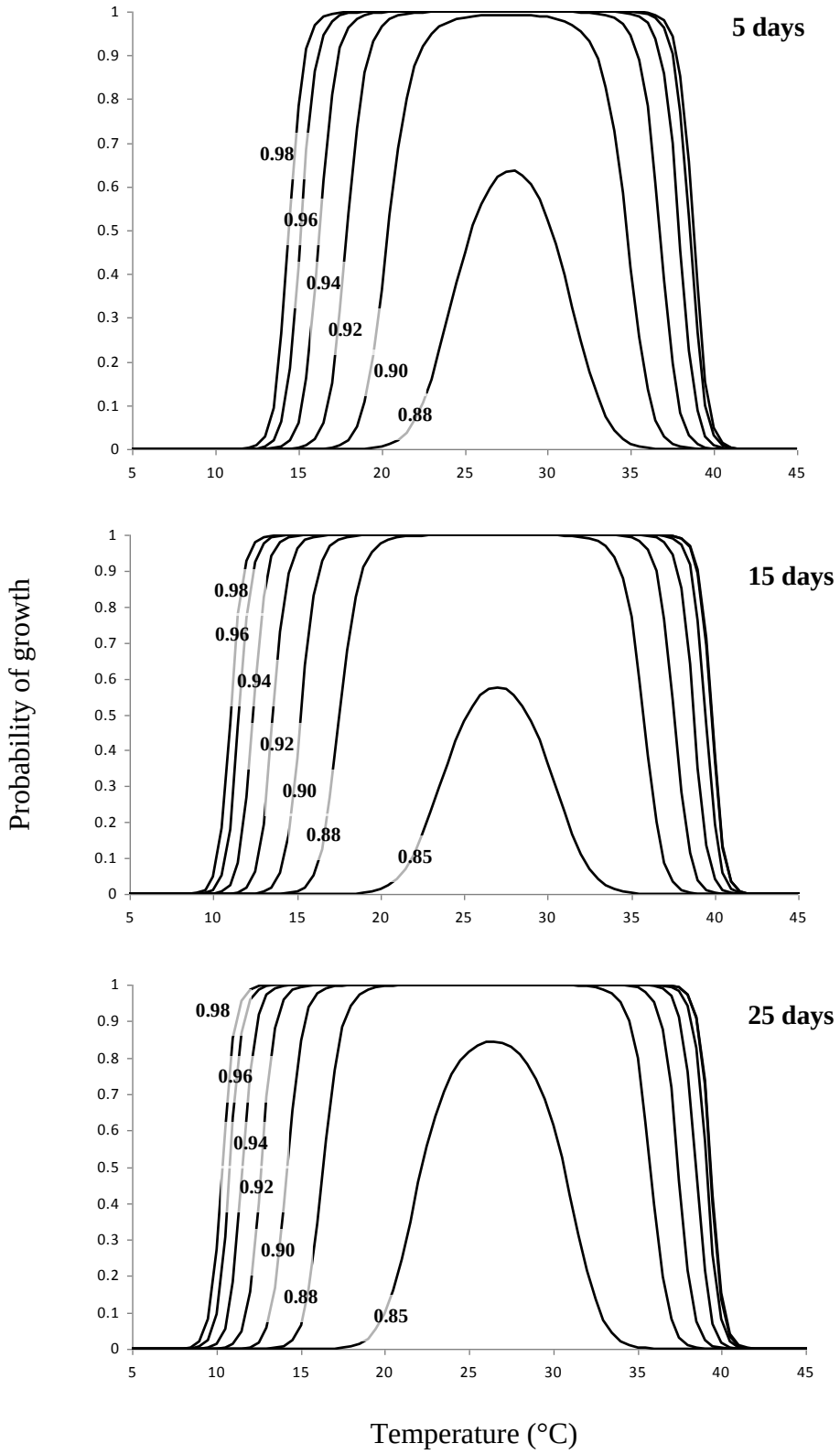

Figure 4.11 The effect of temperature and water activity on the predicted probability of *Aspergillus carbonarius* ATHUM 5659 growth on a synthetic grape juice medium for 5, 15 and 25 days.

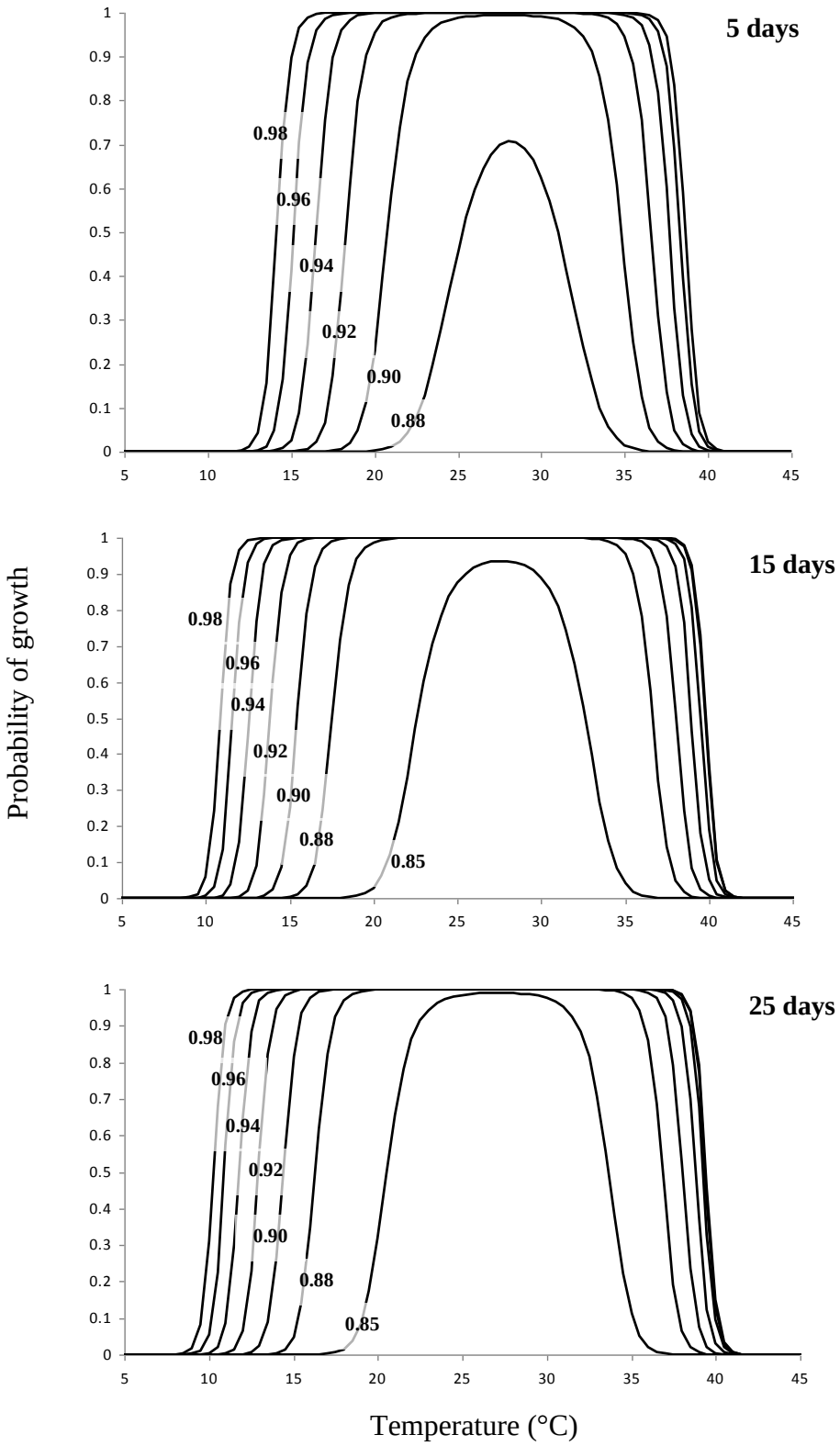

Figure 4.12 The effect of temperature and water activity on the predicted probability of *Aspergillus carbonarius* ATHUM 5660 growth on a synthetic grape juice medium for 5, 15 and 25 days.

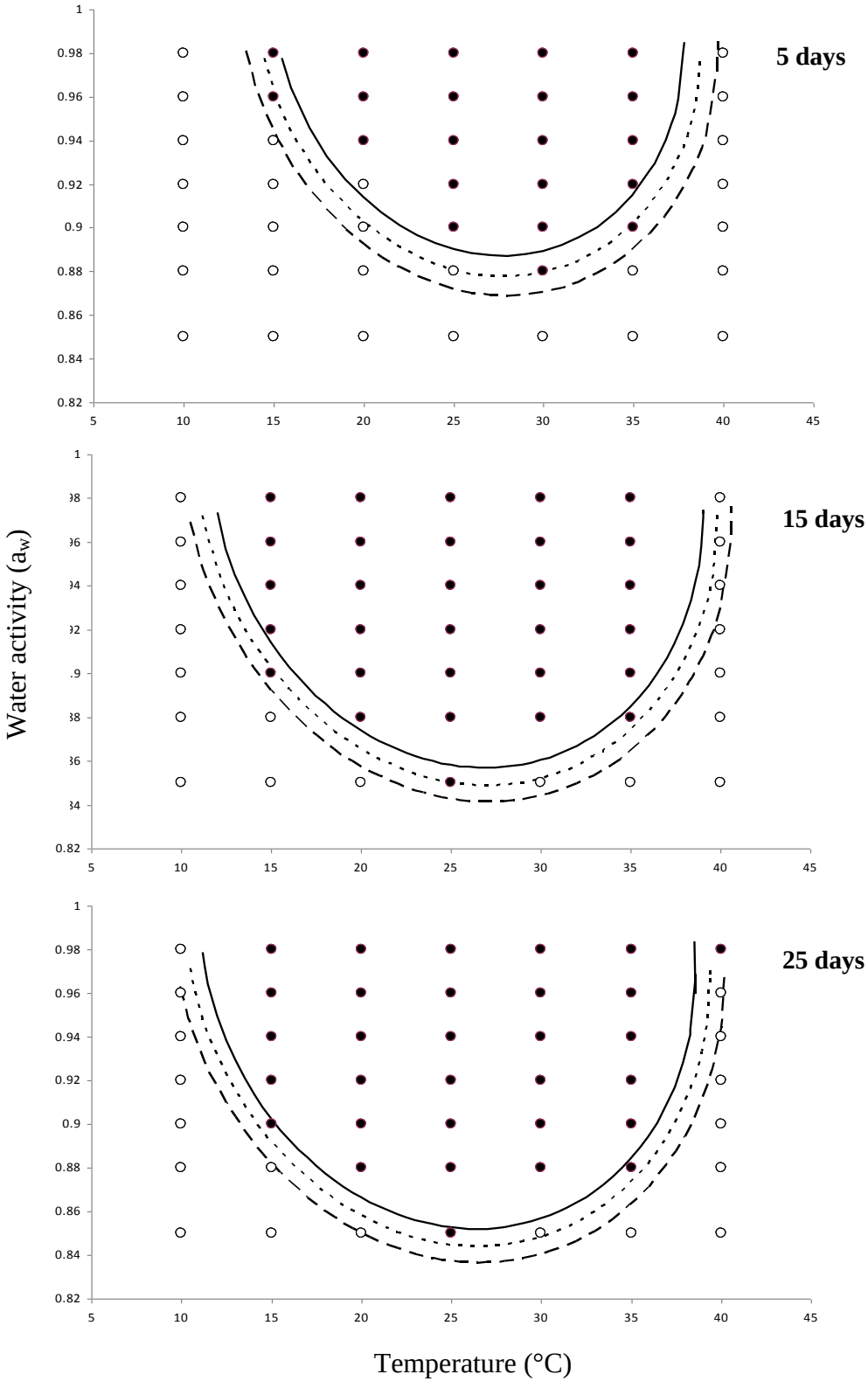

Figure 4.13 Growth/no growth boundaries of *Aspergillus carbonarius* ATHUM 5659 after 5, 15 and 25 days incubation on a synthetic grape juice medium. Solid symbol: growth, open symbol: no growth; solid line $P = 0.9$; dotted line $P = 0.5$; dashed line $P = 0.1$.

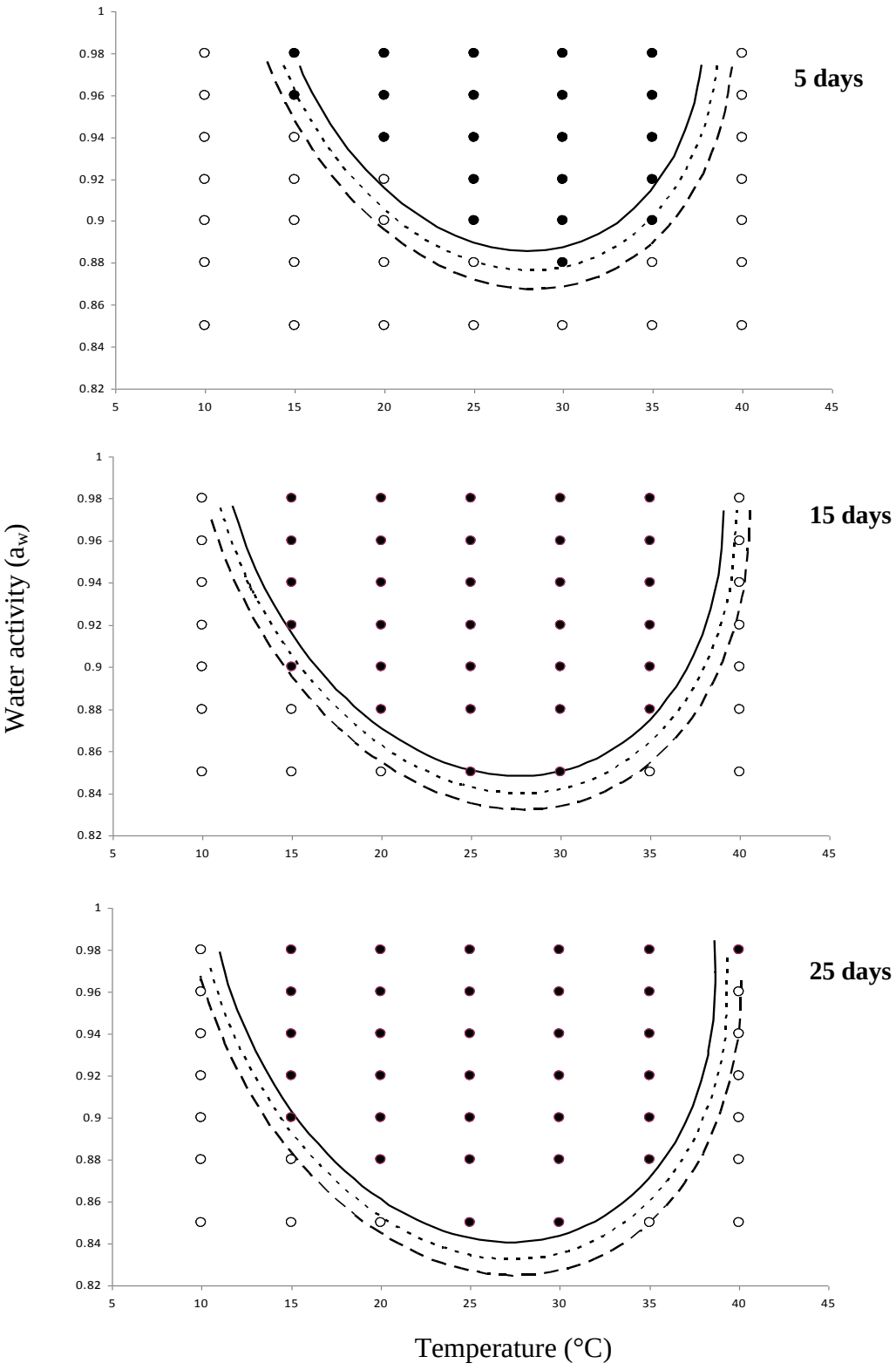

Figure 4.14 Growth/no growth boundaries of *Aspergillus carbonarius* ATHUM 5660 after 5, 15 and 25 days incubation on a synthetic grape juice medium. Solid symbol: growth, open symbol: no growth; solid line $P = 0.9$; dotted line $P = 0.5$; dashed line $P = 0.1$.

A probabilistic approach was also employed for OTA production using a full second order logistic regression model for each fungal isolate. The developed models showed high agreement of prediction with observed probability for OTA production, as was evident from the high concordance rate (98.9-99.1) and the R^2 statistic values (0.832-0.856) (Tables 4.7 and 4.8). Increasing probabilities for OTA were predicted at 15 days, compared to those after 5 days, particularly at the lower a_w levels assayed (0.88-0.94) (Figures 4.15 and 4.16). The lowest probability for OTA production (P = 0.03) was observed at a_w 0.85 and 19-20°C even after 25 days of storage. Probability profiles at 15 and 25 days presented similar patterns with the exception of a_w 0.88 where increased values were estimated for both fungal isolates with respect to time. The predicted OTA interface at probabilities of 0.1, 0.5 and 0.9 is shown in Figures 4.17 and 4.18. No OTA was detected either at 10°C or at 40°C regardless of a_w. Similarly, no OTA was detected at a_w 0.85 at the different temperature levels. At 5 days, predictions with P = 0.5 enclosed all OTA production cases for *A. carbonarius* ATHUM 5960 (Figure 4.18). The same was not observed for the other isolate as three a_w/temperature conditions were left below the interface line (Figure 4.17). With regard to time, the interface shifted to lower a_w values and at 25 days the P = 0.5 interface line enclosed all the OTA production cases for both isolates.

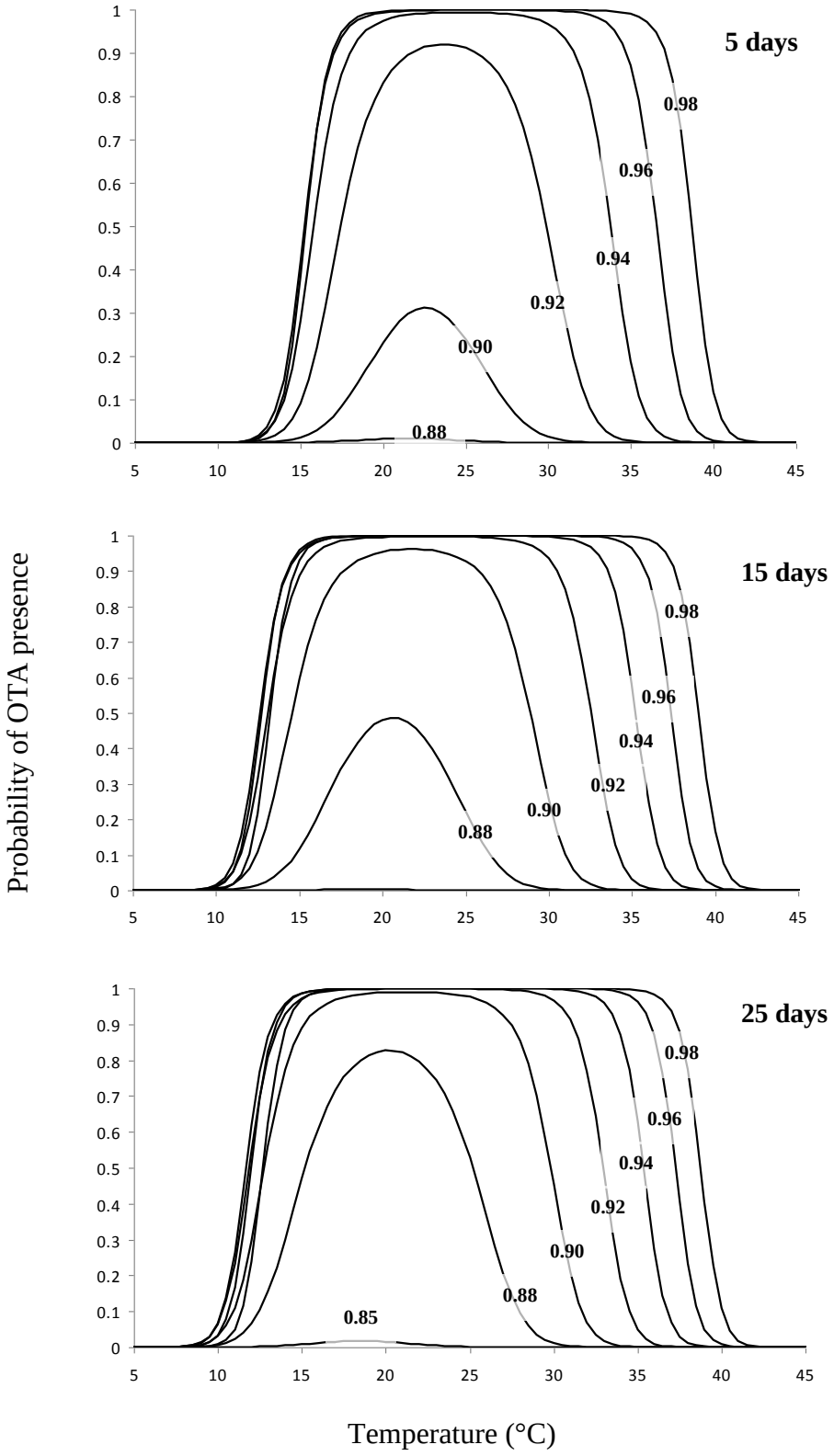

Figure 4.15 The effect of temperature and water activity on the predicted probability of *Aspergillus carbonarius* ATHUM 5659 OTA presence on a synthetic grape juice medium for 5, 15 and 25 days.

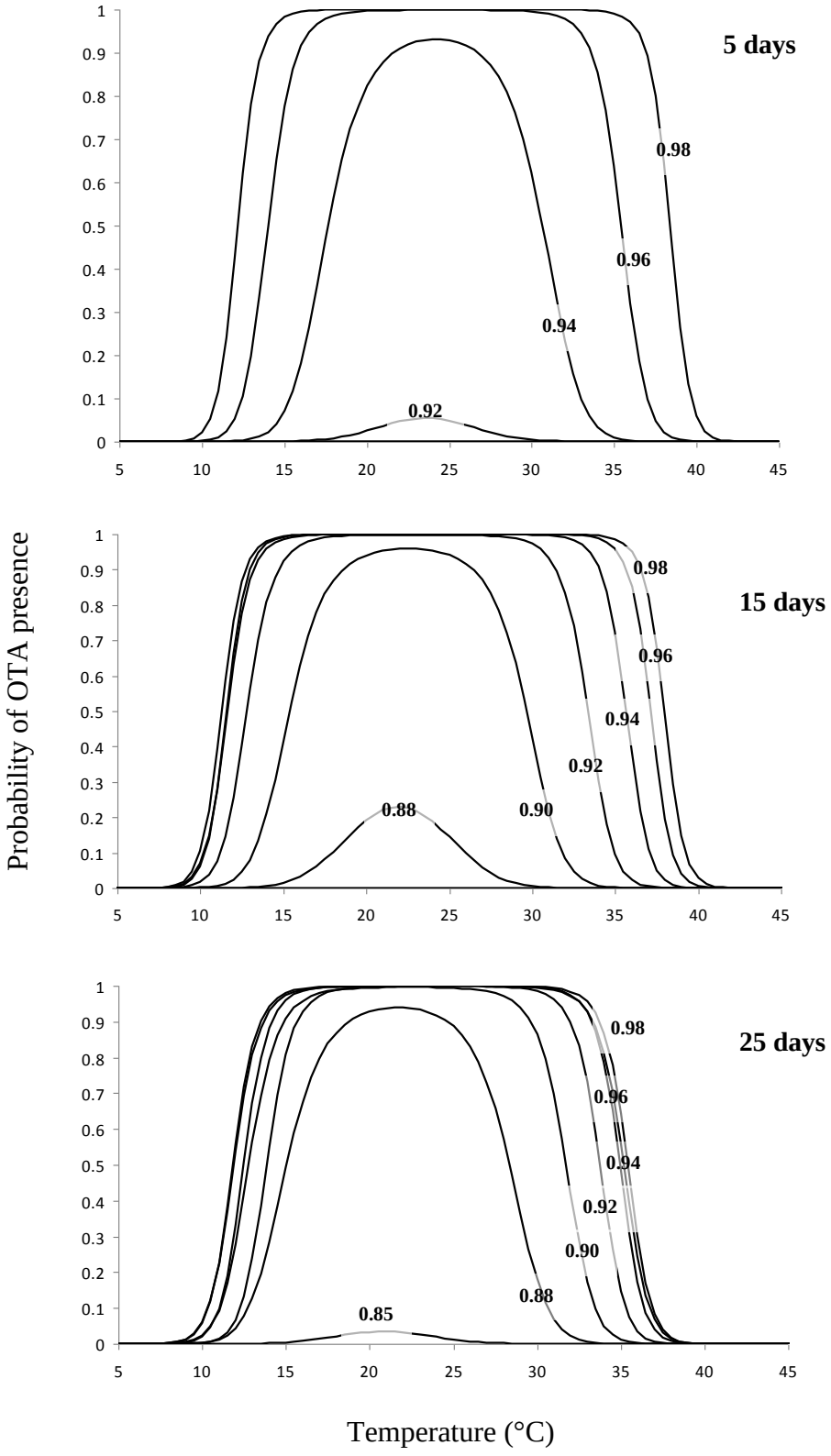

Figure 4.16 The effect of temperature and water activity on the predicted probability of *Aspergillus carbonarius* ATHUM 5660 OTA presence on a synthetic grape juice medium for 5, 15 and 25 days.

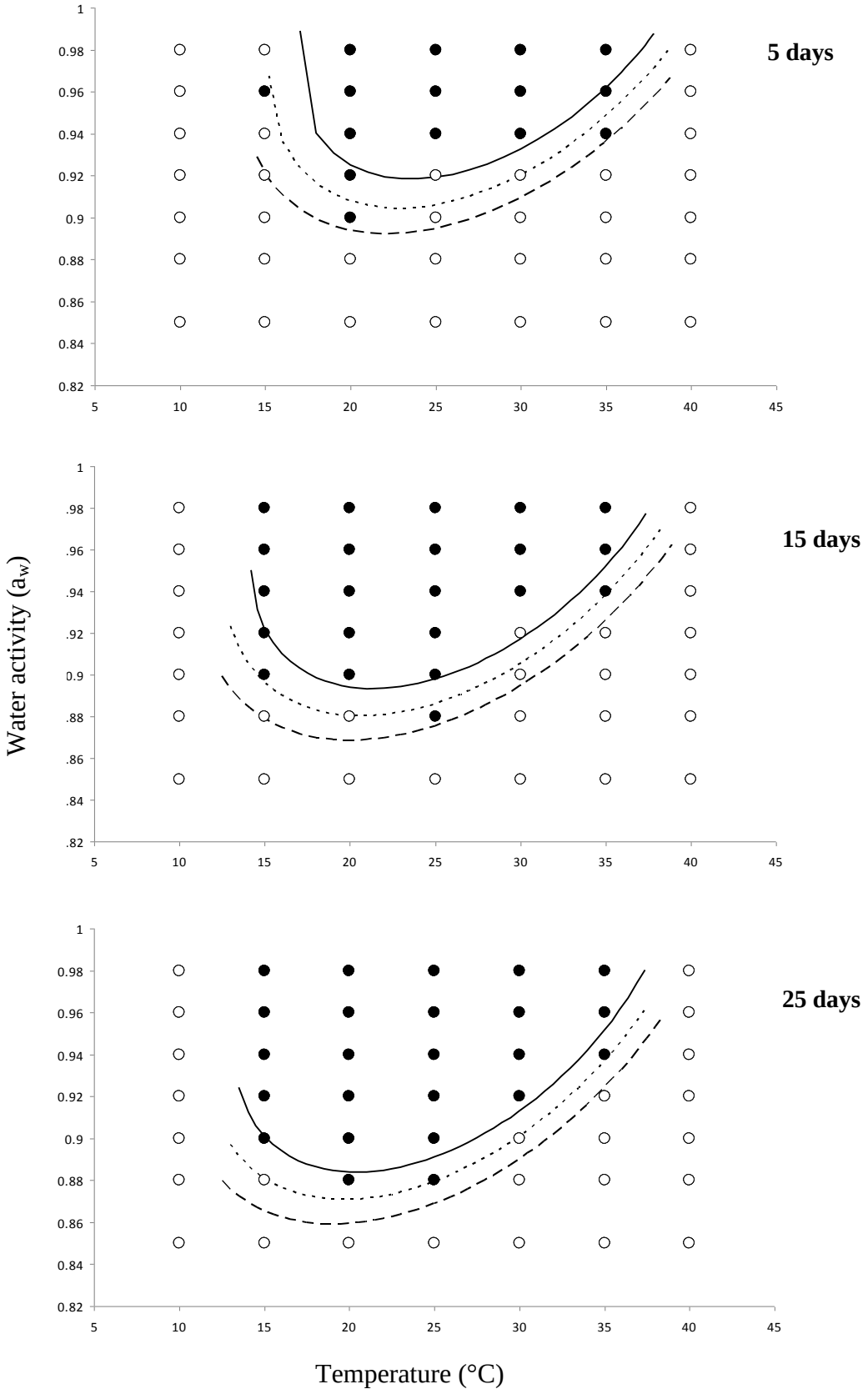

Figure 4.17 OTA production boundaries of *Aspergillus carbonarius* ATHUM 5659 after 5, 15 and 25 days incubation on a synthetic grape juice medium. Solid symbol: OTA presence (>l.d.), open symbol: OTA absence (<l.d.); solid line $P = 0.9$; dotted line $P = 0.5$; dashed line $P = 0.1$.

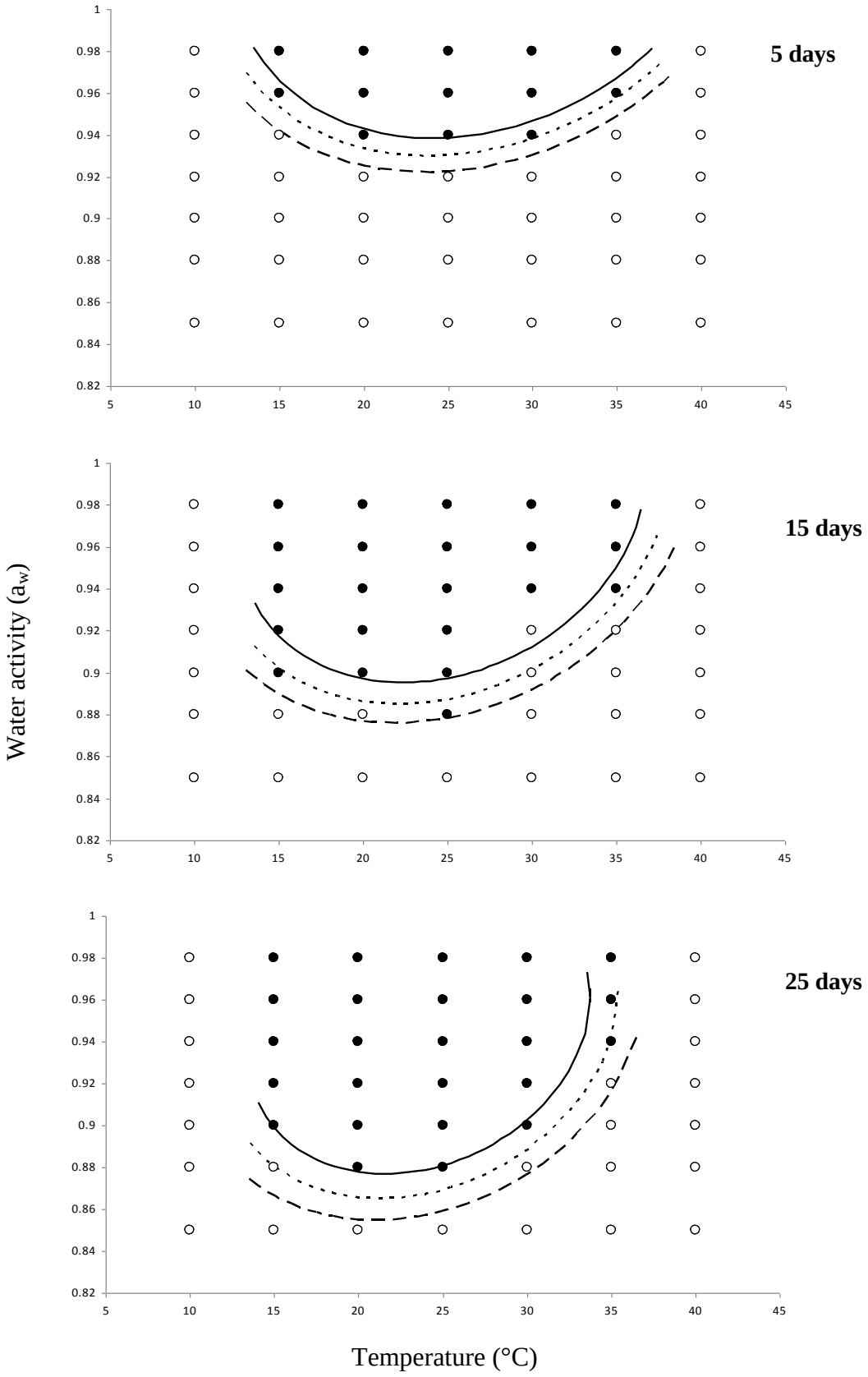

Figure 4.18 OTA production boundaries of *Aspergillus carbonarius* ATHUM 5660 after 5, 15 and 25 days incubation on a synthetic grape juice medium. Solid symbol: OTA presence (>l.d.), open symbol: OTA absence (<l.d.); solid line $P = 0.9$; dotted line $P = 0.5$; dashed line $P = 0.1$.

Table 4.9 Validation of growth/no growth logistic model using independent data of Bellí *et al.* 2005[*]

a_w	Temperature (°C)	Observed growth [b]	Logistic model [c]
0.90	15	0	1
0.93	15	1	1
0.95	15	1	1
0.90	20	1	1
0.93	20	1	1
0.95	20	1	1
0.90	30	1	1
0.93	30	1	1
0.95	30	1	1
0.90	35	1	1
0.93	35	1	1
0.95	35	1	1
0.90	37	1	1
0.93	37	1	1
0.95	37	1	1

[a] Data of eight strains of *A. carbonarius* (W9, W37, W38, W89, W104, W120, W128, W198) for which there was no observed growth at 15°C and 0.90 a_w

[b] After 20 days of incubation, growth = 1; no growth = 0

[c] Growth prediction by logistic model using $P > 0.5$ denotes growth = 1; no growth = 0

Table 4.10 Validation of growth/no growth logistic model using data of Bellí *et al.* (2004)

Strain	a_w	Temperature (°C)	Observed growth [a]	Logistic model [b]
A. carbonarius (36br4)	0.90	10	0	0
	0.93	10	0	0
	0.95	10	1	0
	0.98	10	1	0
	0.90	15	1	1
	0.93	15	1	1
	0.95	15	1	1
	0.98	15	1	1
	0.90	20	1	1
	0.93	20	1	1
	0.95	20	1	1
	0.98	20	1	1
A. carbonarius (A0933)	0.90	25	1	1
	0.93	25	1	1
	0.95	25	1	1
	0.98	25	1	1
A. carbonarius (Mu644)	0.90	30	1	1
	0.93	30	1	1
	0.95	30	1	1
	0.98	30	1	1
	0.90	37	1	1
	0.93	37	1	1
	0.95	37	1	1
	0.98	37	1	1
A. carbonarius (01UAs294)	0.90	10	0	0
	0.93	10	0	0
	0.95	10	0	0
	0.98	10	1	0
	0.90	15	1	1
	0.93	15	1	1
	0.95	15	1	1
	0.98	15	1	1
	0.90	20	1	1
	0.93	20	1	1
	0.95	20	1	1
	0.98	20	1	1
	0.90	25	1	1
	0.93	25	1	1
	0.95	25	1	1
	0.98	25	1	1
	0.90	30	1	1
	0.93	30	1	1
	0.95	30	1	1
	0.98	30	1	1
	0.90	37	1	1
	0.93	37	1	1
	0.95	37	1	1
	0.98	37	1	1

[a] After 20 days of incubation, growth = 1; no growth = 0

[b] Growth prediction by logistic model using $P > 0.5$ denotes growth = 1; no growth = 0

Table 4.11 Validation of logistic model for OTA presence/absence using data of Bellí *et al.* (2005)

Strain	a_w	Temperature (°C)	Observed [a]	Logistic model [b]
A. carbonarius (W9)	0.90	15	0	0
	0.93	15	0	0
	0.95	15	0	1
	0.90	20	0	0
	0.93	20	0	1
	0.95	20	1	1
	0.90	30	0	0
	0.93	30	0	1
A. carbonarius (W38)	0.95	30	1	1
	0.90	35	0	0
	0.93	35	0	0
	0.95	55	0	1
	0.90	37	0	0
	0.93	37	0	0
	0.95	37	0	0
A. carbonarius (W89)	0.90	15	0	0
	0.93	15	0	0
	0.95	15	0	1
	0.90	20	0	0
	0.93	20	0	1
	0.95	20	1	1
	0.90	30	0	0
A. carbonarius (W128)	0.93	30	0	1
	0.95	30	0	1
A. carbonarius (W198)	0.90	35	0	0
	0.93	35	0	0
	0.95	35	0	1
	0.90	37	0	0
	0.93	37	0	0
	0.95	37	0	0
A. carbonarius (W37)	0.90	15	0	0
	0.93	15	0	0
	0.95	15	0	1
	0.90	20	0	0
	0.93	20	1	1
	0.95	20	1	1
	0.90	30	0	0
	0.93	30	0	1
	0.95	30	0	1
	0.90	35	0	0
	0.93	35	0	0
	0.95	35	0	1
	0.90	37	0	0
	0.93	37	0	0
	0.95	37	0	0

[a] After 7 days of incubation, presence (>l.d.) = 1; absence (<l.d.) = 0

[b] Predicted OTA by logistic model using $P > 0.5$ denotes presence = 1; absence = 0

Validation was carried out with literature data from two independent data sets. In the first literature study (Bellí *et al.* 2005) the logistic model predicted growth in all cases (100%), while growth was not actually observed at 0.90 a_w and 15°C for the eight strains of *A. carbonarius* assayed (Table 4.9). A similar situation was observed with the second literature study (Bellí *et al.* 2004) where the model gave three false negative (i.e. no growth predicted when growth was observed) predictions at 10°C and 0.95, 0.98 a_w (Table 4.10) for four strains of the fungus examined. Predicted OTA responses from the first literature study are shown in Table 4.11. There was an overall discordance for 13 a_w/temperature conditions for which the logistic model predicted OTA production above the detection limit, while the observed OTA concentration was below the detection limit. However, all the erroneously predicted cases were on the safe side (fail positive).

4.4 Discussion

4.4.1 Impact of temperature and a_w on lag phase, growth and OTA production of the two *A. carbonarius* isolates

A major part of the South-East Mediterranean region has been linked with a high contamination level of *Aspergillus* section *Nigri* in grapes mainly due to the high day temperatures and the decreasing total rainfall in August. For Greece, there is an increased percentage of *A. carbonarius* isolation during this period of the year (Battilani *et al.* 2006b). The scope of the present study was therefore to investigate the *in vitro* behaviour of Greek isolates under environmental factors that resemble the environmental range of conditions relevant to Greece during the ripening of grapes.

The short lag phases prior to growth found in this study under optimum environmental conditions show how rapidly germination and growth of *A. carbonarius* can occur. These results confirm those with isolates of *A. carbonarius* from Italy, Spain and Israel (Mitchell *et al.* 2004; Magan and Aldred 2005). Indeed, comparison of germination and establishment on grape skin and grape tissue over a range of temperatures and relative humidity suggest that germination is fastest on grape tissue, followed by that *in vitro* and was slowest on undamaged grape skin (Mitchell *et al.*, 2003). The present study and those previously suggest optimum conditions for germination or lag phases prior to growth, is at 25–35°C and a_w range of 0.95–0.98.

The ANOVA of a_w and temperature and their interaction, on growth of the two isolates were highly significant. It was interesting to note that these isolates from Greece were able to grow at 0.85 a_w at 25^{o}C. All previous studies have suggested that growth is inhibited completely at this a_w level (Mitchell *et al.* 2004; Belli *et al.* 2005a; Leong *et al.* 2006b). More isolates would have to be examined to check whether this occurs consistently in Greece as part of adaptation to regional climatic conditions. Although one isolate examined here grew slightly faster than the other, this is not unusual. This was observed for other countries (Mitchell *et al.* 2004; Bellí *et al.* 2005a; Leong *et al.* 2006b). The optimum growth temperature for both Greek *A. carbonarius* isolates was 30-35°C regardless of a_w treatment. Previous studies with isolates from Spain, France, Portugal and Italy reported maximum growth rates of 30-37°C (Mitchell *et al.* 2003; Belli *et al.* 2004a; Mitchell *et al.* 2004; Pardo *et al.* 2005).

The present study found that the optimum a_w for growth of *A. carbonarius* isolates is in the range 0.96–0.98. When the data were processed with quadratic smoothing this provides the contour plot presented. This type of model estimated the whole range of a_w levels above 0.96 as optimum, while for temperature this suggested 25–35°C as optimum instead of 30–35°C that the raw growth data revealed. It may be that quadratic smoothing may slightly overestimate the optimum range for growth. Previous studies on *A. carbonarius* suggest two groups of isolates. Those suggesting an optimum a_w for growth of between 0.95–0.98 (Belli *et al.* 2004a; Mitchell *et al.* 2004) and the other assuming that up to freely available water conditions (a_w >0·96) are the upper limit (Belli *et al.* 2005a; Pardo *et al.* 2005; Magan *et al.* 2005).

In contrast to other studies that found maximum OTA production after 5–10 days incubation (Bellí *et al.* 2004c; Bellí *et al.* 2005a; Mitchell *et al.* 2004), the present study found maximum amounts consistently produced between days 15–25. This supports other studies that suggest maximum OTA production later (Esteban *et al.* 2004; Leong *et al.* 2006a). The subsequent relative decrease of OTA production after 25 days incubation has also been pointed out by other authors (Bejaoui *et al.* 2006; Leong *et al.* 2006a).

The optimum range of the environmental factors for production of OTA was 20°C and 0.93-0.96 a_w. This was followed by treatments at 15°C and 0.93 a_w. In this study OTA production was absent or below the limit of detection at 30 and 35ºC, and at low a_w (0.90). There was also low production (0.02–0.65 μg g^{-1}, 10–15d) at 0.98 a_w regardless temperature. Some authors have reported optimum OTA production when freely available water was present (>0.99 a_w; Bellí *et al.* 2005a; Leong *et al.* 2006b).

The present study and others suggest that intra-isolate differences are very small, especially when are grown under optimum environmental conditions (Bellí *et al.* 2004c; Esteban *et al.* 2004; Mitchell *et al.* 2004; Bellí *et al.* 2005a; Leong *et al.* 2006a). However, at marginal conditions differences may occur.

The models developed and the contour plots obtained can help to establish the interface of OTA production/no production and estimate the cardinal environmental factor values for growth and toxin production by *A. carbonarius*. It is clear that the optima for OTA production maps across to the sub-optimal conditions for growth. The reduction in a_w of berries during ripening (0.98–0.95) and the high temperatures at the end of the véraison period in Greece until vinification (>35ºC) are two factors of major importance influencing OTA contamination. The information here will be valuable in understanding the factors contributing to OTA production in wine grape production in southern Greece and should contribute to development of a more effective prevention strategy.

4.4.2 Predictive models for the effect of temperature and a_w on the growth of *A. carbonarius*

Aspergillus carbonarius is a rather fast growing fungus with a reported temperature range for growth from 10 to 40°C (Esteban *et al.* 2004) and optimum a_w at 0.98-0.99 (Magan and Aldred 2005; Battilani *et al.* 2006). In this study, no growth was observed at marginal conditions of a_w (0.85) and temperatures (10 and 40°C) assayed in the time scale of the experiment. These results are comparable with Mitchell *et al.* (2004), who reported no growth of eight *A. carbonarius* strains from Portugal, Israel, Italy and Greece at 0.88 a_w. The same authors reported no growth of any strain at 10°C, whereas in another study (Bellí *et al.* 2004) minimum growth rates were observed at this temperature.

The effect of temperature and water availability on fungal growth was quantified by two different types of models. One was based on equations with dimensionless (biologically meaningless) parameters (Eqs 2, 3, 5) and the other on models with cardinal values (Eqs 4, 6). A quadratic polynomial model was selected as an empirical modelling approach for fungi (Gibson *et al.* 1994; Valík *et al.* 1999; Valík and Piecková 2001; Pardo *et al.* 2005) and exhibited good performance in terms of R^2 and RMSE (Table 4.3; Figure 4.5) predicting optimal conditions for growth at 35°C and 0.96 a_w. These results are consistent with previous published works (Mitchell *et al.* 2003; Leong *et al.* 2004) reporting optimal temperatures for *A. carbonarius* between 25-35°C, whereas other authors reported lower optimal temperature for growth at 30°C (Battilani *et al.* 2006b).

The $a_{w,opt}$ value predicted by the polynomial model agrees with previous findings (Mitchell *et al.* 2003, 2004) who reported optimum growth rates at intermediate a_w levels. The extended combined model of Parra and Magan (2004) was the second model selected. It combines the parabolic relationship between the natural logarithm of growth rate and a_w developed by Gibson *et al.* (1994), with the square root model showing the relationship between temperature and bacterial growth developed by Ratkowsky *et al.* (1983). The model also presented satisfactory performance as inferred by the relevant statistical indices of R^2 and RMSE (Table 4.3; Figure 4.6). The predicted value lies within the limits reported in the literature for isolates of *Aspergillus* Section *Nigri* obtained from grapes (Bellí *et al.* 2004). The linear Arrhenius-Davey model is an expansion of the original Arrhenius model introduced by Davey (1989) to model the effect of incubation temperature on microbial growth. This model which is widely applied to bacterial growth (Ross and Dalgaard 2004) was selected to determine how well it could describe fungal growth. The model presented satisfactory fitting to the current data (Table 4.3; Figure 4.8) suggesting that this approach can also be expanded to account for fungal growth. The $a_{w,opt}$ was similar to the value predicted by the previous two models. However, the estimated parameters for the $1/T$ and $1/T^2$ terms were not significant confirming previous findings that a_w have a larger effect on fungal growth than temperature (Holmquist *et al.* 1983; Sautour *et al.* 2002; Samapundo *et al.* 2005). Despite the good quality of fit the above models lack biological meaningful parameters and for this reason models with cardinal values were selected. One of the advantages of these models is that they enable easy assessment of initial parameter values and hence, facilitate the convergence procedure. An expanded square root-type model taking into account the entire biokinetic ranges of temperature and water activity was initially tested (Table 4.3; Figure 4.7). The model performed well with the data set and gave realistic estimates for $a_{w,min}$ and $a_{w.max}$ which are in line with published values (Mitchell *et al.* 2003, 2004; Magan and Aldred 2005). However, the model gave an unrealistic value for T_{min} and a higher value for T_{max} than the reported growth range, 10-40°C (Esteban *et al.* 2004). Finally, the complete model of Rosso *et al.* (1995) provided additionally estimates of the optimum values for temperature (T_{opt}) and a_w ($a_{w,opt}$) for growth as well as an estimate for the optimum growth rate (μ_{opt}) (Table 4.3; Figure 4.9). These values are in agreement with previous authors (Magan and Aldred 2005) who reported an optimum radial extension of approximately 10 mm day^{-1} at 30-35°C and 0.99-0.93 a_w. Validation of the developed models with independent data from the literature (Bellí *et al.* 2005) showed reasonably good

correlation among observed and predicted colony growth rates (Table 4.4; Figure 4.10). However, the distribution of the majority of data points above the diagonal line indicated that all models over-estimated fungal growth rates, i.e. the predicted values were higher that the observed. The difference was more intense at higher (35°C) than at lower temperatures (15°C). This was possibly due to differences in the observed growth rates among the different strains of *A. carbonarius* reported by the authors, as there is variation in the growth rates of various strains between countries and also within the same country (Mitchell *et al.* 2004).

In conclusion, the results of this study showed that under the current experimental conditions, the combined effect of temperature and water activity on the growth responses of *A. carbonarius* could be satisfactorily predicted, and the examined models could serve as tools for this purpose. Models with biological interpretable parameters presented good overall performance and may contribute to the literature with cardinal temperatures and a_w values to determine the conditions for germination and growth of *A. carbonarius*. However, in order to build better models, a database is necessary with information from a wide range of strains from different climatic conditions and countries.

4.4.3 Growth and OTA production boundaries of two *A. carbonarius* isolates

The present study describes the applicability of a probabilistic modelling approach for the influence of a_w and temperature on growth and OTA production of two ochratoxigenic isolates of *A. carbonarius* from Greek wine grapes. Models to predict the likelihood of growth of microorganisms as a function of intrinsic and extrinsic factors were first explored in the 1970s (Genigeorgis 1981; Gibson *et al.* 1987), known as "probability" models. Later on it became necessary to manage the risk to consumers from foodborne pathogens and ensure presence/absence of a certain microorganism in a food commodity, thus leading to the development of "growth/no growth boundary" or "interface" modelling (Ratkowsky and Ross 1995). In recent years the need for modelling microbial growth limits has been increasingly recognised (McMeekin *et al.* 2002). Such models can be useful in the development of processes that allow production of safer food products and could also be important for deciding food safety regulations (Schaffnet and Labuza 1997). So far predictive mycology has not received the same level of attention compared to food-borne pathogenic bacteria and only recently the concepts for modelling fungal development have been reviewed (Dantigny *et al.* 2005). Probability models,

although not extensively used in predictive mycology, can provide useful information and define the response of the fungus in boundary conditions of growth and toxin production.

The present logistic model was fitted successfully to the experimental data as the agreement between observed and predicted probabilities was > 99% concordant for fungal growth (Tables 4.7 & 4.8) and > 98% for OTA production (Tables 4.9 & 4.10) for both isolates. It proved difficult to find appropriate literature data to compare our logistic model as no similar approach has been employed so far for *A. carbonarius* in grapes. However, these values are comparable with those reported by Marín *et al.* (2008) for *A. carbonarius* growth and OTA production in pistachio nuts, where the relevant concordant rates were 95.6% (for fungal growth) and 94.6% (for OTA production). The maximum rescaled R^2 of 0.845/0.869 (for growth) and 0.832/0.856 (for OTA) obtained in the present study was higher than that reported in the above mentioned work, i.e. 0.786 and 0.715 for growth and OTA production, respectively. The higher values of R^2 reported in this work could be explained by the fact that our experiment was carried out on a synthetic and well-defined laboratory medium, whereas in the other work the fungus was inoculated directly on pistachio nuts.

With respect to time, the growth boundary shifted to higher temperature levels (25-30°C) (Figures 4.13 & 4.14) whereas the OTA production boundary shifted to lower temperatures (15-25°C) (Figures. 4.17 & 4.18) indicating that OTA production does not occur at its best under the same conditions for growth. The extension of the growth boundary with time was similar for the two isolates, with the lowest a_w for growth at 0.85-0.88 depending on incubation temperature. Growth at these a_w values, although not consistent with literature data (Mitchell *et al.* 2004; Bellí *et al.* 2005; Leong *et al.* 2006), could be attributed to adaptation to regional climatic conditions, thus making these isolates more tolerant to xerophilic conditions. As observed in the probability plots (Figures. 4.11 & 4.12), probabilities of growth over 0.8 were predicted in synthetic grape medium with 0.90-0.98 a_w incubated at 15-35°C in 5 days time. As the a_w in grapes during ripening is 0.95-0.98 and the prevailing temperatures at harvest many vary between 30 and 35°C, or lower depending on regional conditions, there is increased probability of fungal growth and subsequent OTA contamination. The same probability level ($P > 0.8$) for OTA production for the same temperature range and time was attained in growth media with 0.94-0.98 a_w for *A. carbonarius* ATHUM 5659 (Figure 4.17) and 0.96-0.98 a_w for *A. carbonarius* ATHUM 5660 (Figure 4.18), indicating that the range of a_w for OTA production is narrower than that for growth. However, fungal growth and

OTA production is much more complicated under realistic conditions as reported by Marín *et al.* (2006). Environmental fluxes, especially day temperatures may not be appropriate for OTA production but may support hyphal extension increasing the potential for OTA production under lower temperatures at night.

Validation with independent literature data showed that the developed logistic model could adequately predict the growth/no growth cases of other *A. carbonarius* strains at a probability level of 0.5. Some disagreement was only observed with 1 false positive (Table 4.11) and 3 false negative (Table 4.12) predictions out of 68 total growth cases. The false negative cases were not predicted successfully as they were located at the boundaries of the domain of the model. Finally, the model predicted OTA responses reasonably well as the agreement with literature data for OTA absence was 27 out of 40 (67.5%) cases and 5 out of 5 (100%) cases for OTA presence (Table 4.13). The misclassified cases of the model could be attributed to the great variability of different *A. carbonarius* stains in OTA production between countries and also within the same country even under the same environmental conditions (Mitchell *et al.* 2004).

In conclusion, the results of this study indicate that logistic regression models can be successfully employed to predict the boundaries for growth and OTA production of *A. carbonarius* on a synthetic grape juice medium and also for other mycotoxin producing species. However, due to the variability of *A. carbonarius* strains in growth potential and OTA production, further research is needed to develop and validate more extensively such models with additional regional experimental data from grapes and grape products.

Chapter 5: A Survey of Ochratoxin A Occurrence in Greek Wines

5.1 Introduction

Ochratoxin A (OTA) is a toxic metabolite produced by specific fungi that can infect and proliferate on various agricultural commodities in the field and/or during storage (Pitt & Hocking, 1997; Van Egmont, 2000). The occurrence of this toxin in food is influenced by environmental factors such as temperature, humidity, and the extent of rainfall during the pre-harvesting period, the harvesting techniques and post-harvesting handling practices (JEFCA, 2001; FAO, 2007; EFSA, 2006; Magan & Aldred, 2007). OTA has nephrotoxic, immunosuppressive, teratogenic and carcinogenic effects on animals, and is classified as a possible human carcinogen of the 2B Group by the IARC (Heussner *et al.*, 2006; Creppy, 2002; IARC, 1993). Moreover, there are several reports from many countries in the world, describing the high frequencies of OTA in a large number of food groups such as cereals, fruits, meat and their products (Pohland *et al.*, 1992; Murphy *et al.*, 2006). This has resulted in a focus on means of minimizing the risk of exposure of consumers to this mycotoxin in a range of foodstuffs (FAO, 2007; EFSA, 2006; EU, 2002). Grapes, raisins, grape juice and wine can be considered as high risk products which can be contaminated by *Aspergillus carbonarius* (Visconti *et al.*, 2008). It has been suggested that OTA produced by this fungus and the presence in wine accounts for 13-21% of the total human exposure (JEFCA, 2001; EU, 2002). Based on the available scientific data for OTA, the European Union (EU) established maximum permitted limits of 10.0 μg kg^{-1} for dried vine fruits (currants, raisins and sultanas) and 2.0 μg kg^{-1} for wine, grape juice and musts (EU, 2006).

OTA was detected in wine for the first time in 1996 by Zimmerli and Dick (1996), who found that 70% of 118 European table wines were contaminated with OTA. Thereafter, several surveys were conducted worldwide, and these have been reviewed previously and illustrate the severity of the problem (Visconti *et al.*, 2008; Otteneder & Majerus, 2000; Mateo *et al.*, 2007; Visconti *et al.*, 1999). The proportion of positive OTA contaminated wines was very high, exceeding 50% in some countries, especially around the Mediterranean basin. Many authors report a greater contamination of dessert (sweet) wines in contrast with the dry types, probably due to process that is applied before

vinification. A gradient in OTA concentration is usually recognized, correlated with the color, and therefore to the production process of wine, with OTA levels diminishing from red through rosé to white wine. Another, common finding in the majority of the surveys, is the higher level of incidence and concentration of OTA in wines originated from southern, in contrast to those of northern regions of production in the same surveys (Visconti *et al.*, 1999; Otteneder & Majerus, 2000; Soleas *et al.*, 2001; Pietri *et al.*, 2001; EU, 2002; Bellí *et al.*, 2004; Ng *et al.*, 2004; Rosa *et al.*, 2004; Anli *et al.*, 2005; Burdaspal & Legarda, 2007; Mateo *et al.*, 2007; Visconti *et al.*, 2008; Valero *et al.*, 2008). Nevertheless, there are some studies that did not reveal the same conclusions (Shephard *et al.*, 2003; Valero *et al.*, 2003).

With respect to Greek wines, Markaki *et al.* (2001) analyzed 31 samples of red wine, all produced in countries of the Mediterranean basin, with 8 of Greek origin. All the Greek samples contained OTA, with one having the highest contamination reaching 2.35 μg L^{-1}. Soufleros *et al.* (2003) analyzed 35 Greek wines and found a rather high incidence (62.8%) with a maximum level of 3.2 μg L^{-1} OTA but with only 3 samples exceeding 1.0 μg L^{-1}. In another survey for the presence of OTA in 268 Greek wines, Stefanaki *et al.* (2003) found that 40% of the total number of dry wines showed no detectable OTA concentrations, whereas 11.5% of all surveyed Greek wines contained OTA at $\geq$1.0 μg L^{-1}. However, none of these studies examined the possible contamination in different seasons and different vinification systems. The objective of this study was to examine a wide range of wines from the key production areas in Greece produced over the period 1999-2006 in relation to OTA presence based on color, type, origin, variety, production year and whether using traditional or organic cropping systems.

5.2 Materials and methods

5.2.1 Study Area

Greece is located in southern Europe and lies between Italy and Turkey. The Aegean Sea, Ionian Sea and Mediterranean Sea border the country. The terrain of Greece is mostly mountainous with mountain ranges extending into the sea as peninsulas or chains of islands. The climate of Greece is mostly of a temperate kind and has hot, dry summers, followed by a warm and wet autumn, with ample sunlight throughout the year. Seven winemaking regions were chosen for this study, representing more than 80 % of the total

wine producing vineyards of the country. Figure 5.1 show the areas where wine samples originated from. Wines produced from vineyards of northern (Macedonia), western (Epirus), central (Sterea Ellada and Thessaly), southern (Peloponnese and Crete Island) and eastern (Aegean Islands) Greece were evaluated.

Figure 5.1 Incidence of Ochratoxin A in the Greek domain. Numbers indicate positives/total samples.

5.2.2 Wine Samples

Commercial available wines were purchased from supermarkets of Athens and stored at 4 ºC until analysis. A total number of 150 samples was analyzed of which 123 (64 red, 49 white and 10 rosé) were dry and 27 (14 red and 13 white) dessert type (sweet) wines. All wines were produced during the years 1999 to 2006 and were of various viticultural and oenological practices.

5.2.3 Extraction, Detection and OTA Quantification

Analysis was performed always within the year of production of the wine. The extraction and detection procedure of OTA in wine was strictly derived from the European norm (EN 14133, V 03-128) (EU, 2001). A volume of 10 mL of must or wine was diluted in 10 mL of water solution containing PEG (1%) (Acros Organics) and $NaHCO_3$ (5%).The pH value of the result solution corresponds to the range 7.2-7.8. The solution was filtered through Whatman GF/A glass microfiber filter with porosity of 1.6

μm. The filtrate was then passed through an Ochrapep immune-affinity column (IAC) (r-Biopharm, St Didier Au Mont D'Or, France) at a flow rate of 1 drop s^{-1}. The column was successively washed with 5 mL of water solution containing NaCl (2.5%) and $NaHCO_3$ (0.5%) followed by 5 mL of HPLC-grade water at a flow rate of 1-2 drops/s and dried with air. OTA was eluted by 2 mL of HPLC-grade methanol through the IAC at a flow rate of 1 drop/s. The eluate, collected in an HPLC vial (Wheaton; 2 mL), was evaporated under nitrogen stream at 50 °C and reconstituted with 250 μL of mobile phase prior to HPLC analysis.

5.2.4 The Chromatographic System

OTA was detected and quantified by reversed-phase HPLC. The analysis was performed using a chromatographic system (series 1100, Hewlett Packard, Palo Alto, Calif.) equipped with an autosampler (Agilent 1100, G1313A, ALS) and a fluorescence detector (Agilent 1100, G 1321A, FLD) set at 330 nm (excitation) and 460 nm (emission). The system was controlled by Chemstation software. Chromatographic separations were performed with a C18 Spherisorb ODS2 column (5 μm by 250 mm by 4.6 mm; Waters, Milford, Mass.). The flow rate of the mobile phase (acetonitrile–water–acetic acid at 51:47:2) was 1 ml min^{-1}. The injection volume corresponds to 100 μL and the retention time was about 8 min. Under these conditions, the limit of detection of OTA was 0.01 μg L^{-1}. Preparation of samples and further details are referred in paragraphs 3.2.2.3 and 4.2.4.

5.2.5 Statistics

Statistical analysis was performed according to standard analysis of variance (ANOVA) using the Statistica software package (Statsoft, Tulsa, Okla.).

5.3 Results

Of the 150 samples of Greek wines 104 (69%) were found to be positive for OTA presence. Although the percentage of positive samples indicates a rather high incidence of contamination, the majority of the samples tested (91%) had low levels of OTA (n.d. – 1.0 μg L^{-1}) with 14 samples (9%) being between 1.0 and 2.0 μg L^{-1} and only one reaching the E.U. permissible maximum level of 2.0 μg L^{-1}. The incidence and concentration of OTA in red wines were similar (70% positive; mean = 0.21 μg L^{-1}) with that of white (63%

positive; mean = 0.21 μg L^{-1}), and moreover the statistical analysis did not reveal any statistically significant difference between these groups. In contrast, the results support a considerably higher incidence and contamination of dessert type wines (81% positive; mean = 0.57 μg L^{-1}) against dry (67% positive; mean = 0.17 μg L^{-1}), for both red and white wines (Table 5.1). With regards to the origin of the samples there appeared to be a clearly defined gradient, with increasing concentrations of OTA from north to south and western to eastern regions of viticulture (Table 5.2). Finally, conventionally *versus* organically produced wines and year of production did not show any significant differences in terms of mean, median and range of OTA contamination (Table 5.3 and Figure 5.2). Moreover, in Table 5.4 is given an overview of what has been done up today regarding investigation of OTA presence exclusively for Greek wines. This table summarizes the results of 466 Greek wine samples analyzed and published in OTA occurrence surveys.

Table 5.1 Ochratoxin A in μg L^{-1} for dry and dessert Greek wines

Type	No. of positives/total samples[a] (%)	OTA concentration (μg L^{-1})			Incidence of OTA contamination (%)				
		Mean[b]	Median[b]	Range[c]	< 0.05 μg L^{-1}	0.05-0.5 μg L^{-1}	0.5-1.0 μg L^{-1}	1.0-1.5 μg L^{-1}	1.5-2.0 μg L^{-1}
Total Dry wines	**82/123 (66.7)**	**0.17**	**0.06**	**n.d.-1.51**	**43.9**	**44.7**	**4.9**	**5.7**	**0.8**
Red dry wines	45/64 (70.3)	0.21	0.07	n.d.-1.31	40.6	45.3	7.8	6.3	0.0
White dry wines	31/49 (63.3)	0.21	0.08	n.d.-0.51	46.9	42.9	2.0	6.1	2.0
Rosé dry wines	6/10 (60.0)	0.09	0.03	n.d.-0.38	50.0	50.0	0.0	0.0	0.0
Total Dessert wines	**22/27 (81.5)**	**0.57**	**0.40**	**n.d.-2.00**	**18.5**	**37.0**	**22.2**	**18.5**	**3.7**
Red Dessert wines	13/14 (92.9)	0.72	0.57	n.d.-2.00	7.1	35.7	28.6	21.4	7.1
White Dessert wines	9/13 (69.2)	0.41	0.35	n.d.-1.16	30.8	38.5	15.4	15.4	0.0
Total wines	**104/150 (69.4)**	**0.26**	**0.10**	**n.d.-2.00**	**39.3**	**43.3**	**8.0**	**8.0**	**1.3**

[a] In brackets: % precentage.
[b] Concentration of samples with no detectable OTA is considered as LOD/2 (0.005 μg L^{-1}) for the mean and median estimation
[c] n.d. = not detected (<0.01μg L^{-1}).

Table 5.2 Mean OTA concentrations in different types of wine from various regions of Greece

Origin[a]	Dry wines (82/123)[a,b]			Desert wines (22/27)[a,b]		Total[b]
	Red	Rosé	White	Red	White	
North, West & Central Greece (22/43)	**0.10**	**0.06**	**0.06**	**-**	**n.d.[c]**	**0.08**
Macedonia (13/25)	0.13	0.07	0.05	-	-	0.06
Epirus (3/8)	0.13	-	0.01	-	n.d.	0.13
Sterea Ellada (4/6)	0.07	0.04	0.18	-	-	0.10
Thessalia (2/4)	0.04	-	-	-	-	0.04
South Greece (54/77)	**0.13**	**0.17**	**0.21**	**0.72**	**n.d.**	**0.23**
Peloponnese (42/62)	0.12	0.17	0.24	0.72	n.d.	0.30
Crete (12/15)	0.15	-	0.06	-	-	0.12
Aegean Islands (28/30)	**1.05**	**-**	**0.30**	**-**	**0.48**	**0.53**
Overall Greek Regions (104/150)	**0.21**	**0.09**	**0.21**	**0.72**	**0.41**	**0.26**

[a] In brackets: positive/total.
[b] Concentration of samples with no detectable OTA is considered as LOD/2 (0.005 μg L^{-1}) for the mean concentration
[c] n.d. = not detected (<0.01μg L^{-1}).

Table 5.3 Ochratoxin A in μg L^{-1} for conventionally and organically produced Greek wines

Cultivation type	No. of positives/total samples[a]	OTA concentration (μg L^{-1})		
		Mean[b]	Median[b]	Range[c]
Conventional	83/117 (70.9)	0.28	0.09	n.d.-2.00
Organic	21/33 (63.6)	0.20	0.02	n.d.-1.48

[a] In brackets: positive/total.
[b] Concentration of samples with no detectable OTA is considered as LOD/2 (0.005 μg L^{-1}) for the mean concentration.
[c] n.d. = not detected (<0.01µg L^{-1}).

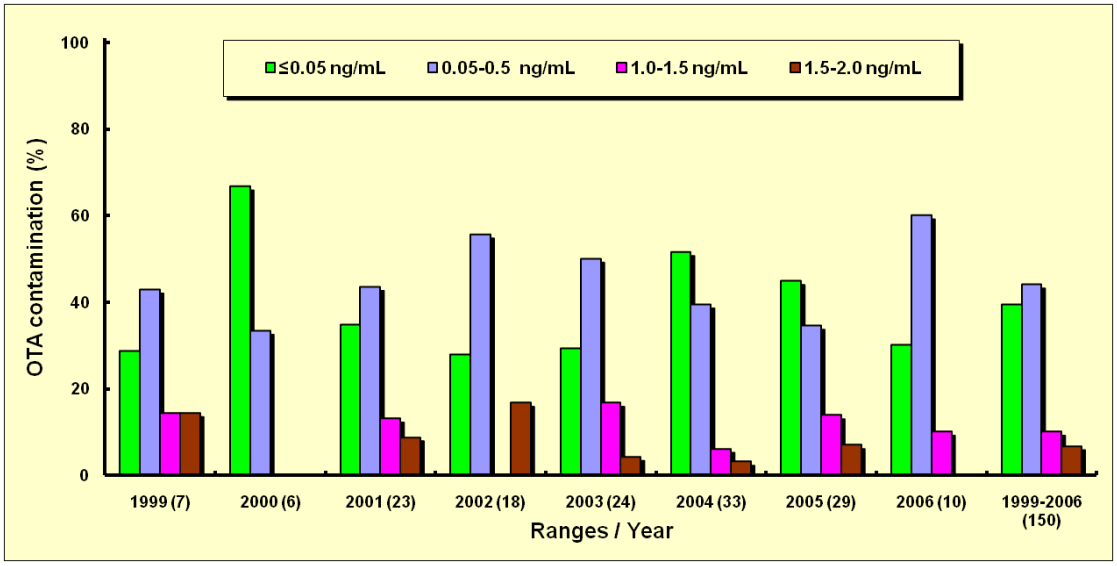

Figure 5.2 Incidence of ochratoxin A contamination of Greek wines produced during the years 1999 to 2006 (In brackets the total number of tested samples).

Table 5.4 Occurrence and levels of OTA in Greek wines

	Wine type											
	Red			Rosé			White			Sweet		
Reference	**Positive / Total**	**Mean**	**Range**	**Positive / Total**	**Mean**	**Range**	**Positive / Total**	**Mean**	**Range**	**Positive / Total**	**Mean**	**Range**
Soleas *et al.*, 2001	4/23[a]	-	<0.05-0.10	-	-	-	2/16[a]	-	<0.05-0.10	-	-	-
Markaki *et al.*, 2001	8/8	-	0.002-2.35	-	-	-	-	-	-	-	-	-
EU, 2002 (*SCOOP*)	21/38	0.16	<0.05-2.61	3/5	0.07	<0.05-0.13	23/45	0.13	<0.05-1.17	6/7	0.54	<0.05-1.68
Stefanaki *et al.*, 2003	71/104	0.34	<0.05-2.69	13/20	0.17	<0.05-1.16	63/118	0.25	<0.05-1.72	15/18	0.58	<0.05-2.82
Soufleros *et al.*, 2003	9/14	0.68	<0.02-2.51	0/1	n.d.	n.d.	7/13	0.27	<0.02-0.87	6/7	0.94	<0.02-3.20
Ng *et al.*, 2004	8/10[a]	-	<0.004-<1.00	-	-	-	9/13[a]	-	<0.004-3.72	-	-	-
Burdaspal *et al.*, 2007	-	-	-	-	-	-	-	-	-	6/6	0.83	0.12-2.95

[a] Authors did not divide sweet from dry type wines.

5.4 Discussion

Our results confirm the hypothesis of a high incidence in the Mediterranean basin and moreover there seems to be a clear gradient within the borders of Greece between the different regions studied. Thus, the northern, western and central Greek regions together, resulted in incidence of 22 contaminated samples out of 43 tested, while the southern Greece and the Aegean Islands had 54 positives out of 77 samples and 28 out of 30, respectively. The main climatic differences between the three former and the two latter regions are the higher temperature and low humidity of the northern Greece during summer periods, and at the same time the fact that in the mainland, either northern or western regions, cultivation takes place usually at higher altitudes. This gradient was also observed in studies in Italy (Pietri *et al.*, 2001; Perrone *et al.*, 2007), Spain (Burdaspal & Legarda, 2007; Valero *et al.*, 2008), and limited surveys in Greece (Stefaniaki *et al.*, 2003; Soufleros *et al.*, 2003; Melletis *et al.*, 2007), and Turkey. However, the latter study in Turkey has shown that those from the Thrace and Aegean coast in the west of the country were more contaminated than those originating from the east mainland of Anatolia (Anli *et al.*, 2005). The factor which may be responsible for this difference could be the climatic conditions after véraison of the grapes and during ripening, where the wet and hotter profile at the Mediterranean coast favors the higher incidence of growth and OTA production on grapes by *A. carbonarius* (Visconti *et al.*, 2008; Blesa *et al.*, 2006; Battilani *et al.*, 2006).

With regards to the color of the wines tested, the gradient in OTA concentration observed between red, rosé and white wines was not evident for the dry type wines. Although the red ones had incidence and range of contamination above those of the white wines, the mean and median concentration did not differ and, moreover, a statistical comparison of the concentrations showed no statistically significant difference. This is in contrast to some other studies for Greek wines (Markaki *et al.*, 2001; Soufleros *et al.*, 2003), but supports some other surveys (Ng *et al.*, 2004; Stefanaki *et al.*, 2003). In previous surveys for other countries, the trend of diminishing OTA contamination from red wines to white has usually been relatively consistent (Visconti *et al.*, 1999; Bellí *et al.*, 2004; Blesa *et al.*, 2004; Rosa *et al.*,

2004; Anli *et al.*, 2005). Although, in other cases was not clear or absent (Zimmerli & Dick, 1996; Otteneder & Majerus, 2000; Ng *et al.*, 2004; Shephard *et al.*, 2003).

Nevertheless, when distinguishing the dessert type wines, the aforementioned increasing gradient of red colored sweet wines versus white is clearly present. The higher contamination of red types against white wines is probably due the longer mash standing followed in red vinification. While white grapes are immediately pressed after being harvested and juice is used for fermentation, red wine grapes are mashed and the skin and juice are put aside for several days. With respect to rosé type wines, the mash standing holds from some hours to one day, according to the desirable tone of the final color. During this processing stage of maceration for the red wines, aerobic conditions and elevated temperatures can favor the growth of the existing mould, and subsequently further OTA production can take place (Otteneder & Majerus, 2000; Visconti *et al.*, 2008; Blesa *et al.*, 2006).

It is well established in the relative literature that dessert (sweet) wines represent the most OTA-contaminated type of wines. In terms of frequency, dessert wines appear to have the highest incidence of OTA presence. In all surveys, from the very first by Zimmerli and Dick (1996) to the most recent by Valero *et al.* (2008), occurrence of OTA in dessert wines usually exceed 90% of the total samples with, sometimes, remarkably high concentration in comparison with dry type wines. Likewise, the present study revealed 22 contaminated samples out of 27 tested (incidence 81%), with 22% of them having > 1.0 μg L^{-1} OTA, while for the dry type wines in this range was only 7%. Some previous studies have reported incidences of between 80 and 100% for dessert wines being contaminated by OTA with similar mean values (Zimmerli & Dick, 1996; Burdaspal & Legarda, 2007; Soufleros *et al.*, 2003; Stefanaki *et al.*, 2003) and sometimes higher (Pietri *et al.*, 2001; Valero *et al.* 2008) than those found in the present study (mean = 0.57 μg L^{-1}, median = 0.40 μg L^{-1}). However, maximum levels in Greek dessert wines in the literature never exceeded the 3.20 μg L^{-1} reported by Soufleros *et al.* (2003). These levels were also never as high as those found by Belli *et al.* (2004) and Valero *et al*. (2008) in Spanish muscatels (15.25 and 15.62 μg L^{-1} respectively). Dessert type wines in Greece are almost always produced by the traditional sun-drying method (insolation). This winemaking procedure is performed by exposing grapes for several days to an open environment in order to lose humidity by sun-drying. When these days are followed by wet and cool nights, damaged grapes become more susceptible to *A. carbonarius*

and OTA contamination (Valero *et al.*, 2008; Soufleros *et al.*, 2003; Gómez *et al.*, 2006).

As regards the influence of the production year, no significant differences in OTA concentrations were found for the tested wines, neither related with the total of samples nor regarding color, type or region groups (data not shown). Nevertheless, it is worth mentioning that the year with the highest incidence and contamination was 1999, followed by 2001, 2002, 2003 and 2005, while the least contaminated samples appear to be produced 2000, followed by those of 2004 and 2006. It should be commented, although, the fact that Stefanaki *et al.* (2003) and Soufleros *et al.* (2003) reported those from the 1999 harvest were the most contaminated. Meteorological data for Greece in that year revealed increased rainfall and relative humidity during the harvesting period, which probably favored growth of OTA-producing fungi.

With respect to conventional and organically produced wines, the statistical examination failed to found any significant differences. The mean, median and the ranges of OTA concentration of the two groups are presented in Table 3 where a slightly increased incidence and contamination for OTA in conventionally produced wines is defined. Tjamos *et al.* (2006) show that the organically grown in Greece varieties Athiri and Cabernet Sauvignon were the less contaminated among the sampled wine grape cultivars and, moreover, the non organically grown Cabernet Sauvignon proved to be highly contaminated by *A. carbonarius* in comparison with the organically grown. In another study in Argentinean wine grapes cultivated under organic and non-organic systems, Ponsone *et al.* (2007) report also lower OTA concentration and *A. carbonarius* presence during harvesting for the organically produced wines. Chiodini *et al.* (2006), in a survey for OTA in wine, compared organically and conventionally produced products, failed to find significant differences between the two types of cropping system. They reported that the use of fungicides in conventional farming may stress molds such that they initiate toxin production. However, as the key factors are the good agricultural, handling, and storage practices required in organic, as well in conventional, agriculture in order to minimize the risk of mold growth and mycotoxin contamination (Blesa *et al.*, 2004; Tjamos *et al.*, 2006; Chiodini *et al.*, 2006).

In conclusion, this study of OTA in wines produced in Greece shows that Greek red, rosé and white wines had lower levels and incidences than wines from other countries of the Mediterranean basin, that all Greek wines were below the proposed

European limit of 2.0 μg L^{-1}, that our results were comparable with published results from EU Member States and, although that the incidence of contamination was rather high, most of the samples contained low levels of contamination with this mycotoxin. The key areas of concern were the results from the southern regions of Greece, mainly Peloponnese and Aegean Islands, especially for the dessert type ones. More detailed surveys should be contacted in the future related to OTA presence in relation to viticultural practices and vinification procedures.

Chapter 6: Microvinification Experiment – Fate of Ochratoxin A during Vinifications and Clarification Procedures

6.1 Introduction

Processing can play an important role in diminishing the potential risks of mycotoxin-contaminated food commodities. Thus, it is important to evaluate the effects of processing on OTA to determine if the toxin level can be managed through post-harvest procedures. The safety of wine can only be assured by monitoring OTA risk throughout the winemaking process. In particular, all stages of the process, starting with grape harvesting, including the intermediate stages in the winery and the distribution of the wine itself should be observed. Information on OTA persistence and transformation during processing would be useful for the development of an effective prevention strategy for OTA contamination (Grazioli *et al.*, 2006).

Several corrective techniques for reducing the incidence of OTA in wines have been tried. The content of OTA has been studied on grape products (Blesa *et al.*, 2004; Leong *et al.*, 2006b; Magnoli *et al.*, 2004; Sera *et al.*, 2004; Shephard *et al.*, 2003; Soufleros *et al.*, 2003; Stefanaki *et al.*, 2003), the conditions and factors required for the development of OTA have also been assessed (Pitt & Hocking, 1997; Sanchis & Magan, 2004; Belli *et al.*, 2006a; Serra *et al.*, 2006), as well as the response to different cropping systems (Chiodini *et al.*, 2006; Cozzi *et al.*, 2007; Ponsone *et al.*, 2007). However, although *A. carbonarius* that produce OTA, the chemical structure of it, and its stability and toxicity are all known, its appearance and metabolic pathway is still uncertain during winemaking. It is not clear how vinification affects OTA content in wines, and only few studies refer on this subject.

During red wine manufacturing, OTA content decreases until the malolactic fermentation preceding its bottling. In white type vinification, were maceration and malolactic fermentation are absent, the decrease taking place before and during fermentation process. The mycotoxin level diminishes, probably due to its adsorption on the *Saccharomyces cerevisiae* yeast surface, to its interaction with metabolites produced by yeast, to its degradation with the lactic bacteria still present in wine or

more likely is associated with removal of spent fractions during winemaking, such as wine lees after fermentation or sediment after racking (Bejaoui *et al.*, 2004a; Cecchini *et al.*, 2006; Grazioli *et al.*, 2006; Fernándes *et al.*, 2007).

For OTA detoxification, chemical compounds or adsorbent materials have been tested such as activated charcoal, cholestyramine, sodium and calcium aluminum silicates (mainly zeolites), bentonite, wood fragments or yeast cells (Leong *et al.*, 2006b; 2006e; Cecchini *et al.*, 2006). Wine fining agents such as potassium caseinate or activated carbon have shown also positive effects on OTA detoxification (reduction up to 82%) but they have also damaged wine quality due the reduction caused in the concentration of some important wine constituents such as polyphenols and aroma compounds (Fernandes *et al.*, 2007; Castellari et al. 2001; Gambouti *et al.*, 2005).

The aim of this work was to study the fate of OTA during the main stages of the winemaking process (crushing, maceration, alcoholic fermentation, racking, and malolactic fermentation) and the influence of wine clarification (bentonite, potassium caseinate, and activated carbon) on OTA concentration, either with the presence of *A. carbonarius* or starter yeast. For these purposes 4 experimental vinifications contacted the 2005 and 2006 vintages, one white and one red vinification every for every year, in laboratory scale but according the instructions of the supplier wineries, in order to imitate as possible the process applied by them in industrial scale.

6.2 Materials and methods

5.2.1 Grape samples

For the micro-vinification trial in the laboratory, grapes (80 kg) were collected at harvest time of the red Agiorgitico cultivar, from Arcadian vineyards of Arkas S.A. (C1; see Chapter 3: Table 3.1 & Figure 3.1). Another vinification assay has been conducted with grapes of Roditis white cultivar, from different vineyards of Achaia Clauss Co. located in Achaea (A1-A5; see Chapter 3: Table 3.1 & Figure 3.1), which were used for the production of the white wine. Roditis cv. was selected among other cultivars for the reason that in mycofloral study (see 3 Chapter) appeared the most contaminated one. The winemaking trials were performed during two vintages, 2005 and 2006. The micro-vinification process performed at the laboratory of Institute of

Technology of Agricultural Products (NAgReF, Lycovrysi, Athens, Greece), immediately after the arrival of the grapes.

6.2.2 Red micro-vinification procedures

Vinification trials started by destemming grapes and crushing them with the help of a manual winepress, yielding a must with pomace (skins and seeds) included. Thereafter, must was separated to 8.0 L batches and placed in food-grade plastic buckets of 10.0 L capacity. Pomace was separated in 1 Kg batches, placed in tulle, sank in each bucket to represent the soaking "pomace cap" of industrial maceration and was immersed twice a day. Each bucket of must with pomace, added with 6.0 g hL^{-1} potassium metabisulfite ($KHSO_2$) in order to generate 30 mg L^{-1} of SO_2. SO_2 was checked every second day and corrected when needed. Temperature, grape-sugar (in Bé density scale) and pH for every case were recorded daily. The pH remained almost constant and within the range of 3.3 to 3.8 and temperature was at 22-26 °C during maceration and kept at 16-18 °C for alcoholic fermentation. Grape sugar density diminished gradually to zero after 18 days when the residual sugars were <1.0 g L^{-1}. After 4 days maceration was completed, drawing off and press of pomace took place, and the received must continued alcoholic fermentation. After the following completion of alcoholic fermentation, a first racking was carried out to remove the lees from the wine. Then, malolactic fermentation (MLF) occurred spontaneously, due to lactic acid bacteria resident in the wine, and after 12 days a second racking took place and wine was stabilized for bottling by the addition of potassium metabisulfite.

6.2.3 White micro-vinification procedures

The procedures followed for white micro-vinification was exactly the same as for the red one with only the following exceptions. Firstly, after grapes were destemmed and pressed, pomace was separated and must proceed to vinification without maceration. Secondly, the end of alcoholic fermentation was after 15 days, malolactic fermentation did not take place and wines were racked with potassium metabisulfite, cold-stabilized for 10 days at 10°C, and bottled. Finally, it must be noted that both years must of white Roditis cv. found contaminated with an OTA concentration of 0.30 and 0.080 $\mu g\ L^{-1}$, respectively for 2005 & 2006, while must of red Agiorgitico cv. was always free of OTA.

6.2.4 Experimental design

Both years the same experiment took place in order to check repeatability of the procedure. Apart from the blanks cases, there have been monitored the presence of 5.0 ppb OTA, of an inoculum of 5×10^4 L^{-1} *A. carbonarius* spore suspension and the presence of starter yeast (200 mg L^{-1} *Saccharomyces bayanus* + 200 mg L^{-1} fermaide), all at the start of the vinification. The outline of the cases studied in both white and red micro-vinification is presented in Table 6.1.

Table 6.1 Experimental design of the micro-vinifications.

Case	Starter yeast	*A. carbonarius*	Ochratoxin A
A	No	No	No
B	No	No	Yes
C	No	Yes	No
D	Yes	No	No
E	Yes	No	Yes
F	Yes	Yes	No

6.2.5 Sampling

Must or wine samples, lees, and pomace for red vinification, were collected on five distinct occasions for OTA determination during the vinification process: after the beginning of the fermentation (1st day), twice during fermentation (5th and 10th days), at the end of fermentation (15th day for the white, 18th for red), at the end of MLF for red (30th day) and at the final product just before bottling. Moreover, microbiological studies performed for both *Saccharomyces* spp. and *A. carbonarius* in order to test their viability during the process.

6.2.6 OTA determination

The methodology for OTA determination was based on the reference method for wines described in the European Standards (EU, 2006b), comprising clean-up by immunoaffinity columns followed by HPLC quantification (see paragraph 5.2.3). For musts, the modification was made: in the clean-up, the initial ratio of must:dilution solution (PEG) was 25:125 ml (instead of the typical 10:10 ml used for wines) (Visconti *et al.*, 2001). All samples were injected in duplicate. The chromatography equipment is presented in paragraphs 5.2.3 & 5.2.4 of the Chapter 5.

6.2.7 Fining Agents and Clarification Experiments

Three enological fining agents (bentonite, potassium caseinate and activated carbon) were purchased in specialized stores and added with double-distilled water as recommended by the cooperated wineries. Clarification experiments were performed by using the fining agents at fixed dosage commonly used in the current winemaking practice. Aliquots of each red wine (50 mL) were poured in cap-vials and added with a single fining agent. Wines were mixed, kept in the dark at +4 °C for 12 h, and then centrifuged for 20 min at 8500 rpm. The supernatant was collected and analyzed for its OTA concentration. The percentage of removed OTA was calculated on the basis of the initial OTA concentration and accounting for the dilution effect caused by the addition of the fining agents.

6.3 Results

6.3.1 Effects of enological practices on OTA

Ochratoxin A levels in wines and the effect of the enological factors essayed are shown in Tables 6.2 and 6.3. Concentration ranges for OTA after finish of vinification were 0.02 to 2.87 μg L^{-1} and not detected to 2.23 μg L^{-1} for white and red wines, respectively. Roditis cv. had a small initial contamination of OTA of 0.30 μg L^{-1}, apart from the added (5 μg L^{-1}), which almost has been detoxified during the white vinification process. Wines from red cultivar Agiorgitico were free of OTA initially and in both OTA adsorption and ranges in final product were better than white, although, for the cases of physically contaminated samples (A & D, Table 6.2), were the amount of OTA was much lesser than the artificially contaminated ones, the toxin almost detoxified at the end of fermentation. Of major importance is also the finding that in both vinifications, when *A. carbonarius* inoculum was added, there have been an increase in OTA for the first days, which these days after started to diminishing. In these cases the % adsorption was measured with the highest concentration reached through the vinification and not the initially present OTA. Very interesting, also, is the fact that pomace analysed after the end of red vinification procedure were highly contaminated either from the artificially contamination or by the presence of *A. carbonarius* with OTA levels up to 4.10 μg L^{-1}. Finally, MLF also decreased the OTA content in wine.

Table 6.2 OTA concentration during white micro-vinification

Roditis cv.				OTA concentration (μg L^{-1})				
Case No	**Starter Yeast**	***A. carbon-arius***	**OTA**	**Vinification process (days)**			**Final product**	**OTA adsorption (%)[1]**
				1	5	10		
A	–	–	–	0.30	0.22	0.18	0.03	90.00
B	–	–	+	4.81	4.29	3.76	2.87	40.33
C	–	+	–	0.83	0.90	0.77	0.63	24.09
D	+	–	–	0.28	0.23	0.19	0.02	92.85
E	+	–	+	5.09	3.71	3.10	2.86	43.81
F	+	+	–	0.73	0.96	0.89	0.83	13.54

[1] % adsorption measured with the highest concentration reached through the vinification and not with the initially present OTA

Table 6.3 OTA concentration during red micro-vinification

Agiorgitico cv.				OTA concentration (μg L^{-1})						
Case No	**Starter Yeast**	***A. carbon-arius***	**OTA**	**Vinification process (days)**				**Final product**	**Pomace**	**OTA adsorption (%)**
				1	5	10	18			
A	–	–	–	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-
B	–	–	+	4.91	3.30	2.90	1.98	1.47	3.10	55.50
C	–	+	–	0.04	0.57	0.23	0.19	0.18	1.34	57.47
D	+	–	–	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-
E	+	–	+	4.87	3.51	3.10	2.67	2.23	4.13	54.21
F	+	+	–	0.10	0.53	0.40	0.22	0.13	1.10	76.48

[1]n.d.= not detected (LOD = 0.01 μg L^{-1})

6.3.2 Adsorptive capacity of fining agents

Finally in following Table 6.4 the performance of 3 common wine fining agents are presented. The adsorbed amount of OTA is calculated from the final concentration at the bottling stage of wines. As it can be seen none of the tested fining agents had satisfactory results.

Table 6.4 Relative amount of OTA (%) adsorbed from wine by the fining agents.

Fining agent	Dosage (g/l)	Application time	OTA adsorption (%)	
			White wine	Red wine
Bentonite	0.40	1-2 hours	19-22	9-14
Potassium caseinate	0.75	1-2 hours	< 5	15-20
Activated carbon	0.10	24 hours	19-21	10-12

6.4 Discussion

The general trend for the evolution of OTA concentration throughout the vinification process is clear. OTA levels decreased markedly in all situations, as can be seen in Tables 6.2 & 6.3. The decrease in OTA of the initial must is more pronounced few days after the start of fermentation, as it has been noted also by others (Ratola *et al.*, 2005; Caridi *et al.*, 2006) and in the present experiment particularly after the fifth day of fermentation. A similar increase was observed from Varga & Kozakiewicz (2006). The microbiological and mycofloral analysis (data not shown) denotes a dramatically increase of yeast dynamics from the 3rd day of fermentation and a coinstantaneous decrease of *A. carbonarius* from that day until the 5th, after which no viable spores were detected. From this point to the end of the fermentation OTA diminished probably due to adsorption from yeast cells and furthermore, for red vinification, due to pomace present, which, due to chemical binding, at the end of maceration, found highly contaminated. In fact, there must be extensive adsorption of OTA to the solid parts of the grapes. Fernandes *et al.* (2003), who reported a decrease in OTA content from must to wine, noted that the presence of biomass could favour such a trend in the must, which could also be due to an adsorption mechanism onto its surface, explained by the overall negative charge in the cell walls and the acidic nature of OTA (Castellari *et al.*, 2001). Otteneder and Majerus (2000) mentioned that grape juices are usually more contaminated than wines, given the absence of a fermentation process.

When the initial contamination was low, either because initially present or produced by *A. carbonarius*, the decrease was greater reaching 90% incidence for the former and 76% for the latter. Nevertheless, the same has not been noticed for the white vinification and *A. carbonarius* presence. In this instance the decrease was relatively poor (13.54 & 23.09% for starter yeast *S. bayanus* present and indigenous yeast, respectively). This should be come from the fact that *A. carbonarius* can proliferate better on the solid "pomace cup", were possibly OTA is bind and removed after maceration, in contrast with white vinification were no maceration taking place.

In addition, the presence of starter yeast did not seem to have any significant performance as compared with the indigenous yeasts. In contrast, many studies have reported better adsorption capacities for *S. cerevisiae* and *S. bayanus* (Caridi *et al.*,

2006; Bejaoui *et al.*, 2004; Fernándes *et al.*, 2007). The yeast cell wall is made up of two principal constituents: *β*-glucans and mannoproteins. Mannoproteins are partially water-soluble components that are released during and, above all, at the end of alcoholic fermentation. The mannoproteins located in the outermost layer of the yeast cell wall give this structure its active properties and have an important role in controlling the wall's porosity. In the low pH range of wine, mannoproteins carry negative charges and, as a consequence, they may establish electrostatic and ionic interactions with the other wine components and therefore bind OTA. The structure of mannoproteins of yeasts walls vary from strain to strain and between species. This structural variability may explain the differences in the binding activity of wine yeasts OTA (Caridi *et al.*, 2006).

After alcoholic fermentation, the MLF causes a significant OTA reduction (Table 6.3). This confirms the positive decontaminating effect of lactic acid bacteria, responsible for the biological deacidification of wines. Trials carried out on lab-scale and industrial scale (Ratola *et al.*, 2005) show that the reduction depends on the bacterial strain and it is inversely related to OTA concentration in wine and independent of the alcohol concentration.

As regards the high concentration of OTA in pomace, data pointed out that the skin contains a large part of OTA either produced by *Aspergillus carbonarius* or added artificially to the must. Indeed, Battilani and Pietri (2002) after investigating the occurrence of the toxin in the skin and the pulp of naturally infected berries reported that the skin of grape berries seems to be the most frequently contaminated tissue. Gambuti *et al*. (2005) observed that intensive pressing of pomace increased the OTA concentration in wine also reported the role of grape skin as a carrier of OTA in wine.

Unfortunately, the wine fining agents did not reveal a significant decrease of OTA, with bentonite having a 19-22 % adsorption for white wine and potassium caseinate a 15-20 % for red (Table 6.4). Indeed, a relatively poor ability of bentonite to bind and precipitate OTA was reported by Castellari *et al.* (2001) during wine fining. These authors found that bentonite was an efficient adsorbent of OTA per unit of surface area; however, at concentrations used in wine fining, the active surface area was small which limits its absorbing efficiency compared with other agents such as activated charcoal. According to Gambuti *et al.* (2005) among several products (natural polysaccharide, cellulose ester, PVPP, cellulose fibre, rind yeast, silica gel,

bentonite, deodorant carbon, de-colorising carbon, high meso-porosity carbon, and high de-colorising carbon) evaluated for the clarifying treatment of contaminated red wines, only enological carbon reduced the OTA concentration. However, according to Leong *et al.* (2006f) bentonite was the most effective fining agent for removing OTA from Semillon wine. These contradictory data are probably due to differences between the fining agent concentrations, the chemical nature of wine components and the OTA concentration in the initial wine.

In winemaking, grapes are subjected to many processing steps, and the final result is a decrease in OTA level. OTA present in grapes is partially released into must during crushing and during maceration. During fermentation, the OTA content decreases in the liquid fraction either in alcoholic or MLF fermentation. The clarification (either natural sedimentation or use of adjuvants, racking), contribute to OTA decrease, because of its adsorption to the sediment. Grape selection is a preventive measure to control safety hazards and good manufacturing practices in winemaking can effectively reduce contamination. Grape crushing is a crucial step and OTA measurement should be done at this stage. Operations unit such as solid liquid separation and the fermentative processes are effective in reducing OTA. In order to manage the hazards of OTA in winemaking and to verify if OTA content in wine is lower than the legal limit of 2 $\mu g\ L^{-1}$ defined by European Commission, OTA analysis in must and in wine at the end of alcoholic fermentation would be enough, since the following phases reduce OTA content.

Chapter 7: General Discussion

7.1 Mycofloral Study

The grape varieties included in this study are representative of a wide range of characteristics. Equally important Greek varieties for wine-making were included, some of them being of V.Q.P.R.D. certification. The position of these varieties (Roditis, Mavrodaphni, Agiorgitico, Muscat Blanc and Cabernet Sauvignon) on the Greek wine making industry and market, and the fact that they produce important quality wines, make the severity of the reported infection from *Aspergillus* section *Nigri* more important.

The occurrence of *A.* section *Nigri* in the total mycoflora of all vineyards tested was always high throughout the 4 years of study (23.9 – 41.7%). However, some samplings of Roditis and Mavrodaphni varieties from Achaea showed incidences >90% for some years. From the *A.* section *Nigri* isolates, *A. carbonarius* represented 20-35%, with the only exception being 2004, where they represented only 10% of the total black Aspergilli isolates.

The study between the two stages of grape maturation, véraison and ripening, revealed that final occurrence of *A.* section *Nigri* in the total mycoflora is proportional to the infection at véraison. Moreover, the main characteristic of the most contaminated samples was the increased proportion of damaged berries, which apart from "sour rot" incidence, may correlate also with the thin berry skin and high sugar proportion of these samples.

The results comparing conventional and organically grown grapes suggest that, when the inhibition of mycoflora by fungicides is not fully successful, the reduction of mycoflora from the chemical treatment allows rapid *A. carbonarius* colonisation to occur, especially when the environmental conditions are favourable.

Definitely, there is an effect of the localisation of vineyards to the population of OTA producing fungi. Localisation of vineyards reflects the weather conditions. High temperatures and relative humidity, favour growth of *A. carbonarius*. Thus, Greece is among the countries that incidence of *A.* Section *Nigri* and *A. carbonarius* at harvesting are particularly high. Varieties grown in the mountainous regions always had lower incidences from the Mediterranean climate localisation. Moreover, from the

study has also revealed for Greek cultivars and wines that the more contaminated were those originating from Mediterranean climates near the sea, like the Aegean Islands and Achaea in contrast with those grown in mountainous climates of Greece. The *Aspergillus* spp. population is increasing with time during grape growing season, while wine cultivars with high amounts of sugar tend to be more infested by *A. carbonarius* than the wine cultivars of high acidity grown for dry wine production. Cultivation practices are considered to be of importance for the occurrence and the population composition of *Aspergillus* spp., since the organically grown vineyards showed very limited infection. On the contrary, vineyards of conventional practices were the most contaminated by *A. carbonarius*, since the applied type of pruning does not prevent contact of canes with the soil. Moreover there is a trend related to fungicides treatments. When used in excess their effectiveness on *A. carbonarius* decreased contrary to isolates of other genera. In addition, the present and many other studies revealed that for Greece organically grown vines present a greater resistance to infestation from ochratoxigenic black *Aspergillus* spp. But the main reasons for this is probably the carefully selected locations of vineyards that assure the good aeration of vines and the thorough application of Good Agricultural Practices that organic culture demands.

7.2 Ecology

Greek *A. carbonarius* strains isolated from the present study presented the shorter lag phases prior to growth under optimum environmental conditions when compared with other isolates of different countries. A similar trend observed as regards the tolerance to xerophilic conditions of these isolates, which seem not losing their spore germination ability at 0.85 a_w. It could be mentioned that although intra-isolate differences are very small, at marginal conditions differences may occur mainly as part of adaptation to regional climatic conditions. As the optimum a_w for OTA production was around 0.95, contamination on the vineyard can occur mainly when grape sugars' diminish, at mature period. Although, at lower temperatures, around 20-25 ºC, OTA production can take place even at véraison.

In addition, the 7 year survey contacted OTA in wines produced in Greece showed that Greek red, rosé and white wines had lower levels and incidences than

wines from other countries of the Mediterranean basin, that all Greek wines were below the proposed European limit of 2.0 μg L^{-1}, that our results were comparable with published results from EU Member States and, although that the incidence of contamination was rather high, most of the samples contained low levels of contamination with this mycotoxin. The key areas of concern were the results from the southern regions of Greece, mainly Peloponnese and Aegean Islands, especially for the dessert type ones. In these areas producers follow the traditional sun-drying method for the production of desert wines, which under the specific environmental condition may favour the production of OTA on the processed grapes. In this instance special attention should be given to the hygiene conditions of the sun-drying areas and time that the process last should be as limited as possible. As regards the micro-vinification experiments, from these a main conclusion is that OTA present in grapes is partially released into must during crushing and during maceration. Consequently, duration of maceration should also be limited or under low and controlled temperatures. Furthermore, during fermentation, the OTA content decreases in the liquid fraction either in alcoholic or MLF fermentation. The clarification (either natural sedimentation or use of adjuvants, racking), contribute to OTA decrease, because of its adsorption to the sediment.

7.3 Predictive Mycology

Several studies have been done to weigh the ecophysiological factors of fungal growth and mycotoxin production on both artificial growth substrates and real foods. In addition, a large number of reports have been published on potential techniques to inhibit fungal growth and mycotoxin production (Garcia *et al.*, 2009). These reported the conditions capable for supporting or inhibiting fungal growth and/or mycotoxin production. The last decade, interest has been increasing for application of predictive modelling techniques to describe fungal growth and mycotoxin production. As predictive mycology continues to grow, even more related models specific for fungal growth will be developed, as currently most models in use today and used in this study are borrowed from predictive microbiology. Moreover, the present study describes the applicability of a probabilistic modelling approach for the influence of a_w and temperature on growth and OTA production of two ochratoxigenic isolates of

A. carbonarius from Greek wine grapes. Models to predict the likelihood of growth and OTA production of mycotoxigenic fungi as a function of intrinsic and extrinsic factors, known as "probability" models, can be very valuable both in prevention strategy and risk assessment planning. The development of predictive mycology to the levels that predictive microbiology has reached requires the development of rapid methods to estimate fungal growth. The current methods of measuring the colony diameters or HPLC for OTA determination are too laborious or time consuming. As an example, methods based on automated image analysis have been used to estimate the growth (hyphal extension) of incubated cultures (Panagou *et al.*, 2005) and ELISA and rapid methods for qualitative assessment of OTA presence have been developed (Saha *et al.*, 2007). Such techniques would significantly reduce the work that has to be done to obtain a sufficient quantity of growth data and OTA production, reduce measurement errors and enable for far more work to be done in the same time. Furthermore, current models need to be extended to account for the consequences of fungal competition, the presence of insects, fungicides and pesticides amongst many others. Once such models can be provided then their predictions can be treated with more confidence than those of current models which only account for the effect of what are considered to be the most important determinants of fungal growth, a_w and temperature. Finally, importance should be noted on inoculum levels and the ability of fungi to grow and contaminate vine grapes with OTA should be thoroughly investigated. Where possible models are incorporating the demonstrated large variation in growth at the individual spore level, should be developed to account for the variation that is not revealed when high inoculum levels are used in model development (Dantigny & Nanguy, 2009).

The results of this study showed that under the current experimental conditions, the combined effect of temperature and water activity on the growth responses of *A. carbonarius* could be satisfactorily predicted, and the examined models could serve as tools for this purpose.

7.4 Use of the gathered information for developing prevention strategies in Greece

7.4.1 Pre-harvesting strategies – Viticulture

Harvesting date should be selected according to the ripeness of grapes, its sanitary level, the meteorological conditions, and always after evaluation of the risk for contamination with ochratoxigenic fungi. In areas of high risk, grapes should be harvested earlier, and when heavily contaminated grapes are detected, they should be rejected from winemaking procedure. Same treatment should be followed for grapes damaged by insects and damaged berries. Transportation of harvested grapes should be as fast as possible and avoid holding periods. Recipients for transportation should be carefully cleaned.

Targeted management strategies require prior identification of vineyards or regions in which grapes and wine are at risk of OTA. Several studies in Europe report the increased isolation of *A. carbonarius* in warm climates and increased humidity. Damage to berries is the primary factor affecting the development of *Aspergillus* bunch rots and the subsequent production of OTA in grapes. Berry damage may occur due to birds, insects or infection by other fungi such as *Botrytis cinerea* (Botrytis rot) or *Erysiphe necator* (powdery mildew). Rain prior to harvest is a common cause of berry damage. During rain, high osmotic pressure within the berries, combined with low evaporative water losses cause the berries to swell and if critical turgor pressure is reached, the berry skin splits. The severity of infection, as assessed by black Aspergillus counts, was highest in years when rain prior to harvest caused berry splitting in grapes grown for drying (Leong *et al.*, 2006b). An increase in counts during fruit drying was also observed, which is indicative of continued growth by *A. carbonarius* and possible concomitant OTA production during the early stages of drying (Magan & Aldred, 2005). It may be possible to modulate the incidence of A. carbonarius in soil through vineyard management techniques. Vineyards in which the soil was tilled regularly showed greater *A. carbonarius* counts in the soil than vineyards which had minimal soil cultivation. Similarly, grapes from a vineyard with frequent soil cultivation displayed a higher incidence of black Aspergillus spp. than grapes from a vineyard with minimal cultivation (Leong *et al.*, 2006b). Moreover, Tjamos *et al.* (2006) noted that vineyards located on hilly, sunny and well aerated

sites, and were applied with linear cultivation system that facilitates aeration and reduces relative humidity, had lower incidences of *A. carbonarius*. Considering the aforementioned, the choice of organic cultivation, under Good Agricultural Practices that necessitate in this cropping type, poses a partial solution for *A. carbonarius* and OTA contamination problem. Finally, an effective strategy on the field should definitely include a narrow monitoring of vineyards for fungi incidence and berries status from early véraison until harvest. If toxigenic black *Aspergilli* spp. were frequently identified in a vineyard in the months before harvest, application of fungicidal sprays may reduce the incidence of *Aspergillus* rot and OTA contamination (Leong *et al.*, 2006b; Tjamos *et al.*, 2004; Batillani *et al.*, 2005b).

7.4.2 Enological practices

Grape selection is a preventive measure to control safety hazards and good manufacturing practices in winemaking can effectively reduce contamination. Grape crushing is a crucial step and OTA measurement should be done at this stage. Next, in the final stage of the maceration process, good conditions exist for mould growth, as long as there is no fermentation and there are aerobic conditions. This is a point that control of the process should be taken e.g. keeping temperature in low ranges. Moreover maceration type with pomace in free air contact on the top of the must should be avoided.

Yeast is predominant in natural flora on the surface of fresh grapes and is significant in winemaking because it carries out the alcoholic fermentation and its autolysis products may affect the sensory quality products and the growth of spoilage bacteria. Ochratoxigenic fungi are inhibited by ethanol and the generally anaerobic conditions. Moreover, the primal mode of removal of OTA during vinification was adsorption rather than degradation. In particular, binding of OTA to yeast cells during fermentation appeared to play an important role in OTA reduction. Many studies report yeast strains with high “binding” capacity to OTA, either through the fermentation process or to adsorption on lees. Although, due to quality assurance issues wineries is difficult to differentiate the strain that ensure the characteristics of their final product. Also, the lactic acid fermentation can contribute, by the same way of cell binding, to OTA decontamination.

Enological fining agents have been shown to reduce the OTA level in wine during the ordinary clarification practice. In particular, activated carbon and

potassium caseinate have a good capacity to absorb OTA in model solutions, whereas bentonite has demonstrated a low affinity for OTA. Although, agents such as bentonite may remove proteins to which OTA are already bind, whereas proteinaceous material such as gelatine or potassium caseinate can provide the OTA to be bound on. Nevertheless, the higher reduction on OTA reported, in most studies is the step of pressing of grapes and of manipulation of solid and liquid parts during vinification (e.g. maceration, fining). Concluding, there must always taking place during vinification a close monitoring of crushing, maceration and fermentation processes, which could perfectly serve also as CCP of an implemented HACCP system for wineries.

7.5 Suggestions for future work

- Research to clarify whether the primary mode of infestation is opportunistic, latent, or otherwise is still required.
- The identification of genes preside for OTA production, are still pending, and moreover, it should be elucidated the interaction of *A. carbonarius* strains of different origin, with other mycoflora, fungicides, and environmental factors.
- Current models of predictive mycology need to be extended in order to account also of fungal competition, the presence of insects, fungicides and pesticides amongst many others.
- Research for new varieties less susceptible to fungal infection.
- Innovative vinification procedures.
- Molecular detection techniques. Indeed, late years, it has been shown that induction of OTA biosynthesis genes, which can be measured by real-time PCR and microarray, some time before the mycotoxin identification by analytical methods, and thus, the activation of these genes can be used as an early indicator for mycotoxin biosynthesis (Schmidt-Heydt & Geisen, 2007; Schmidt-Heydt *et al.*, 2008). However only some genes of a given mycotoxin biosynthesis pathway can be regarded as key genes, whose activation is directly coupled to the mycotoxin biosynthesis. In this direction evolution is rapid and good promising.

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