

Cranfield University
Applied Mycology Group, Cranfield Health

PhD Thesis

**THE INFLUENCE OF FARMING SYSTEMS
ON THE INCIDENCE OF *ASPERGILLUS*
CARBONARIUS IN VITICULTURAL PRODUCTS**

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ABSTRACT

In viticulture, the most effective way to prevent or reduce the ochratoxin A (OTA) contamination of viticultural products is the control of ochratoxigenic fungal species, and especially *Aspergillus carbonarius*, the predominant producer of ochratoxin A in these products.

In the present study, conducted in the years 2010, 2011 and 2012, in Heraklion Prefecture (Crete, Greece), the influence of the different farming systems that are applied in the Cretan vineyards, on the incidence of *A. carbonarius* in grapes and soil, and hence the potential of OTA contamination of viticultural products, were examined. The impact of different cultivar characteristics, cultivation techniques (trellis system, nutrition and irrigation management, fungicides treatments) and area of cultivation was also investigated.

The results showed that the different farming systems had a significant influence on the incidence of *A. carbonarius*, with the integrated farming system being the most effective regarding the control of *A. carbonarius* and prevention/reduction of OTA occurrence in viticultural products. Nevertheless, the grape cultivar had a significant impact on *A. carbonarius* incidence on grapes. Differences among the cultivars grown under the same farming system were related to the different sensitivity of the cultivars, the relative intensity of the applied cultivation practices (e.g. crop protection system employed) and cultivar-related techniques (trellis system, infected berry trimming). The most important factor which differentiated the incidence of *A. carbonarius* independent of the farming system was the altitude at which cultivation was done which was a critical factor.

ABSTRACT

A correlation between *A. carbonarius* incidence on grapes and in soil, as well as a correlation between *A. carbonarius* incidence on grapes and OTA concentration in wines, was found. Finally, a differential effect of fungicides used in viticulture, against other fungi and on *A. carbonarius* growth was found, with the fungicides used against *Botrytis cinerea* and *Plasmopara viticola* being more effective against *A. carbonarius*, compared to fungicides used against *Uncinula necator*.

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ABBREVIATIONS

a_w	Water Activity
CCA	Coconut Cream Agar
CFS	Conventional Farming System
CFUs	Colony forming units
CYA	Czapek Yeast Agar
DRBC	Dichloran Rose Bengal Chloramphenicol
EC	European Commission
EtOTB	Ethylochratoxin B
EU	European Union
g	grams
HPLC	High Performance Liquid Chromatography
IAC	immunoaffinity columns
IFS	Integrated Farming System
L	litre
LOD	Limit of Detection
LOQ	Limit of Quantification
MEA	Malt Extract Agar
MEA-B	Malt Extract Agar - Boscalid

MeOTA	Methylochratoxin A
MeOTB	Methylochratoxin B
mg	micrograms
mg	milligrams
min	minute
ml	millilitres
NAGREF	National Agricultural Research Foundation
ng	nanograms
nm	nanometres
OFS	Organic Farming System
OTA	Ochratoxin A
OTB	Ochratoxin B
OTC	Ochratoxin C
PBS	Phosphate Buffer Saline
PPI	Plant Protection Institute
ppm	Parts per million
UV	Ultra Violet
VQPRD	Vin de Qualité Produit Dans Une Région Déterminée
µg	micrograms
µl	microlitres

CHAPTER ONE

GENERAL INTRODUCTION - LITERATURE REVIEW

1.1. GENERAL INTRODUCTION

In recent years there has been a continuous increasing concern regarding the health of consumers and their exposure to foods containing chemical residues and natural toxins, such as mycotoxins (Balazs and Schepers, 2007; Arvanitoyannis, 2008). Mycotoxin is a secondary metabolite produced by organisms of the fungus kingdom, including mushrooms, molds and yeasts, possibly as a defensive reaction, against environmental conditions, other fungi and bacteria, which can reduce their growth rate (Benbrook, 2005) (Plate 1.1). Mycotoxins considered most relevant for human health, and having the greatest agro-economic importance are aflatoxins, ochratoxins, trichothecenes, zearelenone, fumonisins, tremorgenic toxins, and ergot alkaloids (Hussein and Brasel, 2001; Serra *et al.*, 2005).

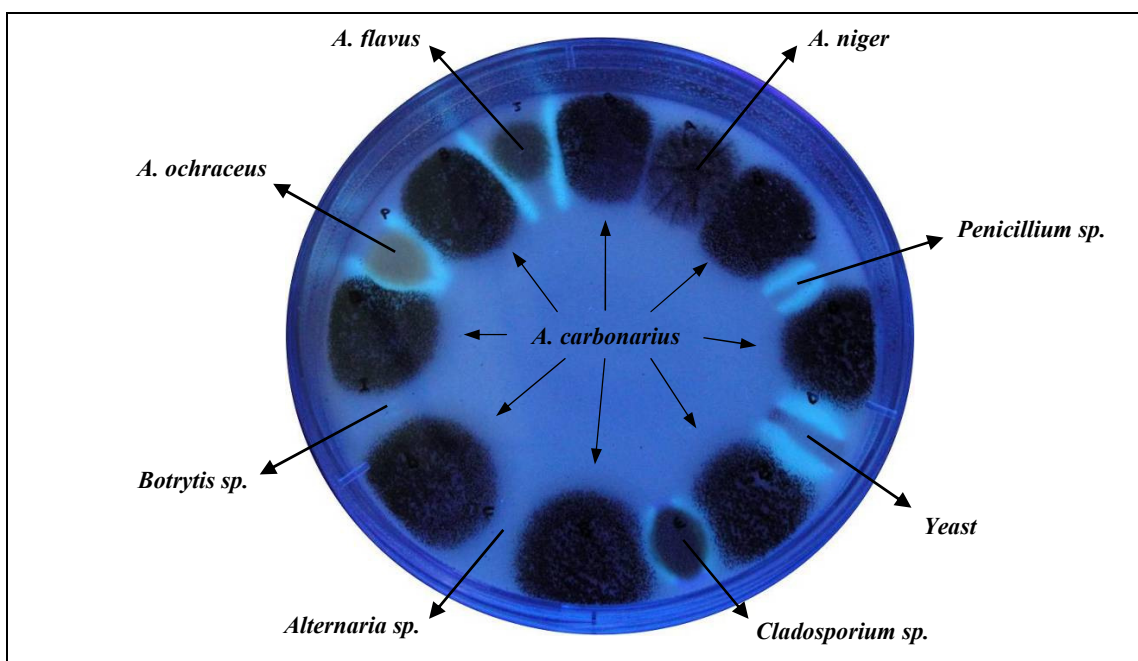


Plate 1.1. Ochratoxin A production by *Aspergillus carbonarius*, as a defence against other fungal species, but not against *A. niger*. Ochratoxin A appears as blue-fluorescence under UV (365 nm) light on CCA 50% cultures.

During grape cultivation and wine production, many fungi may occur until harvest, but the most important genera which cause mycotoxin contamination are the black Aspergilli (*Aspergillus* section *Nigri*), especially *Aspergillus carbonarius* and *A. niger* (Battilani *et al.*, 2003; Hocking *et al.*, 2007), which are the predominant producers of Ochratoxin A (OTA). This is the main mycotoxin of concern in vine products, such as grapes, wine and dried vine fruits (raisins).

OTA may be found in a wide variety of food products, such as cereals, grapes, wine, dried fruits, nuts, spices, coffee, cocoa, meat (Varga and Kozakiewicz, 2006), as well as olives, olive oil, herbal teas and medical plants, liquorices and vinegar (Duarte *et al.*, 2009). Additionally, airborne movement of *Aspergillus spp.* spores is a possible source for human infection, through the respiratory system (Rigby and Caceres, 2001; Brera *et al.*, 2002; Duarte *et al.*, 2009; Smith and McGinnis, 2009).

The importance of black Aspergilli contamination of food products and OTA occurrence relies on the confirmed nephrotoxic, hepatotoxic, genotoxic, neurotoxic, immunotoxic, mutagenic, embriotoxic and teratogenic effects and suspected carcinogenicity (Zollner and Mayer-Helm, 2006; Brera *et al.*, 2008; Duarte *et al.*, 2009). Human are exposed to mycotoxins directly and indirectly, by consumption of contaminated ingredients and foodstuffs, and by infected animals destined for human consumption respectively (Pitt *et al.*, 2000), while the ability of OTA to move up the food chain is aided by its long half-life in humans and certain edible animal species (Turner *et al.*, 2009).

Several surveys are conducted worldwide aiming at the reduction of black Aspergilli contamination and OTA occurrence in food products, in order to minimize the health

risk of humans. The EU has taken measures to minimise contaminants in foodstuffs by setting maximum levels for certain contaminants. Specifically, for OTA, maximum levels have been laid down for cereal and cereal products, dried vine fruit, coffee, wine, grape juice, processed cereal based foods and baby foods for infants and young children, dietary foods for special medical purposes specifically for infants (European Commission, Commission Regulation (EC) No 1881/2006). Commission Regulation No 401/2006 lays down the provisions for methods of sampling and analysis for the official control of OTA (European Commission, Commission Regulation (EC) No 401/2006).

The objective of this study was to investigate the influence of the different farming systems (Integrated, Organic and Conventional) that are applied in the Cretan vineyards (Greece), on the incidence of *A. carbonarius*, the predominant producer of OTA in viticultural products. The influence of different grape cultivars and the effects of the growing areas of the vineyards, mainly due to the different climatic conditions and altitudes were also considered. The final aim was the determination of the most efficient farming system and cultivation techniques to minimise the incidence of *Aspergillus spp.* and the production of OTA in viticultural products.

1.2. FUNGI AND MYCOTOXINS IN FOOD

Several fungi infect a wide range of food products at pre-harvest, harvest and post-harvest, causing not only the quantitative and qualitative devaluation of products, but also they render these foods dangerous for the health of consumers, due to toxic secondary metabolites, the mycotoxins, which they secrete (Jackson and Taher, 2008). Some fungi produce more than one mycotoxin and some mycotoxins are produced by more than one fungal species (Brera *et al.*, 2008), whereas not all fungi are toxigenic and not all secondary metabolites are toxic (Hussein and Brasel, 2001).

Mycotoxins are characterised by their relative potency and the legislative limits reflect this. Thus the major class included aflatoxins, ochratoxins, trichothecenes, fumonisins, patuline and zearalenone which are the most important mycotoxins from the viewpoint of human and animal health (Brera *et al.*, 2008). Table 1.1, shows the most commonly affected commodities and the main toxic effects of the major mycotoxins while in Table 1.2 the most important major and minor classes of mycotoxins and the dominant fungal species that produce them are presented.

Mycotoxins occur in a wide range of food products, such as cereals, spices, fruits, coffee beans, corn, rice and viticultural products which have consequences for not only human and animal health, but have significant economic impacts that include health care, reduction of crop and livestock production, disposal of contaminated foods and feeds, as well as investment in research for minimising mycotoxins contamination problems (Hussein and Brasel, 2001).

The mycotoxin of main concern, regarding the viticultural products, such as grapes, wine and vine dried fruits, is OTA, produced mainly by fungi belonging to *Aspergillus* Section *Nigri*, also known as "black aspergilli". Other species in the *Aspergillus* section *Circumdati* and *Penicillium verrucosum* and *P. nordicum*.

Table 1.1. Major mycotoxins and the most commonly affected commodities.

Mycotoxins	Affected commodities	Reference
Aflatoxins	Cereals, spices, cottonseed, copra, dried fruits and nuts; among processed food, eggs, pasta, bread and milk and cheese	Brera <i>et al.</i> , 2008
Ochratoxin A	Cereals, grapes, wine, dried fruits, nuts, spices, coffee, cocoa, meat, olives, olive oil, herb teas and medical plants, liquorices, vinegar	Varga and Kozakiewicz, 2006; Duarte <i>et al.</i> , 2009
Trichothecenes	Corn, wheat, barley, oats, rice, rye	Richard, 2007
Zearalenone	Corn wheat, barley, sorghum and rye	Richard, 2007
Fumonisin	Corn, sorghum and rice	Richard, 2007
Patulin	Fresh fruit, Apple juice, Pear juice, Grape juice, Cider, Purees, Baby foods, Tomatoes	Brera <i>et al.</i> , 2008

Table 1.2. Major and minor mycotoxins and their production fungal species (Brera *et al.*, 2008).

Mycotoxins		Fungal species
Major mycotoxins	Aflatoxins	<i>Aspergillus flavus</i> , <i>Aspergillus parasiticus</i> , <i>Aspergillus nomius</i> , <i>Aspergillus pseudotamarii</i>
	Ochratoxins	<i>Aspergillus</i> spp., <i>Penicillium</i> spp.
	Trichothecenes	<i>Fusarium</i> spp. (in particular <i>Fusarium langsethiae</i> , <i>Fusarium poae</i> and <i>Fusarium sporotrichioides</i>), <i>Myrothecium</i> spp., <i>Stachybotrys</i> spp., <i>Trichoderma</i> spp., <i>Cephalosporium</i> spp., <i>Trichothecium</i> spp., <i>Verticimonosporium</i> spp.
	Zearalenone	<i>Fusarium graminearum</i> , <i>Fusarium culmorum</i> , <i>Fusarium sacchari</i>
	Fumonisin	<i>Fusarium verticillioides</i> [Sacc.] Nirenberg (former <i>F. moniliforme</i> Sheldon), <i>Fusarium proliferatum</i>
	Patulin	<i>Penicillium</i> spp., <i>Aspergillus</i> spp., <i>Byssosclamyces</i> spp.
Minor mycotoxins	Ergot alkaloids	<i>Claviceps</i> spp.
	Citrinin	<i>A. ochraceus</i> , <i>P. citrinum</i>
	Cyclopiazonic acid	<i>Penicillium cyclopium</i> , <i>A. flavus</i>
	Sterigmatocystin	<i>Aspergillus nidulans</i> and <i>Aspergillus versicolor</i>
	Moniliformin	<i>F. moniliforme</i> Sheldon (<i>F. verticillioides</i> [Sacc.] Nirenberg), <i>F. avenaceum</i> , <i>F. subglutinans</i> , <i>F. proliferatum</i>
	Gliotoxin	<i>Gliocladium fimbriatum</i> , <i>Aspergillus fumigatus</i> , <i>Candida albicans</i> .
	Citreoviridin	<i>Penicillium citreonigrum</i> Biourge (synonyms: <i>Penicillium citreoviride</i> and <i>Penicillium toxicarium</i>), <i>P. ochrosalmoneum</i>
	Tremorgenic mycotoxins	<i>Penicillium</i> spp., <i>Aspergillus</i> spp., <i>Claviceps</i> spp., <i>Acremonium</i> spp.
	Penicillic acid	<i>Aspergillus</i> spp., <i>Penicillium</i> spp. (in particular <i>P. cyclopium</i> and <i>P. canescens</i>)
	Roquefortine	<i>Penicillium roqueforti</i>
	3-Nitropropionic acid	<i>Astragalus</i> spp., <i>Arthrimum</i> spp.
	Fusaproliferin	<i>Fusarium proliferatum</i> , <i>Fusarium subglutinans</i>

1.3. THE GENUS *ASPERGILLUS*

The genus *Aspergillus* contains more than 100 recognized species (Chang *et al.*, 1999) belongs to the family of Moniliaceae, of Deuteromycetes class. The fungi classified in *Aspergillus* Section *Nigri*, also known as "black aspergilli" due to rots black colour (black conidia), are widespread saprophytes in soils around the world (Leong *et al.*, 2006b).

Aspergillus spp. can be found in a wide range of foods and feeds, such as corn, rice, nuts, spices, coffee, fruits, cheeses, dried meat and fishes (Chang *et al.*, 1999), as well as viticultural products (grapes, wine, dried vine fruits and vinegar), where *A. carbonarius* is predominantly responsible for OTA contamination (Battilani *et al.*, 2006d) (Plate 1.2).



Plate 1.2. *Aspergillus carbonarius* growing on a grape berry, where are visible the conidiophores and the black spores.

The conidiophores of *Aspergillus spp.* consists of the aspergillum, the stipe and the "foot cell". Aspergillum is a specialized structure on which are produced the conidia, which are borne by specialized cells called phialides (uniseriate aspergillum), and maybe a second layer of specialized cells called metulae (biseriate aspergillum). The aspergillum is borne by the stipe, in the basal part of which is formed the *Aspergillus spp.* characteristic "foot cell" (Chang *et al.* 1999) (Plate 1.3).

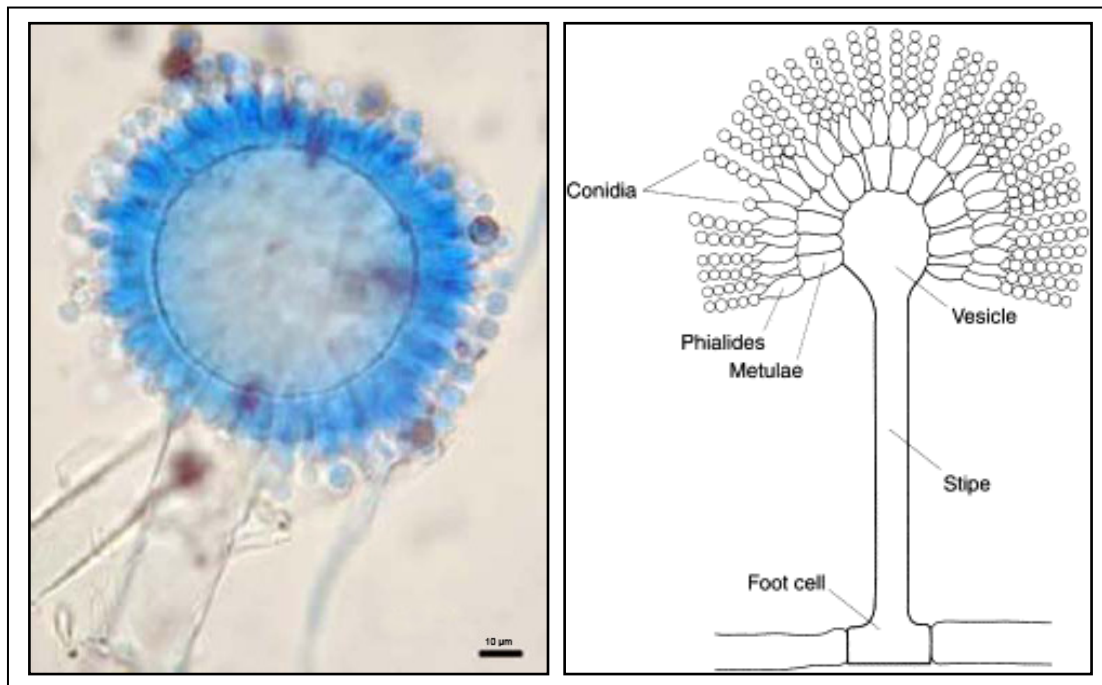


Plate 1.3. *A. carbonarius* conidiophore (left) (Serra *et al.*, 2005) and basic morphological structures of *Aspergillus spp.* (right) (Chang *et al.*, 1999).

1.4. OCHRATOXINS

Ochratoxins are secondary metabolites produced mainly by *Penicillium verucosum* and *Aspergillus ochraceus* in cereals and members of the *Aspergillus* section *Nigri*, especially *A. carbonarius* in grapes, wine and vine fruits (Magan, 2006).

Ochratoxin A (OTA), B (OTB) and C (OTC) (Figure 1.1) are compounds which have in common a dihydroisocoumarin moiety linked over a 7-carboxy group to l-phenylalanine via a peptide bond (Zollner and Mayer-Helm, 2006), while OTA, the toxin of most concern, also contains a chlorine atom on the aromatic ring, which accounts for its toxicity. On acid hydrolysis, OTA yields phenylalanine and an optically active lactone acid, Ochratoxin α (OT α) which is less toxic (Brera *et al.*, 2008).

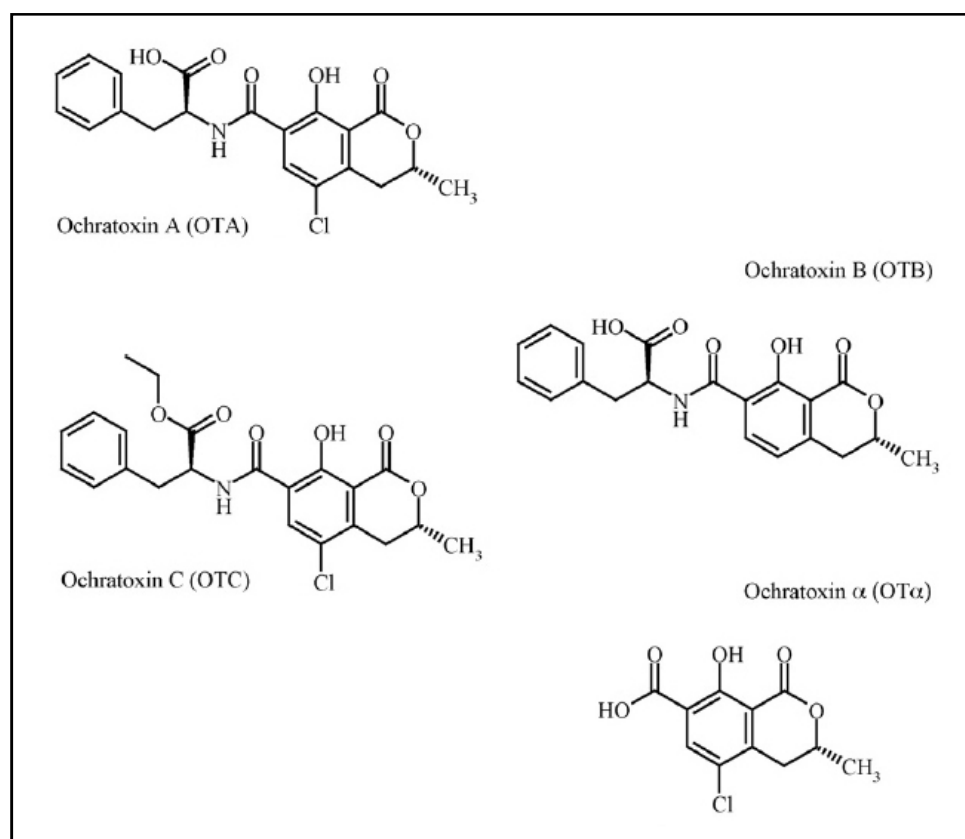


Figure 1.1. Structures of Ochratoxin A (OTA), B (OTB), C (OTC) and α (OT α).

1.5. BLACK ASPERGILLI AND OCHRATOXIN A IN VITICULTURAL PRODUCTS

Several fungi may occur in a vineyard during one growing cycle, such as powdery mildew (*Uncinula necator*), downy mildew (*Plasmopara viticola*), and grey rot (*Botrytis cinerea*). However, regarding contamination with mycotoxin the main concern are the black Aspergilli (Serra *et al.*, 2003). While *A. niger* is the most frequently reported species (Samson *et al.*, 2006), *A. carbonarius* is the key fungus responsible for OTA on grapes, wine, dried vine fruits and other grape products (Bau *et al.*, 2005; Barkai-Golan, 2008).

One of the primary sources of black Aspergilli is the soil, and especially the surface soil (0-1 cm) and soil directly beneath vines (Leong *et al.*, 2006b; Hocking *et al.*, 2007), perhaps mainly due to damaged and dried berries falling to the soil, that provide a sugar-rich medium for the growth of saprophytic *Aspergillus spp.* Another equally important source of black Aspergilli is the vine remnants (residue) on the soil, such as dried berries, dead canes and vine barks (Hocking *et al.*, 2007). However, the spores of black Aspergilli disperse by the air, as well by insects, from the aforementioned sources onto the ripening berry surfaces.

In order to minimize the contamination of black Aspergilli and OTA occurrence on grapes, wine and other grape products, it is important to know the factors that induce the infection and development of black Aspergilli, as well as their interactions and inter-relationships.

1.6. BLACK ASPERGILLI IN SOIL

A large number of fungal species, more than 80000 as estimated, occur in soil, during their life cycle, having either an active role as saprophytes involved in degradation of dead material, or survive for a period of their life cycle as resting spores or mycelial fragments in dead debris (Bridge and Spooner, 2001).

Indeed, the surface soil of vineyards (0-5 cm depth) beneath the vines is considered the primary source of contamination of grape products by *Aspergillus* species. The incidence of *A. carbonarius* is greater at the surface than in deeper soil in undisturbed grounds (untilled). In regularly tilled soils they are found beneath vines rather than between rows, due to the vine trash and berries falling on the ground (Leong *et al.*, 2006b).

The dispersal of conidia and their transfer to vine parts comes through the air, while the concentration of conidia in the air decreases, as the height from the soil increases (Leong *et al.*, 2006b).

The diversity and population of some fungal species and strains can be affected by physical factors, such as the temperature of soil surface (Jaime-Garcia and Cotty, 2010), but also by anthropogenic factors relative to the type of cultivation.

The farming system could affect the incidence of some fungal species in soil, mainly by the use of synthetic fertilizers and pesticides (Klingen *et al.* 2002). Herbicides, insecticides and especially fungicides (Kuthubutheen and Pugh, 1979) falling on the ground during sprayings, could have an effect on the incidence of fungal species whether directly, or indirectly by affecting the microbial community and relative populations of fungal species.

1.7. BIOTIC AND ABIOTIC FACTORS

The growth of *Aspergillus spp.* and the production of OTA can be affected by many biotic and abiotic factors, during the growing season, as well as after harvest in storage.

Temperature, which varies among viticultural regions, seasons and stages of grape maturity, is one of the most important factors, affecting the fungal growth and OTA production (Chiotta *et al.*, 2009). Another equally important factor is **water activity (a_w)**, which is a measure of the energy status of the water in a system (Astoreca *et al.*, 2007b; Romero *et al.*, 2007). Water activity within berries decreases during maturation due to increase of berry sugars, and furthermore while berries dry (Leong *et al.*, 2006a).

Aspergillus carbonarius is able to grow and produce OTA in a wide range of temperatures (Esteban *et al.*, 2004) and water activities (Esteban *et al.*, 2006b). However optimum conditions for growth are very different from those for OTA production (Mitchell *et al.*, 2004). The optimum temperatures and water activities, although they differs among several researchers, as reported are for growth between 30-35 °C, with no growth below 15 °C and above 45 °C (Magan, 2006), and 0.93-0.987 a_w (depending on the strain), and for OTA production 15-20 °C and 0.95-0.98 a_w , respectively (Mitchell *et al.*, 2004; Astoreca *et al.*, 2009).

Another factor similar to water activity is the actual **relative humidity (RH)** of the air, which also affects fungal growth and OTA production. Indeed the higher relative humidity (up to 100%) increases the berry infection by *Aspergillus spp.* and OTA production, especially under optimum temperature conditions and the relative level of damage to the berries (Pardo *et al.*, 2005; Belli *et al.*, 2007).

Skin damage of berries is also a significant factor which influences *Aspergillus spp.* growth and OTA production, since the lesions of berries skin are used as entries for the penetration of fungus to the interior of berries (Somma *et al.*, 2012).

Injury to the berry skin may be caused by mechanical damages, chemical treatments, or splitting during rainy weather (Belli *et al.*, 2007; Clouvel *et al.*, 2008). A very common reason for skin damage is insect attacks by the grape berry moth (*Lobesia botrana*) (Cozzi *et al.*, 2006; Clouvel *et al.*, 2008) and Mediterranean fruit fly (*Ceratitis capitata*), as well as infections by other fungi, such as *Botrytis spp.* and *Erysiphe necator* (Leong *et al.*, 2006a; Clouvel *et al.*, 2008).

In addition, *Aspergillus carbonarius*, besides penetration through damaged skin, could access interior of berries, via other routes such as the berry stalk (Belli *et al.*, 2007). According to Battilani and Pietri (2002) *A. carbonarius* is able to colonize and penetrate berries even without any skin damage.

The ability of *A. carbonarius* to penetrate into undamaged berries differs among grape cultivars and viticultural regions, with an increase from white grapes to red, and from northern regions to southern (Battilani and Pietri, 2002). Additionally, Battilani *et al.* (2004), reported differences in the incidence of colonized berries and their OTA contents among twelve grape cultivars in Italy, differences which are not explained by the characteristics of grape cultivars.

In contrast, Leong *et al.* (2006a) suggested that some grape cultivars may be more susceptible to *Aspergillus spp.* rot, because of their susceptibility to berry splitting, perhaps due to their different skin hardness and thickness (Belli *et al.*, 2007), or their different times of maturity, as the sugar content increases and berry skin softens (Belli

et al., 2005; Leong *et al.* 2006a). *Aspergillus* rot development is low in immature berries, and increases in maturing berries (Ponsone *et al.*, 2007). Similarly, the formation of OTA increases when sugar-content is greater than 12-13° Brix (Leong *et al.*, 2006c). Generally, the potential for *Aspergillus spp.* development and OTA formation, increase from berry set until harvest, as the tartaric acid decreases and sugar-content increases.

Meteorological conditions (Battilani *et al.*, 2006b) but also **climatic and geographical differences** can affect the incidence of *Aspergillus spp.* and consequently OTA production in different regions (Battilani *et al.*, 2006d; Varga and Kozakiewicz, 2006; Valero *et al.*, 2008). **Latitude** and **longitude** have influences in the growing of *Aspergillus spp.* and OTA production, perhaps due to different climatic conditions (temperature, rain), with a positive gradient from West to East and from North to South (Battilani *et al.*, 2006a). Indeed, as reported by Battilani *et al.* (2006b), the incidence of *A. carbonarius* has a positive correlation with the summation of **degree-day** and a negative correlation with the summation of **rain** between veraison and ripening. **Altitude** of areas is another factor affecting the incidence of *Aspergillus spp.* with a positive gradient from higher areas to the lower areas until sea level (Pateraki, 2008).

Photoperiod and day-night temperatures could also affect the growth of *Aspergillus spp.* (Belli *et al.*, 2006c), while **UV radiation** may be an inhibitory factor of the germination of *A. carbonarius* spores on berries, particularly on immature green berries (Leong *et al.*, 2006e; Hocking *et al.*, 2007). Despite the black colour which provides some protection of sun light and UV radiation (Esteban *et al.*, 2006a; Esteban

et al., 2006b), *A. carbonarius* is susceptible to prolonged sun exposure (Leong *et al.*, 2006a).

Vineyard management techniques play a great role in the incidence and growth of *Aspergillus spp.*, and in OTA production, the most important of them are the soil tillage, pruning, irrigation, as well as chemical treatments.

The **soil tillage** and **irrigation** have significant impacts on the incidence of *A. carbonarius* in the soil and on the grapes, given that the soil in the vineyards, mainly the surface soil and the vine trash beneath vines, is the primary source of *A. carbonarius*. The regular tillage has positive effects in the incidence of *A. carbonarius* in the soil and on grapes, while minimal soil tillage practices reduce that impact. The irrigation of the vineyards can also affect the presence of *A. carbonarius*, since in moist soils, the counts of this species seems to be reduced (Mitchell *et al.*, 2004; Leong *et al.*, 2006b; Hocking *et al.*, 2007).

The **vine pruning** process can also have significant effects on the incidence and the growth of *A. carbonarius*, as well as OTA contamination of grapes, since a sparse canopy allows greater air movement among grapes, a greater exposure to sunlight and UV radiation, as well as a greater penetration of fungicide sprays (Leong *et al.*, 2006b; Varga and Kozakiewicz, 2006).

Several of fungicides which are used to control other fungal infections are being investigated for their efficacy against *Aspergillus spp.*, (Marin *et al.*, 2008). **Chemical applications** with some of those fungicides seem to have significant effects in reducing the occurrence of *Aspergillus spp.* (Tjamos *et al.*, 2004), while on the contrary, applications with other fungicides, are not only ineffective, but also have the opposite

effects. Suboptimal concentration of some fungicides can result in stimulation of OTA production (Magan, 2006), since they promote *A. carbonarius* predominance over other less ochratoxigenic species (Medina *et al.*, 2007).

Several studies have been conducted on the efficacy of **biological control agents**, as alternatives to pesticide usage or in combination with fungicides application, where some of the organisms tested (atoxigenic Aspergilli, yeasts, bacteria and fungi), were found to be effective against *A. carbonarius* growth and OTA contamination on grapes (Tsitsigiannis *et al.*, 2012). Indeed, there are several antagonistic yeasts, with the potential to control *Aspergillus spp.*, which could be used for biological control, as alternatives to pesticides usage (Bleve *et al.*, 2006; Dimakopoulou *et al.*, 2008).

1.8. FARMING SYSTEMS

The intensification of agriculture in the last decade or so has had positive effects (increase of agricultural production etc.), had also some negative effects. Thus an increase in energy, water, fertilizers and plant-protection product inputs have increased the costs of production. However, they have also drastically changed natural ecosystems with unfavourable effects on the environment, as well impacted on producers and consumer health. While the development of sustainable agricultural production systems (Pacini *et al.*, 2003) has become a priority generally, the impact on production of food which is highly nutritious in value and quality has also impacted on the environment. Generally, there is a movement from the Conventional Farming System (CFS) to the Integrated Farming System (IFS) and the Organic Farming System (OFS) (Goumenaki and Kapetanakis, 2008).

The **Organic Farming System (OFS)** is "a production system that sustains the health of soils, ecosystems and people. It relies on ecological processes, biodiversity and cycles adapted to local conditions, rather than the use of inputs with adverse effects. Organic agriculture combines tradition, innovation and science to benefit the shared environment and promote fair relationships and a good quality of life for all involved" (IFOAM, 2009). It is a holistic production management system which promotes agro-ecosystem health, whereas it emphasizes in the use of, where possible, cultural, biological and mechanical methods, in preference to the use of off-farm inputs (Codex Alimentarius Commission, 1999), aiming in the production of qualitative products with not at all chemical residues, according to the Council Regulation (EEC) No 2092/91 of 24 June 1991, on organic production of agricultural products and indications referring

thereto on agricultural products and foodstuffs (European Commission, Council Regulation (EEC) No 2092/91).

The **Integrated Farming System (IFS)** is an environmentally sensitive and economically viable production system or process which uses the latest available techniques to produce high quality food in an efficient manner (Morris and Winter, 1999). "Integrated Farming meets the potentially conflicting challenges of Sustainable development which must encompass food production alongside conservation of finite resources and protection of the natural environment, at farm level, in a manner that balances food production, profitability, safety, animal welfare, social responsibility and environmental care. Integrated Farming seeks to reinforce the positive influences of agricultural production whilst reducing its negative impacts. It is a means of achieving a sustainable agriculture and an indispensable part of sustainable development" (European Commission, DG Environment, Agra CEAS Consulting, 2002).

The **Conventional Farming System (CFS)** or non-organic farming system, as opposed to organic farming system is, often, according to the FAO (2007) definition, industrialized agriculture, characterized by mechanization, monocultures, and the use of synthetic inputs such as chemical fertilizers and pesticides, with an emphasis on maximizing productivity and profitability.

1.8.1. Influence of farming systems on the incidence of *Aspergillus spp.* and OTA production.

Which of the farming system is more efficient with regard to the prevention of *Aspergillus spp.* incidence and the production of OTA in viticulture (and other) products, is a question without any satisfactory answer. This needs to be examined in more detail.

Regarding the Organic Farming System, the absence of chemical pesticides (insecticides, fungicides), as well the minimal use of fertilisers (nitrogen), which has as result the rise of berry sugar contents, can lead to increase of berries susceptibility against fungi.

In contrast, the use of several pesticides, could be not only inefficient, but may affect the microbial balance in the ecosystem, favouring the dominance of *Aspergillus spp.* over other fungi, while the increased use of fertilizers could results in thinner cell walls of the berries skins, rendering them more susceptible to fungal colonisation.

From several researches that have been carried out with regard to the influence of farming systems on the incidence of *Aspergillus spp.* and the production of OTA in various cultures, it is clear that there are needed more detailed studies to inform the available data.

Juan *et al.* (2008) in a study of organic and non-organic cereals and cereal products from Spain and Portugal, found higher levels of OTA in organic compared to non-organic cereals. Magkos *et al.* (2006) in a critical review of the literature, about organic and conventional food safety, compared the recent studies on OTA occurrence in

cereals produced organically and conventionally, and observed that the OTA contamination was higher in organic than in conventionally produced cereals in only 25% of studies, while in the others similar contamination levels were found.

Chiodini *et al.* (2006) in a study of 44 organically and conventionally produced wines, report no difference in the concentration of OTA between wines produced under organic and conventional systems. In contrast, Ponsone *et al.* (2007) report a significant influence of the cultivation systems on the colonization of *Aspergillus* section *Nigri* of wine grapes produced under organic and non-organic systems.

1.9. IDENTIFICATION OF *ASPERGILLUS* SPECIES

For the detection, identification and enumeration of *Aspergillus spp.* in foods and feeds, two of the methods that are commonly used are the dilution plating and direct plating (Plate 1.4). The direct plating method is more efficient in determining the existence of fungal species, while the dilution plating method gives information on the population size. However, a combination of these methods gives a more complete picture of the status of the fungal contamination using traditional techniques.

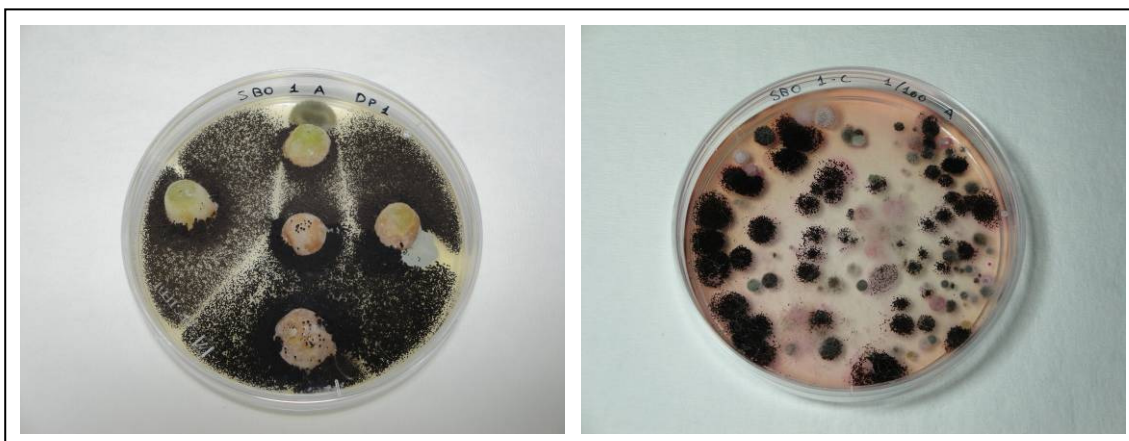


Plate 1.4. Direct plating (left) of grapes and serial dilution plate from a grape sample plated on malt extract agar medium.

The culture plates, containing agar (usually Potato Dextrose Agar or Malt Extract Agar) and maybe additives such as acids or antibiotics (bacterial growth control), are inoculated and generally incubated at 25 °C for 5-7 days in the dark (anamorphic species) or for 10-14 days (teleomorphic species). The *Aspergillus* genus may be recognized even with low power magnification using a 10x-30x objective, while the further distinction between *Aspergillus spp.* is based on morphological characteristics, such as shape and colour of conidial heads, the presence of metulae, the size and shape of conidia. (Chang *et al.*, 1999) (Plates 1.5 and 1.6).

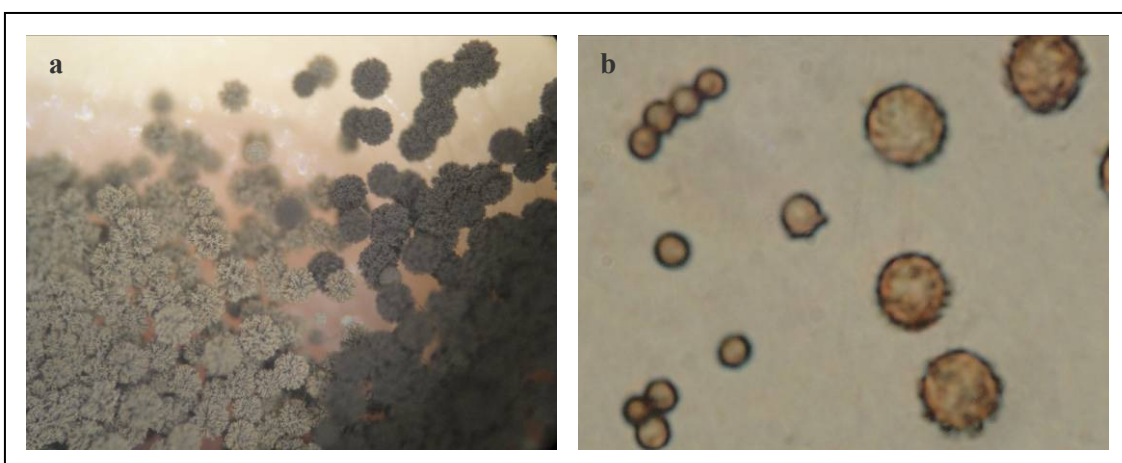


Plate 1.5. *A. niger* (left) and *A. carbonarius* (right) in macro (a) and micro (b) view.

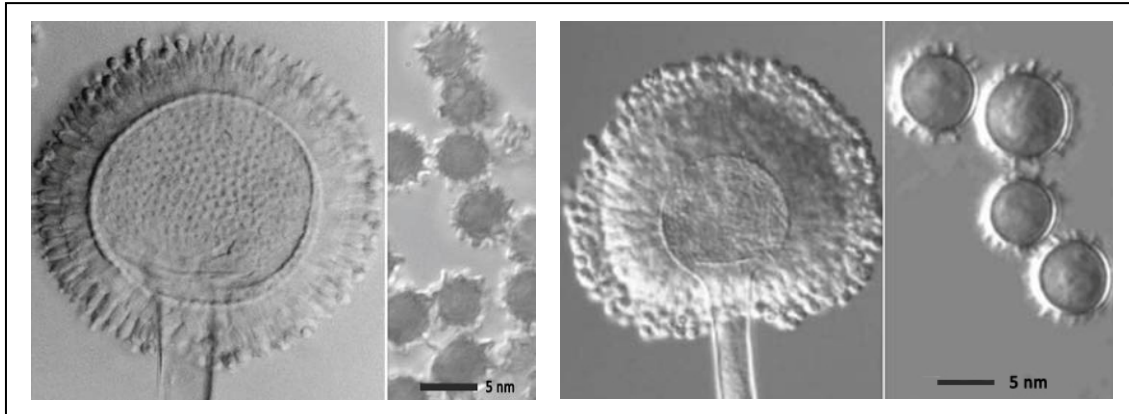


Plate 1.6. *A. niger* (left) and *A. carbonarius* (right) in micro-view (Pitt and Hocking, 2009).

Recently, apart from the culturing techniques, DNA based techniques such as real time PCR are used for the detection and quantification of fungal species. The advantages of these techniques are the specific detection of fungal species among other and the rapid quantification of them, in contrast to the time consumable culturing techniques and optical identification and quantification, based on morphological characteristics. Quantitative real time PCR techniques are available for the rapid detection and quantification of *A. carbonarius* in grapes, predicting the potential ochratoxigenic risk in viticultural products (Mule *et al.*, 2006; Selma *et al.*, 2008).

1.10. METHODS FOR THE DETERMINATION OF MYCOTOXINS

Since most mycotoxins are toxic at very low concentrations, sensitive and reliable methods are required to monitor and quantify mycotoxins rapidly. It is of course also important to take a representative sample of the treatment/field for mycotoxins quantification (Magan and Aldred, 2007; Venancio, 2008; Turner *et al.*, 2009).

The EU has specified (Commission Regulation (EC) 401/2006) the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs (European Commission, 2006).

Several methods have been developed for the qualitative and quantitative analysis of mycotoxins, while most of these methods include five main stages: sampling, sample preparation, extraction, clean-up and quantification (Venancio, 2008).

Sampling plays a crucial role in the precision of the determination of the levels of mycotoxins, as the samples taken must represent the whole lot (European Commission, Commission Regulation (EC) No 401/2006), while without the implementation of a good sampling plan a misclassification of the lot can easily occur (Smith and McGinnis, 2009).

In the following Tables, the number of the samples that must be taken depending on the weight of the lot, for the determination of OTA in dried vine fruits (currants, raisins and sultanas) (Table 1.3), and in wine, grape juice and grape must (Table 1.4), according to the Commission Regulation (EC) No 401/2006 for the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs are presented (European Commission, 2006).

Table 1.3. Number of incremental samples to be taken depending on the weight of the lot of dried fruits (European Commission, Commission Regulation (EC) No 401/2006).

Lot weight (tonnes)	Number of incremental samples	Aggregate sample weight (Kg)
≤ 0.1	10	1
$> 0.1 - \leq 0.2$	15	1.5
$> 0.2 - \leq 0.5$	20	2
$> 0.5 - \leq 1.0$	30	3
$> 1.0 - \leq 2.0$	40	4
$> 2.0 - \leq 5.0$	60	6
$> 5.0 - \leq 10.0$	80	8
$> 10.0 - \leq 15.0$	100	10

Table 1.4. Minimum number of incremental samples to be taken from the lot of wine, grape juice and grape must (European Commission, Commission Regulation (EC) No 401/2006).

Form of commercialisation	Volume of lot (in litres)	Minimum number of incremental samples to be taken	Minimum volume of the aggregate sample (in litres)
Bulk (fruit juice, spirit drinks, cider, wine)	-	3	1
Bottles/packages (fruit juice, spirit drinks, cider)	≤ 50	3	1
Bottles/packages (fruit juice, spirit drinks, cider)	50 to 500	5	1
Bottles/packages (fruit juice, spirit drinks, cider)	> 500	10	1
Bottles/packages wine	≤ 50	1	1
Bottles/packages wine	50 to 500	2	1
Bottles/packages wine	> 500	3	1

The **sampling** procedure includes the collection of representative samples from single places of the lot, the combining and homogenization of all samples in an aggregate sample, and finally the removal of a portion of the aggregate sample to be used as a laboratory sample for analyses (Venancio, 2008).

The **extraction** stage aims at removing the mycotoxin from the biological matrix and dissolving it in a solution which will be used for the analysis. The extraction method is dependent on the structure of the mycotoxin and the kind of matrix (Turner *et al.*, 2009). Some of the most common solutions used for the extraction are water, ethyl acetate, chloroform, methanol-water, acetonitrile-water, hexane-acetonitrile, and dichloromethane-ethanol mixtures with sodium hydrogen carbonate, phosphoric acid or magnesium chloride (Zollner and Mayer-Helm, 2006).

The **clean-up** stage is required for the separation of the mycotoxin from other co-extracted compounds, while the extent of the required clean-up is depended to the specificity and sensitivity of the quantification.

Some of the clean-up methods used widely are: the Liquid-Liquid Extraction (LLE), the Supercritical Fluid Extraction (SFE), and the Solid Phase Extraction (SPE) which is a technique used to absorb selectively a substance from a liquid solution into solid matrix. Other less common clean-up methods are: the Lateral Flow Devices (LFD), Immunoaffinity Columns (IACs), Molecular Imprinted Polymers (MIP), Array Biosensors, and Liquid-Phase Micro-Extraction (LPME) (Venancio, 2008).

The **separation** of the mycotoxins is essential for the quantification, in order to be free of other interferences, and there are several chromatographic methods which are used for the mycotoxins separation.

High Performance Liquid Chromatography (HPLC) is one of the most common methods, which is used in a wide range of mycotoxins, and is characterized by excellent performance, low detection levels, and safety for the operator. HPLC usually is followed by Fluorescence Detection (OTA) or UV detection (Brera *et al.*, 2008).

Thin Layer Chromatography (TLC) is also a common separation method used for mycotoxins analysis, which have the potential to screen large numbers of samples economically (Turner *et al.*, 2009).

Other chromatographic separation methods are the: **Capillary Electrophoresis (CE)** which integrate fast development, high efficiency and low consumption of solvents and reagents (Gonzalez-Penas, 2006), **Liquid Chromatography (LC)** (Zollner and Mayer-Helm, 2006), **Gas Chromatography (GC)** (Nielsen and Thrane, 2001) etc..

Besides the chromatographic methods, there is also an immunologic method, the **Enzyme-Linked ImmunoSorbent Assay (ELISA)** which is based on the ability of a specific antibody to distinguish the three-dimensional structure of a specific mycotoxin (Brera *et al.*, 2008). ELISA is suitable for rapid screenings of a large sample numbers (Zollner and Mayer-Helm, 2006); however there are possibilities to obtain false positive results due to cross-reaction of the antibodies with other mycotoxins, or false negative results due to low levels of contamination (Brera *et al.*, 2008).

1.11. EUROPEAN COMMISSION LEGISLATIONS AND MAXIMUM LEVELS OF OTA IN FOODSTUFFS

Many countries worldwide, in order to protect the consumer from the negative impact of mycotoxins have established regulations, setting limits of the toxins in a wide range of foods (Egmond and Jonker, 2008). Because of the increasing concern about the consequences of OTA exposure in humans and animals, including carcinogenicity, nephrotoxicity, teratogenicity, immunotoxicity and possibly neurotoxicity, the European Commission (EC), with the Commission Regulation (EC) No 1881/2006 (European Commission, 2006) about the "setting of maximum levels for certain contaminants in foodstuffs", apart from other contaminants, maximum levels of OTA have been set for cereals, cereal products, dried vine fruit, roasted coffee, wine, grape juice and foods for infants and young children were set (Table 1.5), based on: "Opinion of the Scientific Committee on Food on Ochratoxin A" (expressed on 17 September 1998), "Assessment of dietary intake of Ochratoxin A by the population of EU Member States" (Miraglia and Brera, 2002), and "Opinion of the Scientific Panel on contaminants in the Food Chain on a request from the Commission related to Ochratoxin A in food" of the European Food Safety Authority (EFSA, 2006).

Table 1.5. Maximum levels for Ochratoxin A in foodstuffs (European Commission, Commission Regulation (EC) No 1881/2006).

Foodstuffs	Ochratoxin A maximum levels (µg/kg)
Unprocessed cereals	5.0
All products derived from unprocessed cereals, including processed cereal 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 and concentrated grape must as reconstituted, intended for direct human consumption	2.0
Processed cereal-based foods and baby foods for infants and young children	0.5
Dietary foods for special medical purposes intended specifically for infants	0.5
Green coffee, dried fruit other than dried vine fruit, beer, cocoa and cocoa products, liqueur wines, meat products, spices and liquorice	—

1.12. METHODS OF PREVENTING OR REDUCING OTA IN WINE

Aiming at the absence of OTA in foods and feeds, or at least to be at levels beneath the maximum permitted, there are several strategies, from which, probably the best is the prevention of the infection. The protection of grapes from mechanical damages, insect attacks and fungi infections, by using cultivation methods and chemical treatments are the main measures that could be taken at pre-harvest, while during harvest, it's important to use the appropriate equipment, in optimum season and optimum conditions. At postharvest, the storage and transport under well-controlled conditions are also important, while good results could have the various treatments with fungicides, essential oils etc. (Magan, 2006; Amezcqueta *et al.*, 2009).

Besides of preventing measures, and when the products are already contaminated, although OTA is a moderately stable molecule which can survive from most food processing (Varga *et al.*, 2000), several physical, chemical and microbiological methods have been developed and could be used, aiming at their detoxification, with the presupposition that they will not influence the quality of the products (Amezqueta *et al.*, 2009). A treatment of OTA contaminated wine with activated carbon or sodium bentonite seems to reduce the OTA levels (Var *et al.*, 2008). The choice of the appropriate yeasts can also reduce the OTA contamination of must, during fermentation, as they absorb to their cell walls a part of the toxin, to an extent which depends on the yeast strain (Caridi, *et al.*, 2006; Cecchini *et al.*, 2006; Meca *et al.*, 2010). Also, a recent technique, of repassage high OTA levels wine over uncontaminated pomaces from the same or other grape cultivars, seems to have good results, at least in red wines OTA decontamination (Solfrizzo *et al.*, 2010).

Apart from the chemical and physical methods of detoxification, also important is the degradation of OTA by organisms such as bacteria, yeasts and filamentous fungi (Ciconova *et al.*, 2010), as well as by several *Aspergillus spp.*, which cleave the OTA molecule to Ochratoxin α , a less toxic moiety (Varga *et al.*, 2000). It is thought that those species use OTA degradation as an alternative nitrogen source, when amounts of available nutrients are limited (Leong *et al.*, 2006d; Astoreca *et al.*, 2007a; Hocking *et al.*, 2007) and especially when fungal fast growth favoured by optimum temperatures (Marin *et al.*, 2006), but it is not known if this process occurs also in field where nutrient sources may be unlimited.

1.13. OCHRATOXIN A IN WINE

Several studies have been carried out on the OTA levels in wines in relation to factors such as the geographical location, due to different climates, the grape cultivars, the type of wine, the cultivation and winemaking techniques in Europe (SCOOP, 2002).

OTA levels seem to be higher in red, than in rose and white dry wines (Visconti *et al.*, 1999; Brera *et al.*, 2008; Spadaro *et al.*, 2010), probably due to the different winemaking techniques, while higher are the levels in sweet – dessert wines than in dry wines. Also, an increase of OTA levels seems to exist in wines from southern regions in relation with wines from northern regions (Pietri *et al.*, 2001; Perrone *et al.*, 2007; Brera *et al.*, 2008), due to the different climates.

In Spain, during the 1997 and 1998, Lopez de Cerain *et al.* (2002), in an analysis of 28 red and 12 white wines (20 wines of 1997 and 20 wine of 1998) found differences between the two years were attributed to the bad climate in 1997. Quintela *et al.* (2011) analysed 100 wines (1995-2008) from two areas of Rioja Alavesa (Spain) and found that the percentage of contaminated with OTA wines was higher in the semi-arid area (71%) than in the dry area (43%), which was attributed to the less rainfalls and the higher temperature in the semi-arid area.

In Greece, Stefanaki *et al.* (2003), in a survey for OTA contamination of 268 Greek wines (of years 1997, 1998 and 1999), found a slight increase in OTA levels from white, to rose, to red dry wines, but with no significant differences, while there was an increase in red dry wines from northern to southern Greece. In a similar research, Soufleros *et al.* (2003), in an analysis of 35 Greek wines (14 red, 13 white and one rose

dry wines and three red and four white sweet wines) of years 1995 to 1999, found a slightly higher level of OTA contamination in red wines than in white wines.

However, in a recent comparative research, Remiro *et al.* (2013) examined 96 red wines of eight Mediterranean countries: Spain, France, Italy, Croatia, Greece, Turkey, Israel and North Africa (12 wines of each country), for their co-occurrence of Ochratoxin A and five compounds related with OTA: Ochratoxin B (OTB), Ochratoxin C (OTC), Methylochratoxin A (MeOTA), Methylochratoxin B (MeOTB) and Ethylochratoxin B (EtOTB).

Ochratoxin A was found in 99% of the wines, due to the low limits of detection, and although, with the exception of North Africa, no statistical significant differences found among the countries, according to the OTA median concentrations, the order of the Countries, from lower to higher concentrations was: France < Croatia < Israel < Italy < Turkey < Spain < Greece < North Africa.

The OTA levels, as the OTB, OTC, MeOTA, MeOTB and EtOTB levels (minimum, maximum, mean and median) that found in the 96 wines of the research, are shown per Country, in the Table 1.6.

Table 1.6. Ochratoxin levels found in Mediterranean red wines (Remiro *et al.*, 2013).

	Spain	France	Italy	Croatia	Greece	Turkey	Israel	North Africa
Ochratoxin A (ng L⁻¹)								
Positives ^a (%)	100	91.7	100	100	100	100	100	100
Minimum	1.05	<LOD	5.18	0.36 ^c	4.35	2.91	3.63	84.4
Maximum	104	88.3	286	61.3	212	101	65.4	455
Mean ^b	37.2	33.6	53.9	18.8	59.4	31.1	18.5	195
Median	25.1	3.56	20	7.65	31.7	20.5	11.7	171
Ochratoxin B (ng L⁻¹)								
Positives ^a (%)	100	100	100	100	100	100	100	100
Minimum	3.78	2.05	3.12	2.6	6.28	2.06	7.17	36.6
Maximum	48.5	39.1	119	27	69.8	50.3	43.8	92.7
Mean ^b	18	13.4	22.5	9.82	28.5	13.3	19	63.9
Median	12.4	8.53	10.1	4.95	21.8	8.71	16.8	65.1
Methylochratoxin A (ng L⁻¹)								
Positives ^a (%)	75	58.3	75	16.7	83.3	41.7	100	50
Minimum	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.56	<LOD
Maximum	2.14	2.37	48.2	0.59	1.33	1.03	3.64	0.71
Mean ^b	1.08	1.22	31.4	0.59	0.78	0.74	1.82	0.66
Median	0.63	0.38 ^c	0.40 ^c	<LOD	0.48 ^c	<LOD	1.73	0.28 ^c
Ochratoxin C (ng L⁻¹)								
Positives ^a (%)	91.7	66.7	100	58.3	100	100	100	100
Minimum	<LOD	<LOD	0.91	<LOD	0.53	0.27 ^c	0.40 ^c	5.92
Maximum	14.3	7.33	23.5	3.01	23.5	11.2	4.41	31.5
Mean ^b	3.93	3.12	5.09	1.71	6.04	3.63	1.91	14.2
Median	1.8	0.97	3.26	0.74	2.92	1.66	1.2	12.5
Methylochratoxin B (ng L⁻¹)								
Positives ^a (%)	100	58.3	100	66.7	100	100	100	41.7
Minimum	0.34 ^c	<LOD	1.04	<LOD	0.29 ^c	0.48 ^c	0.40 ^c	<LOD
Maximum	3.34	1.91	4.25	3.32	3.64	4.3	8.11	4.56
Mean ^b	1.45	0.9	2.07	1.62	1.43	1.38	3.38	2.6
Median	0.98	0.41 ^c	1.77	0.30 ^c	1.06	1.02	2.93	<LOD
Ethylochratoxin B (ng L⁻¹)								
Positives ^a (%)	91.7	50	66.7	91.7	100	100	66.7	100
Minimum	<LOD	<LOD	<LOD	<LOD	0.21 ^c	0.19 ^c	<LOD	1.16
Maximum	2.49	1.31	4.2	2.33	1.81	3.04	2.65	3.08
Mean ^b	1.12	0.89	1.43	1.04	0.89	1.52	1.41	1.94
Median	0.49 ^c	0.19 ^c	0.26 ^c	0.59	0.56	0.94	0.39 ^c	1.77

^a: >LOD; ^b: >LOQ; ^c: LOD – LOQ; LOD: Limit Of Detection; LOQ: Limit of Quantification

1.14. OVERALL OBJECTIVES AND PROGRAMME OF WORK

The main objective of this study was to investigate the influence of the different farming systems that are applied in the Cretan vineyards (Greece), on the incidence of *A. carbonarius*, the predominant producer of OTA in viticultural products.

The influence of different grape cultivars, either for wine or table grape production, on the incidence of *A. carbonarius*, as it may be affected by the differences in sensitivity, cultivation techniques (trellis system, pruning, nutrition and irrigation management, chemical treatments) and other factors were studied. The effects of the growing areas of the vineyards were also considered.

The influence of the farming system in the incidence of *A. carbonarius* in soil, as well as, the possible correlation between the incidences of *A. carbonarius* on the grapes and in the soil of the vineyards was also considered in this study.

The OTA concentration in wines produced in wineries located in the experimental area, from grapes cultivated under the different farming systems, were examined and the possible correlations with the incidence of *A. carbonarius* in the relevant areas were also examined.

The effect of the fungicides and other crop protection products used in viticulture, as well as, the effect of essential oils on the growth of *A. carbonarius* were finally tested.

The final aim of the present study was the determination of the most efficient farming system and cultivation techniques to minimise the incidence of *Aspergillus spp.* and the production of OTA in viticultural products.

Objectives of this study were:

- The influence of the farming systems on the incidence of *A. carbonarius* on grapes and in soil of vineyards.
- The correlation of *A. carbonarius* incidence on grapes and in soil of vineyards.
- The effect of the different cultivars on the incidence of *A. carbonarius*, due to the different characteristics of grapes, and cultivation techniques.
- The effect of different areas of cultivation, due to the different climates and altitudes.
- The effect of the fungicides, other crop protection products and essential oils on the growth of *A. carbonarius*.
- The OTA in wines produced from organic and integrated farming systems grapes from the experimental area and correlation with the incidence of *A. carbonarius*.

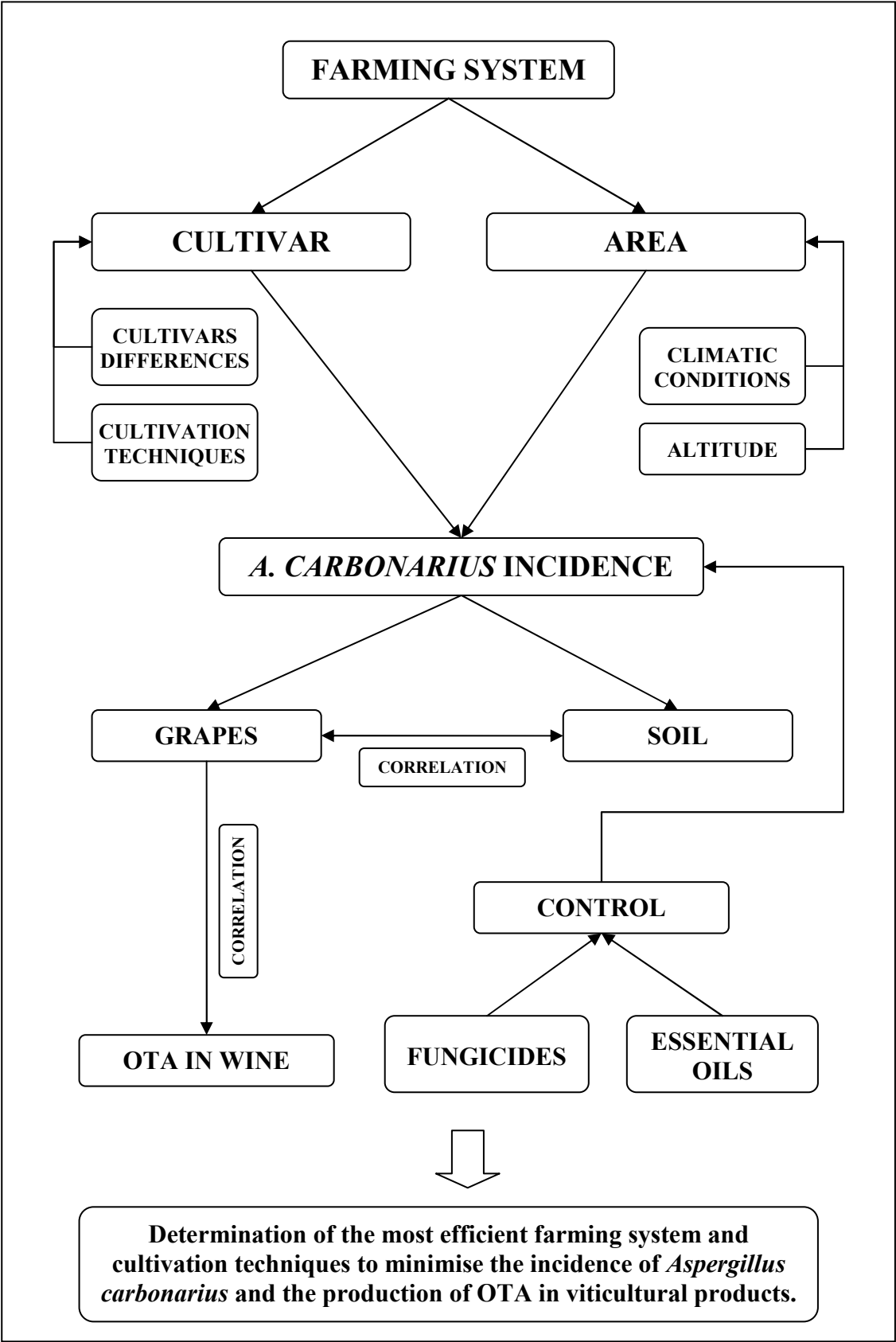


Figure 1.2. Flow chart of different components of thesis studies.

CHAPTER TWO

MATERIALS AND METHODS

2.1. EXPERIMENTAL PLAN

For the aims of the research of the first year (2010 growing season) 27 vineyards of table and wine cultivars were chosen, from the three main viticultural areas of Heraklion Prefecture of Crete (Greece), grown under the three farming systems: Organic Farming System - OFS, Integrated Farming System – IFS and Conventional Farming System - CFS.

Nine vineyards of Kotsifali cultivar (three of each farming system) in Area A (Peza), nine vineyards of Soultanina cultivar in Area B (Malevizi) and nine vineyards of Soultanina cultivar in Area C (Messara) were chosen (Plate 2.1 and Table 2.1) (map made with ArcGIS 9.2 software - ESRI). All vineyards were chosen to be as representative as possible of the cultivar and the cultivation (in each farming system); every vineyard was separated in five parts (5 blocks) for statistical analysis purposes (Plate 2.2), while all vineyards had the same size, about 3500 m² (700 m² per block).

More analytically, all vineyards of Soultanina cultivar were chosen to be tillaged, irrigated, and with the same trellis system (V-type) (Plate A.3), while all vineyards of Kotsifali cultivar were chosen to be tillaged, not-irrigated and with the same trellis system (vertical-type) (Plate A.4). All the vineyards of each area were chosen to be at the same as possible altitude, with an average altitude of 430 m for area A, 205 m for area B and 250 m for area C.

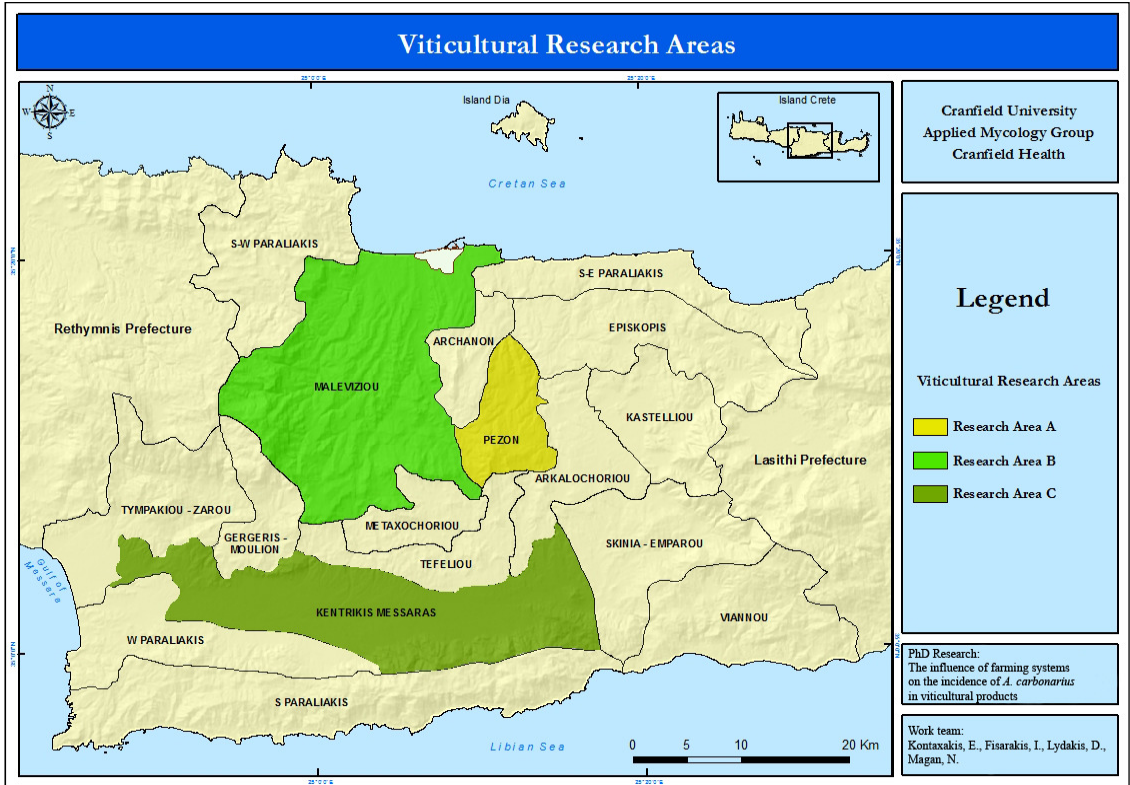


Plate 2.1. Map of viticultural research areas in Heraklion prefecture.



Plate 2.2. Satellite view of SBI3 vineyard (GeoEye, NOAA, U.S. Navy, GEBCO, 2011).

Table 2.1. Experimental vineyards of the first year project.

S/N	Code	Area	Cultivar	Farming System
1	KAC1	A - Peza	Kotsifali	Conventional
2	KAC2	A - Peza	Kotsifali	Conventional
3	KAC3	A - Peza	Kotsifali	Conventional
4	KAI1	A - Peza	Kotsifali	Integrated
5	KAI2	A - Peza	Kotsifali	Integrated
6	KAI3	A - Peza	Kotsifali	Integrated
7	KA01	A - Peza	Kotsifali	Organic
8	KA02	A - Peza	Kotsifali	Organic
9	KA03	A - Peza	Kotsifali	Organic
10	SBC1	B - Malevizi	Soultanina	Conventional
11	SBC2	B - Malevizi	Soultanina	Conventional
12	SBC3	B - Malevizi	Soultanina	Conventional
13	SBI1	B - Malevizi	Soultanina	Integrated
14	SBI2	B - Malevizi	Soultanina	Integrated
15	SBI3	B - Malevizi	Soultanina	Integrated
16	SBO1	B - Malevizi	Soultanina	Organic
17	SBO2	B - Malevizi	Soultanina	Organic
18	SBO3	B - Malevizi	Soultanina	Organic
19	SCC1	C - Messara	Soultanina	Conventional
20	SCC2	C - Messara	Soultanina	Conventional
21	SCC3	C - Messara	Soultanina	Conventional
22	SCI1	C - Messara	Soultanina	Integrated
23	SCI2	C - Messara	Soultanina	Integrated
24	SCI3	C - Messara	Soultanina	Integrated
25	SCO1	C - Messara	Soultanina	Organic
26	SCO2	C - Messara	Soultanina	Organic
27	SCO3	C - Messara	Soultanina	Organic

According to the first year research results, the project was redesigned for the next two years research. For the new project eight vineyards were chosen in each of the three areas of research (24 vineyards totally), cultivated under the Organic (OFS) and Integrated (IFS) farming systems, while the Conventional Farming System was abandoned, as the results of the first year, were very variable with no discernible patterns.

Eight vineyards of Kotsifali cultivar in Area A (Peza), eight vineyards of Soultanina cultivar in Area B (Malevisi) and eight vineyards of Soultanina cultivar in Area C (Messara) were selected. Those cultivars, Kotsifali and Soultanina, are the main wine and table grape cultivars, grown respectively in the aforementioned areas (Figure 2.1 and Table 2.2).

Table 2.2. Experimental vineyards of 2011-2012 project.

S/N	Code	Area	Cultivar	Farming System
1	KA01	A - Peza	Kotsifali	Organic
2	KA02	A - Peza	Kotsifali	Organic
3	KA03	A - Peza	Kotsifali	Organic
4	KA04	A - Peza	Kotsifali	Organic
5	KAI1	A - Peza	Kotsifali	Integrated
6	KAI2	A - Peza	Kotsifali	Integrated
7	KAI3	A - Peza	Kotsifali	Integrated
8	KAI4	A - Peza	Kotsifali	Integrated
9	SBO1	B - Malevizi	Soultanina	Organic
10	SBO2	B - Malevizi	Soultanina	Organic
11	SBO3	B - Malevizi	Soultanina	Organic
12	SBO4	B - Malevizi	Soultanina	Organic
13	SBI1	B - Malevizi	Soultanina	Integrated
14	SBI2	B - Malevizi	Soultanina	Integrated
15	SBI3	B - Malevizi	Soultanina	Integrated
16	SBI4	B - Malevizi	Soultanina	Integrated
17	SCO1	C - Messara	Soultanina	Organic
18	SCO2	C - Messara	Soultanina	Organic
19	SCO3	C - Messara	Soultanina	Organic
20	SCO4	C - Messara	Soultanina	Organic
21	SCI1	C - Messara	Soultanina	Integrated
22	SCI2	C - Messara	Soultanina	Integrated
23	SCI3	C - Messara	Soultanina	Integrated
24	SCI4	C - Messara	Soultanina	Integrated

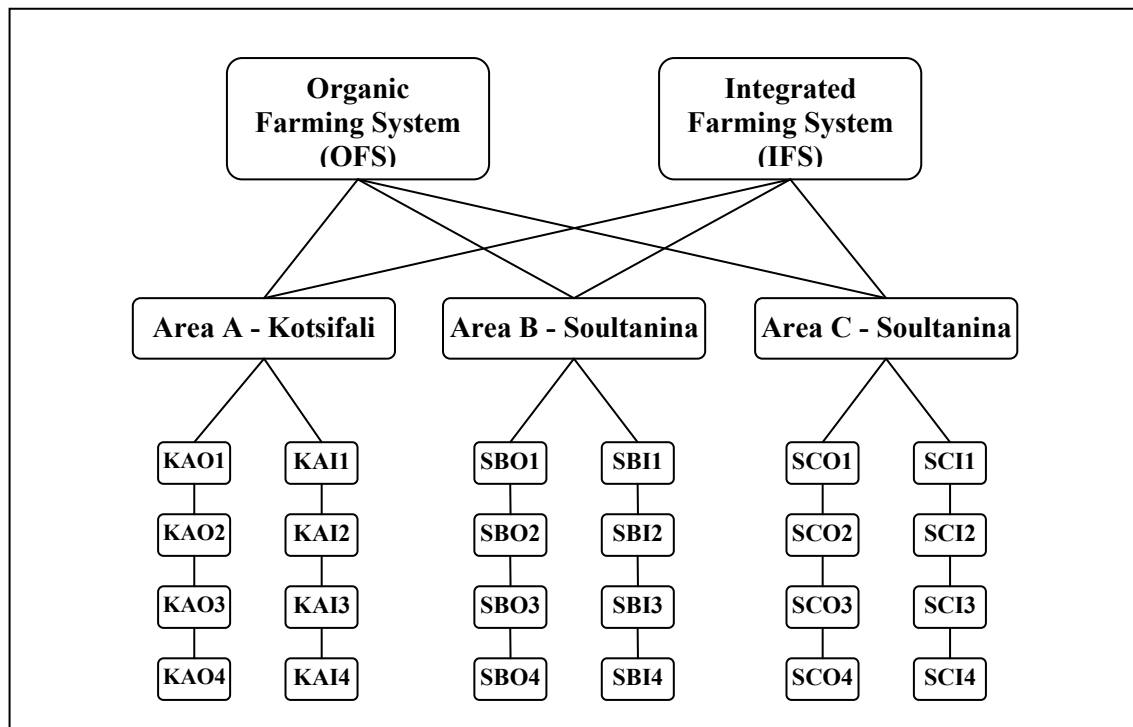


Figure 2.1. Experimental plan of Vineyards in the three areas of research.

Kotsifali (*Vitis vinifera* L. cv. Kotsifali) is a colour local wine cultivar, grown mainly in Heraklion Prefecture, which gives excellent quality wines, while it's co-vinification with Mandilari cultivar gives the V.Q.P.R.D. wines "Peza" and "Archanes". Soultanina (*Vitis vinifera* L. cv. Soultanina) is a white table cultivar, which represents the 80% of total grape growing in Heraklion Prefecture, of which the 47.5% is in Malevisi and the 12.66% in Messara viticultural zones (areas). Soultanina grapes are used for table grapes (main cultivar of Crete exports), raisins and in some cases for wine making (Fisarakis *et al.*, 2008).

2.2. MYCOLOGICAL ANALYSIS OF GRAPES

2.2.1. Grape Sampling

Grape berry samples of about 200 g were taken from each of five blocks of all the vineyards, in the stage of maturity, a few days before harvest. The samples were put in sterilized bags, and these were placed in an electric portable refrigerator to keep them cool for transport to the laboratories. The samples were kept in appropriate conditions (2-3°C) until their elaboration (<1 days). For mycological analysis, only intact, without obvious infections and insect injured berries were used.

2.2.2. Mycological analysis methods

For the mycological analysis of grapes two methods were used: the Direct Plating and the Dilution Plating (Serial Dilutions) method.

The identification of all fungus species and genera isolated from the directly plated berries and from the washings of the berries were determined after seven days incubation, according to Pitt and Hocking (2009), by microscopic and stereoscopic observation based mainly on morphologic characteristics (McClenny, 2005). These included colour, shape, and size of the colonies, conidiophores, conidia and reference made to type cultures of the different species. For the further distinguishing of some fungal species the isolates were cultured on Czapek Yeast Extract medium and identified according to Pitt and Hocking (2009), due to their specific colony shape and their spore morphology.

The results of direct plating method are reported as the percentage of isolation (%) of the fungal species and genera from the berries, while the fungal species and genera in the serial dilution plating method are reported as Colony Forming Units (CFUs) g⁻¹ of berries.

Additional to optical identification of *A. carbonarius*, all isolates were cultivated in a semi-selective medium (Malt Extract Agar – Boscalid [MEA-B]) (Pollastro *et al.*, 2006), as a confirmation of the identification. That medium allows the growth of *A. carbonarius* and prevents the growth of other *Aspergillus spp.* (Plate 2.3).

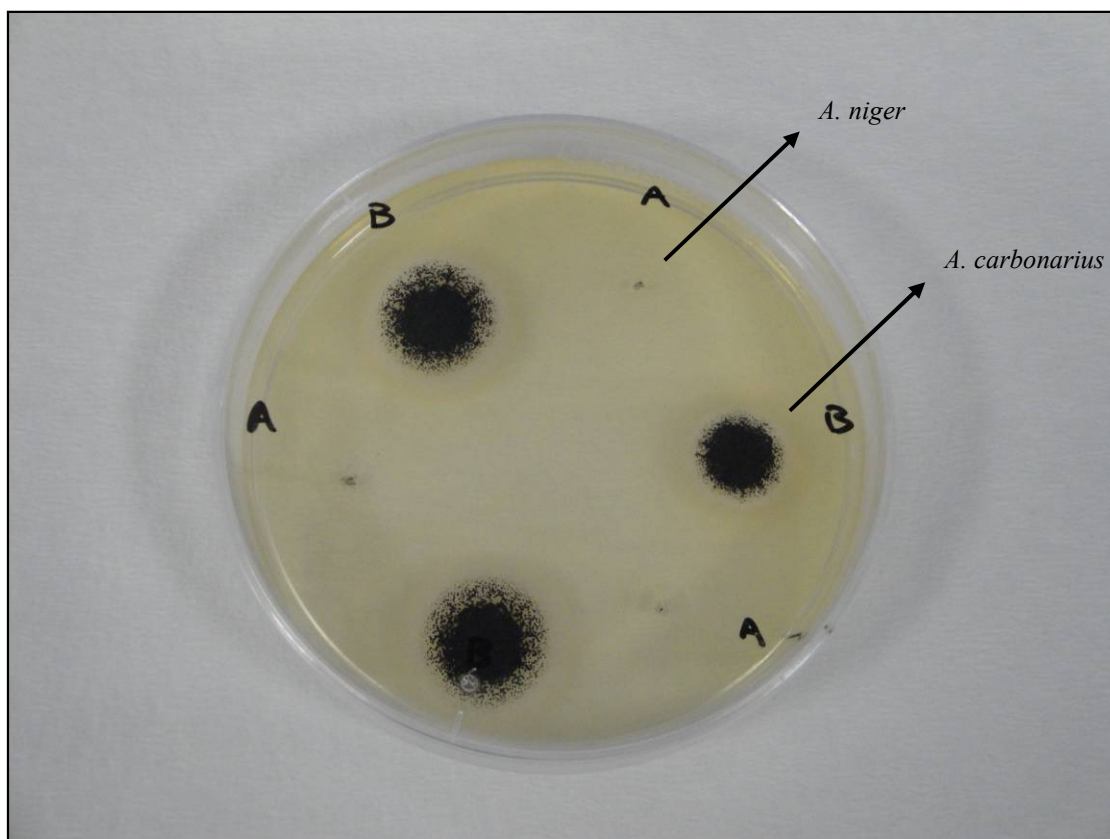


Plate 2.3. *A. niger* (A) and *A. carbonarius* (B) cultivation on MEA-B semi-selective medium, where only *A. carbonarius* had the ability to grow.

2.2.2.1. Direct plating method

In direct plating, grape berries are placed directly on the surface of nutrient media. The results from direct plating method are expressed as a percentage infection of berries. The technique does not give direct evidence of the extent of fungal invasion in individual berries. However, it is reasonable to assume that a high rate of infection is associated with an extensive invasion of the berries and a higher risk of developing OTA contamination (Pitt and Hocking, 2009).

In this study ten half berries were randomly selected from each sample, plated onto the MEA 0.96 a_w nutrient medium in Petri plates, and incubated in the dark at 25 °C for seven days.

2.2.2.2. Serial dilution plating method

Dilution plating was suitable to obtain information of the total fungal populations and that of individual fungal genera/species isolated from, washings of the berries. These were presented as Colony Forming Units (CFUs) g^{-1} of berries. For these studies 50 g grape berries, from each sample, were weighed and suspended in 150 ml sterile 0.1 % peptone solution and homogenized in a Seward Stomacher[®] 80, at the normal speed for 120 sec. The mixture was serially diluted in sterile 0.1% peptone solution (decimal), and 0.2 ml of each dilution transferred with a plunger-operated pipette (Eppendorf Research[®], Germany) and spread onto the surface of MEA 0.96 a_w medium in 3 Petri plates (3 replications), with a sterile glass spreader. The cultures were incubated in the dark, at 25 °C for seven days.

2.3 MYCOLOGICAL ANALYSIS OF SOIL

Given that the soil is the main source of contamination of vine parts by *Aspergillus* and other fungal species, it was important to determine any effect of the farming system on populations present in the soil. Thus small soil samples were serially diluted as described previously and spread plated onto the agar media.

2.3.1. Soil Sampling

A sample of 1 Kg soil (5 sub-samples of 200 g), were taken from each of the 24 vineyards (Organic and Integrated Farming Systems), in the three areas of research, beneath vines to a depth of 0-5 cm.

The samples were left to dry at ambient temperature, in the dark, for 48 hours and then homogenized and sieved through a 1.8 mm soil sieve.

2.3.2. Mycological analysis method

A representative sample of 10 g soil was mixed with 90 ml sterile 0.1 % peptone solution, and homogenized in a Seward Stomacher[®] 80, at normal speed for 60 sec. The mixture was serially diluted in sterile 0.1% peptone solution (decimal), and 0.1 ml of each dilution transferred with a plunger-operated pipette (Eppendorf Research[®], Germany) and spread onto the surface of Dichloran Rose Bengal Chloramphenicol Agar (DRBC) medium in three Petri plates (3 replications) with a sterile glass spreader. The cultures were incubated in the dark, at 25 °C for seven days.

Although only about 17 % of the known fungal species, are able to grow in culture (Bridge and Spooner, 2001), the fungal species that have concern in our study, can be grown successfully in a wide range of culture media.

In this study, Dichloran Rose Bengal Chloramphenicol Agar (DRBC) medium was used, as all dominant fungal species could growth successfully, but also for its ability to reduce the size of colonies, which resulted in easier identification and enumeration of colonies (Plate 2.4).

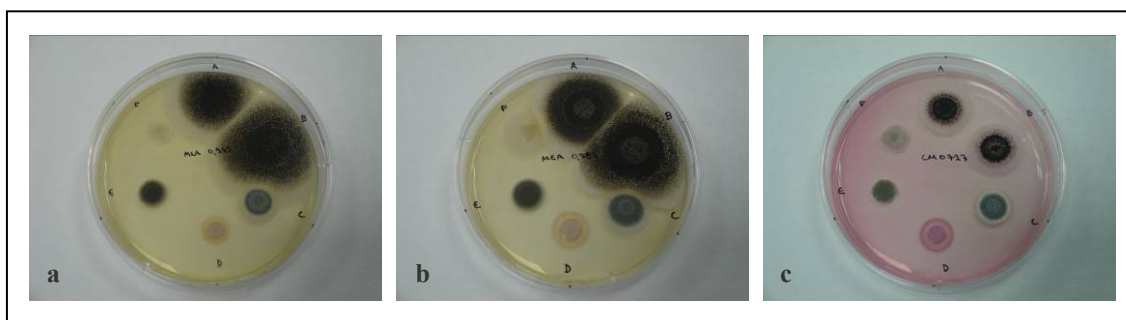


Plate 2.4. Dominant fungal species grown on different media and at different water activities (a_w) [a. Malt Extract Agar (0.965), b. Malt Extract Agar (0.982), c. DRBC (CM0727 - Oxoid)].

Similar to mycological analysis of grapes, after incubation, fungal identification and enumeration of colonies was carried out, by microscopic and stereoscopic observation based on morphologic characteristics, and the results expressed as Colony Forming Units (CFUs) g^{-1} of soil.

2.4. EVALUATION OF OCHRATOXIN A PRODUCTION ABILITY OF *A. CARBONARIUS* ISOLATES

Aspergillus carbonarius isolates from the mycological analysis of grapes and soil, were tested for their ability to produce Ochratoxin A. All isolates were cultivated on Coconut Cream Agar (CCA 50%) medium, and incubated in the dark, at 25 °C for seven days. The reverse side of cultures was examined for blue-fluorescence (due to toxins) under UV light (365 nm) (Davis *et al.*, 1987; Dyer and McCammon, 1994).

2.5. PHYSICOCHEMICAL ANALYSIS OF GRAPES

2.5.1. Sugar content of grape samples

The sugar content of grape samples juice, were measured by an automatic electronic refractometer (ATAGO CO. LTD, Japan) and the results reported as the percentage of sugars in the juice (°Brix).

2.5.2. Total Acidity of grape samples

For the total acidity determination, 5 ml of grape juice were placed into 250 ml Erlenmeyer flask, with 10 ml distilled water and 5 drops bromothymol blue indicator, followed by titration with 0.1 N NaOH until the colour change endpoint. The result was expressed as tartaric acid (g l⁻¹), calculated according to the following formula:

$$R(g/l) = \frac{V(titr) \times C(titr) \times 150.09}{V(samp) \times 2}$$

$R(g/l) = \text{Tartaric Acid } (g\ l^{-1})$

$V(\text{titr}) = \text{total volume (ml) of titrant to reach the endpoint}$

$C(\text{titr}) = \text{titrant concentration in eq } l^{-1} \text{ (currently 0.1)}$

$V(\text{samp}) = \text{sample volume (ml)}$

$150.09 = \text{molecular weight of tartaric acid } [HOOC(CHOH)_2COOH]$

$2 = \text{Ratio between titrant and sample (2 titrants react with 1 sample)}$

2.5.3. pH of grape samples

The pH of the grape samples juice was measured by an electronic pH-meter (Crison Instruments S.A., Spain).

2.6. EFFECT OF CROP PROTECTION PRODUCTS ON *ASPERGILLUS CARBONARIUS* GROWTH

A wide range of products are used in viticulture against diseases, insect enemies and nutrition disorders. A large number of them, mostly chemical compounds, are available for use in integrated farming. In organic farming systems, only a few products are approved for use, mainly sulphur and copper compounds, as well as organic compounds from algal and fungal extracts, with amino-acids and microelements, mainly to enhance plant resistance.

In order to evaluate the influence on *A. carbonarius* incidence of various spraying applications in vineyards, with chemicals, organic compounds and plant growth regulators, the most common substances used by the producers mainly against other

fungi, such as *Plasmopara viticola*, *Uncinula necator*, *Botrytis cinerea*, *Phomopsis viticola* and *Elsinoe ampelina* were selected.

Twenty three chemical fungicides used only in integrated farming system, and seven fungicides and organic compounds approved for use in organic farming systems (one brand name per active substance) were tested for their impact on *A. carbonarius* growth. Sodium bicarbonate was tested, as it is used, in some cases, against *Uncinula necator* in organic farming systems, although it is not a fungicide registered for use. Gibberellic acid, a plant growth regulator, commonly used in table grapes vineyards for increasing the size of berries, was also tested (Table 2.3).

Two ochratoxigenic strains of *A. carbonarius*, BA1211 and BB1111 (held in Plant Pathology laboratory of Technological Educational Institute of Crete), isolated from grapes of the experimental vineyards, were used to inoculate centrally, with a dilution of 10^6 spores ml^{-1} , Petri dishes with 20 ml MEA 0.98 a_w medium containing the fungicides and other products that were tested.

The MEA medium, autoclaved at 121 °C for 15 minutes, and left until the temperature fell to 50 °C. All substances were added to the medium aseptically, in the doses recommended by the manufactures (table 2.3), homogenized and plated to the petri dishes. Where a range of recommended doses was given, the higher dose was used. Pure Malt Extract Agar medium was used for the control. Both of *A. carbonarius* strains were cultured in triplicate. The cultures were incubated in the dark at 25 °C for seven days, and the diameters of colonies were measured after three, five and seven days.

Table 2.3. Crop protection products tested, their active substances, the manufacture company, the target of use and their recommended doses.

Code	Product Name	Active Substance(s)	Producer	Target	Organic	Dose 100 L ⁻¹
S1	ACROBAT 7,5/66,7 WG	Dimethomorph / Mancozeb	BASF	<i>Plasmopara viticola</i>	-	200-250 g
S2	ALIETTE 80 WG	Fosetyl-Al	Bayer CropScience	<i>Plasmopara viticola</i>	-	250-300 g
S3	ALPER 4/64 WP	Cymoxanil / Maneb	DuPont	<i>Plasmopara viticola</i>	-	250 g
S4	ANTRACOL 70 WP	Propineb	Bayer CropScience	<i>Plasmopara viticola</i>	-	200 g
S5	ANTRACOL COMBI X 65/2 WP	Propineb / triadimenol	Bayer CropScience	<i>Plasmopara viticola</i> / <i>Uncinula necator</i>	-	200 g
S6	BAYFIDAN 250 EC	Triadimenol	Bayer CropScience	<i>Uncinula necator</i>	-	10-20 ml
S7	BORDEAUX MIXTURE 50 WP	Calcium copper sulphate	Agrochemicals of Crete S.A.	<i>Plasmopara viticola</i> / <i>Elsinoe ampelina</i>	+	550 - 650 g
S8	COLLIS 20/10 SC	Kresoxim - methyl / Boscalid	BASF	<i>Uncinula necator</i>	-	40 ml
S9	COPPER OXYCHLORIDE 50 WP	Copper oxychloride	Agrochemicals of Crete S.A.	<i>Plasmopara viticola</i> / <i>Elsinoe ampelina</i>	+	260 g
S10	DITHANE M-45 80 WP	Mancozeb	Dow AgroSciences	<i>Plasmopara viticola</i>	-	200 g
S11	DOURO 10 EC	Penconazole	SAPEC AGRO S.A.	<i>Uncinula necator</i>	-	25 - 40 ml
S12	EQUATION PRO 22,5/30 WG	Famoxadone / Cymoxanil	DuPont	<i>Plasmopara viticola</i>	-	40 g
S13	FLINT 50 WG	Trifloxystrobin	Bayer CropScience	<i>Uncinula necator</i>	-	12.5 g
S14	FOLICUR 250 EW	Tebuconazole	Bayer CropScience	<i>Uncinula necator</i>	-	40 ml
S15	KARATHANE STAR 35 EC	Meptyldinocap	Dow AgroSciences	<i>Uncinula necator</i>	-	60 ml
S16	KUMULUS 80 WG	Sulfur	BASF	<i>Uncinula necator</i>	+	100 - 400 g
S17	KU-RAME	SO ₃ , Cu, K ₂ O, Fe, B, Aminoacids	IKO-HYDRO SRL	Plant mechanical resistance	+	400 - 600 g
S18	MILDEX 4,44/66,7 WG	Fenamidone / Fosetyl-Al	Bayer CropScience	<i>Plasmopara viticola</i>	-	250 g
S19	NIMROD 25 EC	Bupirimate	ALFA AGR. SUPPL. S.A.	<i>Uncinula necator</i>	-	150 - 180 ml
S20	POLYRAM 80 WG	Metiram	BASF	<i>Plasmopara viticola</i> / <i>Phomopsis viticola</i>	-	150 - 200 g
S21	PROFILER 71.1 WG	Fluopicolide / Fosetyl-Al	Bayer CropScience	<i>Plasmopara viticola</i>	-	250 - 300 g
S22	PROSPER 500 EC	Spiroxamine	Bayer CropScience	<i>Uncinula necator</i>	-	60 ml
S23	RENOVATION LADA	Algae extract	Agrolaboratorios nutricionales	Plant growth regulator	+	300 ml
S24	RIDOMIL GOLD MZ 68 WG	Metalaxyl M / Mancozeb	Syngenta	<i>Plasmopara viticola</i>	-	200 g
S25	ROVRAL 75 WG	Iprodione	BASF	<i>Botrytis cinerea</i>	-	100 g
S26	SCALA 40 SC	Pyrimethanil	BASF	<i>Botrytis cinerea</i>	-	150 - 200 ml
S27	SODIUM BICARBONATE	NaHCO ₃		<i>Uncinula necator</i>	+	400 - 500 g
S28	SWITCH 25/37.5 WG	Fludioxonil / Cyprodinil	Syngenta	<i>Botrytis cinerea</i> / <i>Aspergillus spp.</i>	-	80 - 100 g
S29	SYSTHANE 24 EC	Myclobutanil	Dow AgroSciences	<i>Uncinula necator</i>	-	8 - 12 ml
S30	TALOCUPER	Copper 5.6%	LIDA	Nutriprotection / Plant resistance increase	+	100 - 150 ml
S31	VIO-GIBB 5 SL	Gibberilic Acid	VIORYL	Plant growth regulator	+	120 ml

+ Approved use in organic farming system, - Not approved in organic farming system

2.7. EXAMINATION OF ESSENTIAL OILS INFLUENCE ON *A. CARBONARIUS* GROWTH

In the framework of organic control of *A. carbonarius*, mainly post-harvest, because of the difficulty in applying in the field, five essential oils were tested for their ability to prevent or reduce the *A. carbonarius* growth, preferably post-harvest.

Essential oils were extracted by hydrodistillation for three hours using a Clevenger type apparatus (Clevenger, 1928), from *Eucalyptus globulus*, *Salvia officinalis*, *Laurus nobilis*, *Origanum vulgare* and *Origanum dictamnus* (dittany, Crete endemic herbal).

Two ochratoxigenic strains of *A. carbonarius*, BA1211 and BB1111 (held in Plant Pathology laboratory of Technological Educational Institute of Crete), isolated from the experimental vineyards, were used as an inoculum. With using a concentration of 10^6 spores ml^{-1} , the Petri dishes containing 20 ml MEA 0.98 a_w medium + essential oils were inoculated centrally. Four concentrations of each essential oil (25 ppm, 75 ppm, 150 ppm and 250 ppm) were tested and compared with the controls.

The MEA medium was autoclaved at 121 °C for 15 minutes. The essential oils were added aseptically when the medium cooled (< 50 °C) in order to not evaporate, and the media poured into 9 cm Petri dishes. Controls were used excluding the essential oil treatments. Both of *A. carbonarius* strains were cultured in triplicate. The cultures incubated in the dark at 25 °C for seven days, and the diameters of the colonies were measured after three, five and seven days.

2.8. FARMING SYSTEM INFLUENCE IN OCHRATOXIN A OCCURRENCE IN WINE

In order to evaluate the farming system influence in OTA occurrence in wine, 20 wines from grapes of organic farming system vineyards and 20 from grapes of integrated farming system vineyards were analyzed. All the wines, independent of the farming system were consisted of Kotsifali cultivar 100% or co-vinificated Kotsifali and Mandilari cultivars, 80% and 20% respectively (according to the legislation for the VQPRD wines Peza). The samples were collected from the wineries of Peza region (Area A) of Heraklion Prefecture (Crete, Greece). Representative wine samples (according to the European Commission Regulation (EC) No 401/2006) were taken from the tanks of wineries before bottling, the harvest years 2011 and 2012.

2.8.1. Wine samples preparation and immunoaffinity column clean-up

A modified method of direct clean-up method of Stefanaki *et al.* (2003), according and the instructions of Immunoaffinity Columns (IAC) manufacturer (Romer Labs Inc., USA), was used for the analysis of the wine.

The wine samples were filtered through Whatman filter papers. Ten millilitres of the filtered wine were diluted with ten millilitres of Phosphate Buffer Saline (PBS) [P4417 - Sigma-Aldrich, Germany], and the mixture was shaken vigorously for one minute. The pH of the mixture was measured and corrected to 7.4 with NaOH. Ten millilitres of the mixture were passed through the immunoaffinity columns (Ochrastar IAC, Romer Labs Inc., USA) with pressure on the top, using an Immunoaffinity Column Accessory Pack (R-Biopharm Rhone Ltd, Scotland), at a flow rate of 1 ml min⁻¹.

After the dilution completely passed through, the container and the columns washed twice with 10 ml of PBS (2 x 10 ml), while any remaining liquid, removed by air pressure on the top of the column.

Ochratoxin A was eluted with 1.5 ml of methanol / acetic acid 98:2 (v/v) (three portions of 0.5 ml). During the elution, three times back flushing of desorption solution was used to ensure complete elution of the Ochratoxin A. The eluate was collected, dried in a water bath at 40 °C under nitrogen and redissolved immediately in 500 µl of mobile phase before HPLC analysis.

2.8.2. Ochratoxin A standard solution

Crystalline powder of Ochratoxin A standard was purchased from Sigma-Aldrich, USA (O1877). The crystalline standard was reconstituted with benzene : acetic acid (99:1) v/v to give a stock concentrate of approximately 1 mg ml⁻¹, and left overnight at room temperature to ensure the completely dissolution of OTA. The OTA concentration of the standard was determined by measurement of the absorbance of the solution at 333 nm, using a UV spectrophotometer, blanked against benzene : acetic acid (99:1) v/v, in triplicate.

The calculation of the concentration of the Ochratoxin A was calculated using the following equation: [OTA], µg ml⁻¹ = (Abs (333nm) x Molecular Weight x 1000) / OTA Extinction coefficient = (Abs (333nm) x 403.81 x 1000) / 5550 = Abs (333nm) x 72.76. The standard dilutions that used for the calibration curves, were prepared by dilution of the aforementioned standard, with (methanol : acetic acid, 98:2) - water (1:1) v/v.

2.8.3. High Performance Liquid Chromatography coupled with Fluorescence Detector (HPLC – FLD) method

Ochratoxin A analysis was performed using reverse-phase High Performance Liquid Chromatography with fluorescence detection, consisted of an Agilent 1200 Degasser (G1379B), an Agilent 1200 Bin Pump (G1312A), an Agilent 1200 Autosampler (G1329A), an Agilent 1200 Thermostatted Column Compartment (G1316A) and an Agilent 1200 Fluorescence Detector (G1321A).

The samples were separated using an Agilent Zorbax Eclipse XDB-C18 column (150 mm x 4.6 mm, 5 μ m). An excitation wavelength of 333 nm and an emission wavelength at 477 nm were used for UV detection. The mobile phase consisted of acetonitrile : water : acetic acid (49 : 49 : 2) v/v, and the flow rate was 1 ml min⁻¹. Run time for samples was 10 minutes with OTA being detected at about 5 min (Figure A.1 – appendix A). The limit of quantification (LOQ) was estimated at 0.05 μ g l⁻¹. Several standard solutions from 0.1 to 5 ng 3 ml⁻¹ (0.033 to 1.666 μ g l⁻¹) used in order to build a calibration curve.

2.8.4. IAC clean-up and HPLC solvents

The methanol (34860 - Sigma-Aldrich, Germany), the acetic acid glacial (AC0346 - Scharlau, Spain), the Benzene (270709 – Sigma Aldrich, Germany), the Acetonitrile (34851 – Sigma Aldrich, Germany) and the Water (34877 – Sigma Aldrich, Germany), used for IAC clean-up, OTA standard solutions and HPLC analysis, were HPLC grade.

2.9. NUTRIENT MEDIA AND REAGENTS

2.9.1. Malt Extract Agar (MEA)

- 50 g Malt Extract Agar (Sigma-Aldrich - 70145 Fluka, Germany)
- 0.1 g Chloramphenicol (Sigma-Aldrich - C0378, Germany)
- Glycerol (Sigma-Aldrich - 33224, Germany)
- 1 l distilled water

*Sterilized by autoclaving at 121 °C for 15 min.

**The amount of glycerol added for the modification of media water activity (a_w) was established according to Dallyn and Fox (1980) (Table 2.4).

Table 2.4. Modification of media water activity by adding glycerol (Dallyn and Fox, 1980).

Water Activity (a_w)	Glycerol (g ml ⁻¹ water)
0.995	0.0276
0.991	0.0461
0.982	0.0920
0.971	0.1472
0.964	0.1840
0.955	0.2300
0.937	0.3220
0.928	0.3680
0.919	0.4140
0.910	0.4600
0.901	0.5060
0.892	0.5520
0.884	0.5980
0.867	0.6900
0.850	0.7820

2.9.2. Dichloran Rose-Bengal Chloramphenicol Agar (DRBC)

- 15.75 g DRBC AGAR BASE (Oxoid - CM0727, Cambridge, UK)
- 0.1 g Chloramphenicol (Sigma-Aldrich - C0378, Germany)
- 0.5 l distilled water

*Sterilized by autoclaving at 121 °C for 15 min.

2.9.3. Coconut Cream Agar (CCA 50%)

- 15 g Agar-Agar Purified (Merck - 1.01614.1000, Germany)
- 500 g Coconut Cream (Blue Dragon Brand, UK)
- 500 ml distilled water

*Sterilized by autoclaving at 121 °C for 15 min.

2.9.4. Malt Extract Agar – Boscalid (MEA-B)

- 50 g Malt Extract Agar (Sigma-Aldrich - 70145 Fluka, Germany)
- 0.1 g Chloramphenicol (Sigma-Aldrich - C0378, Germany)
- 10 mg Boscalid (Cantus 50WG, BASF)
- Glycerol (Sigma-Aldrich - 33224, Germany)
- 1 l distilled water

*Sterilized by autoclaving at 121 °C for 15 min.

**The boscalid added to medium aseptically, after autoclaving (<50 °C).

2.9.5. Peptone 1% (Diluent)

- 1 g Mycological Peptone (Sigma-Aldrich - 77199 Fluka, Germany)
- 0.005% Tween 80 (Sigma-Aldrich - P1754, Germany)
- 1 l distilled water

*Sterilized by autoclaving at 121 °C for 15 min

2.9.6. Czapek Yeast Extract Agar (CYA)

- K_2HPO_4 1 g
- Czapek concentrate 10 ml
- Trace metal solution 1 ml
- Yeast extract, powdered 5 g
- Sucrose 30 g
- Agar 15 g
- Water, distilled 1 l

*Sterilised by autoclaving at 121°C for 15 min.

**Final pH = 6.7

2.9.6.1. Czapek concentrate

- NaNO_3 30 g
- KCl 5 g
- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 5 g
- $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1 g
- Water, distilled 100 ml

2.9.6.2. Trace metal solution

- $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.5 g
- $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 1 g
- Water, distilled 100 ml

2.10. STATISTICAL ANALYSIS

For the statistical analysis of the results, Univariate Analysis of Variance (ANOVA), Multivariate Analysis of Variance (MANOVA) and Analysis of Covariance (ANCOVA) were used. One-way ANOVA, was used even for two groups' analysis, instead of T-Test, as it gives exactly the same results (Howitt and Cramer, 2008).

The correlation of the data from the mycological analysis of grapes (dilution plating method) and soil was calculated, in order to establish any correlation between the occurrence of *A. carbonarius* on grapes and in soil, by using Pearson product-moment correlation coefficient measure.

All the statistical analysis and handling of the data performed by Statistical Programme for Society Sciences (IBM SPSS Statistics, version 19). The graph plotting was carried out using Microsoft Excel 2003 and GraphPad Prism 5. The data from the mycological analysis of grapes and soil results were transformed to $\text{Log}_{10} \text{ CFUs g}^{-1}$ prior to analysis in order to homogenise the variance.

CHAPTER THREE

RESULTS

MYCOLOGICAL ANALYSIS OF GRAPES

RESULTS (2010)

DIRECT PLATING METHOD

3.1. FARMING SYSTEM EFFECTS ON THE INCIDENCE OF THE MOST DOMINANT FUNGAL SPECIES AND GENERA

3.1.1. Farming system effects on Kotsifali cultivar vineyards in Area A

Figure 3.1 shows the effect of the farming system on the frequency of isolation of the most dominant fungal species and genera from grape samples of Kotsifali cultivar, Area A vineyards. Multivariate analysis of variance (MANOVA) showed that the effect of the farming system on the incidence of the most dominant species / genera was significant (*Pillai's Trace* = 0.421, $F_{10,78} = 2.080$, $p = 0.036$, *partial eta-square* = 0.210), whereas there was no significant differences in fungal species frequency of isolation among the farming systems [*A. niger* ($F_{2,42} = 0.468$, $p = 0.629$), *A. carbonarius* ($F_{2,42} = 1.534$, $p = 0.227$), *Penicillium spp.* ($F_{2,42} = 1.748$, $p = 0.187$), Yeasts ($F_{2,42} = 1.155$, $p = 0.325$), *Cladosporium spp.* ($F_{2,42} = 2.373$, $p = 0.106$)].

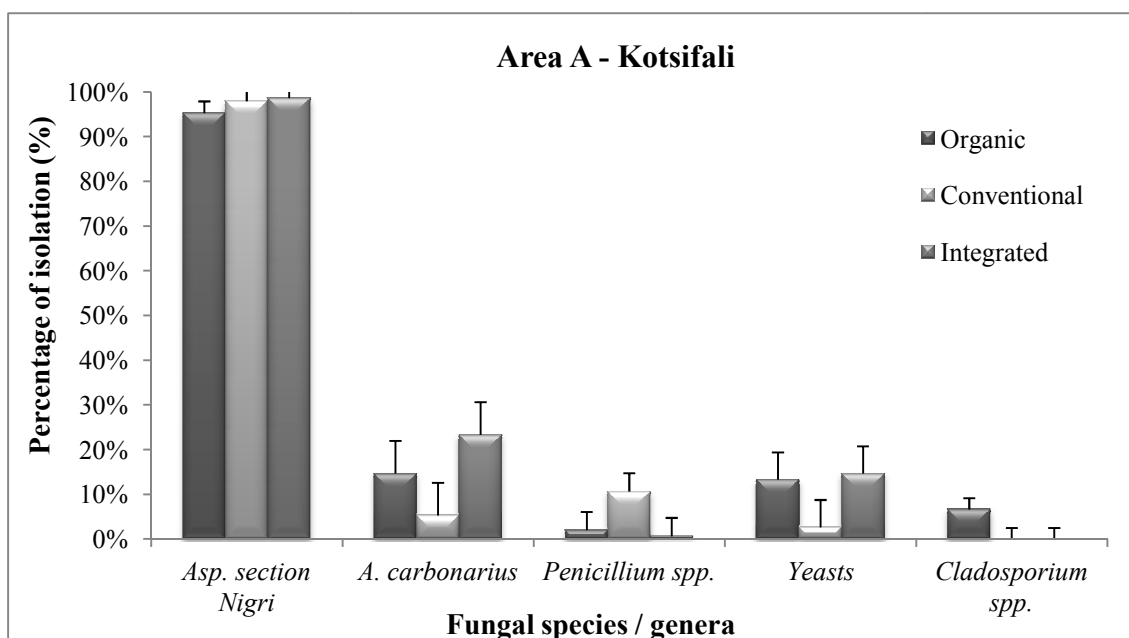


Figure 3.1. Frequency of isolation of the most dominant fungal species / genera on directly plated grape samples on MEA96, from Kotsifali Area A vineyards, from different farming systems. The error bars represent the standard errors of the means.

3.1.2. Farming system effects on Soultanina cultivar vineyards in Area B

Figure 3.2 shows the effect of the farming system on the frequency of isolation of the most dominant fungal species and genera from grape samples of Soultanina cultivar, Area B vineyards. Multivariate analysis of variance (MANOVA) showed that the effect of the farming system to the incidence of the most dominant species / genera was significant (*Pillai's Trace* = 0.495, $F_{6,82} = 4.490$, $p = 0.001$, *partial eta-square* = 0.247), although only *A. carbonarius* ($F_{2,42} = 8.703$, $p = 0.001$) and yeasts had significant differences in incidence among the three Farming Systems

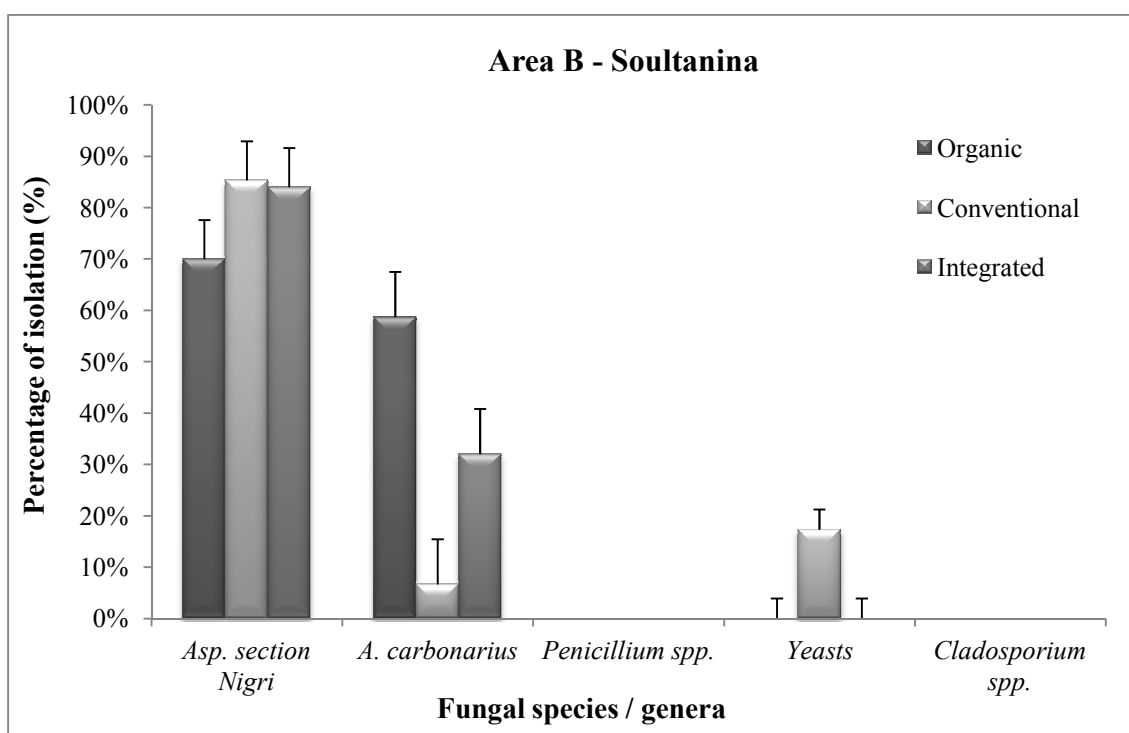


Figure 3.2. Frequency of isolation of the most dominant fungal species / genera on directly plated grapes on MEA96, from the Soultanina Area B vineyards in the three farming systems. The error bars represent the standard errors of the means.

3.1.3. Farming system effects on Soultanina cultivar vineyards in Area C

Figure 3.3 shows the effect of the farming system on the frequency of isolation of the most dominant fungal species and genera from grape samples of Soultanina cultivar, Area C vineyards. Multivariate analysis of variance (MANOVA) showed that the effect of the farming system to the incidence of the most dominant species / genera was significant (*Pillai's Trace* = 0.756, $F_{10,78} = 4.742$, $p = 0.000$, *partial eta-square* = 0.378). *Aspergillus* section *Nigri* species ($F_{2,42} = 6.302$, $p = 0.004$), *Penicillium* spp. ($F_{2,42} = 8.544$, $p = 0.001$) and Yeasts ($F_{2,42} = 7.213$, $p = 0.002$) had significant differences in frequency of isolation among the three farming systems, while *A. carbonarius* ($F_{2,42} = 2.303$, $p = 0.112$) and *Cladosporium* spp. ($F_{2,42} = 0.110$, $p = 0.896$) had no significant differences.

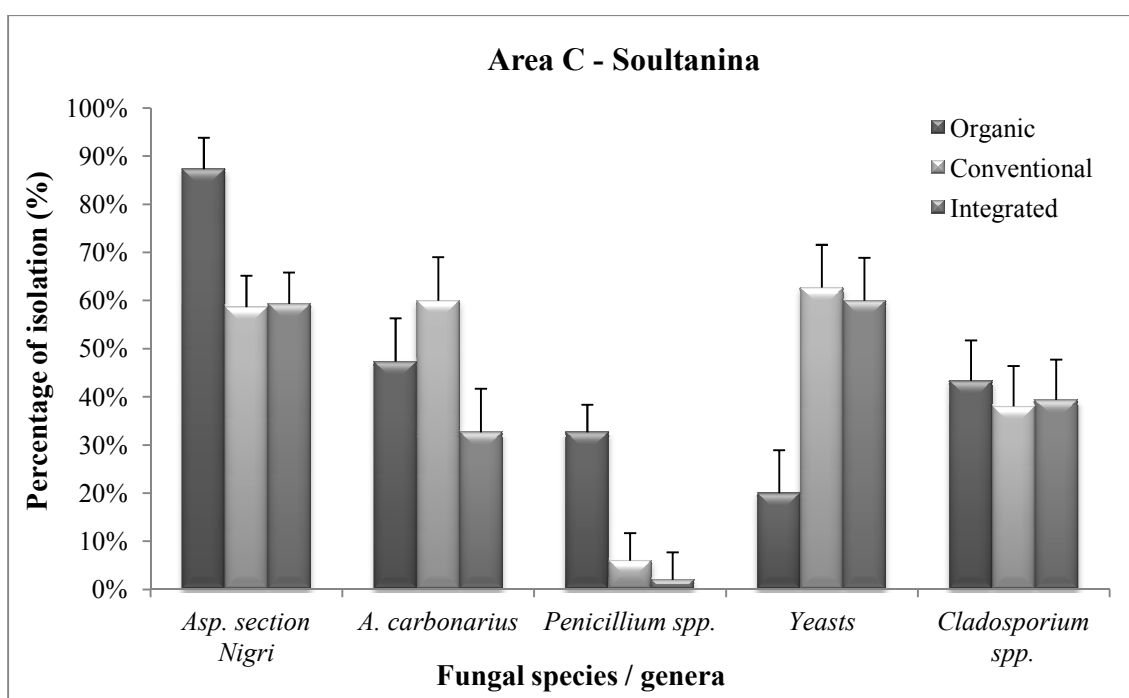


Figure 3.3. Comparison of the frequency of isolation of the most dominant fungal species / genera on directly plated grapes on MEA96, from Soultanina Area C vineyards from the different farming systems. The error bars represent the standard errors of the means.

3.2. INCIDENCE OF *ASPERGILLUS CARBONARIUS* IN THE THREE AREAS OF RESEARCH

Figure 3.4 shows the overall incidence (percentage of isolation %) of *Aspergillus carbonarius* in the three areas of research, regardless of the farming system and the cultivar. The isolation of *A. carbonarius* was more frequent in the grape samples of Area C (46.67%), followed by Area B (32.44%), while Area A had significantly lower incidence of *A. carbonarius* than the other two areas (14.44%).

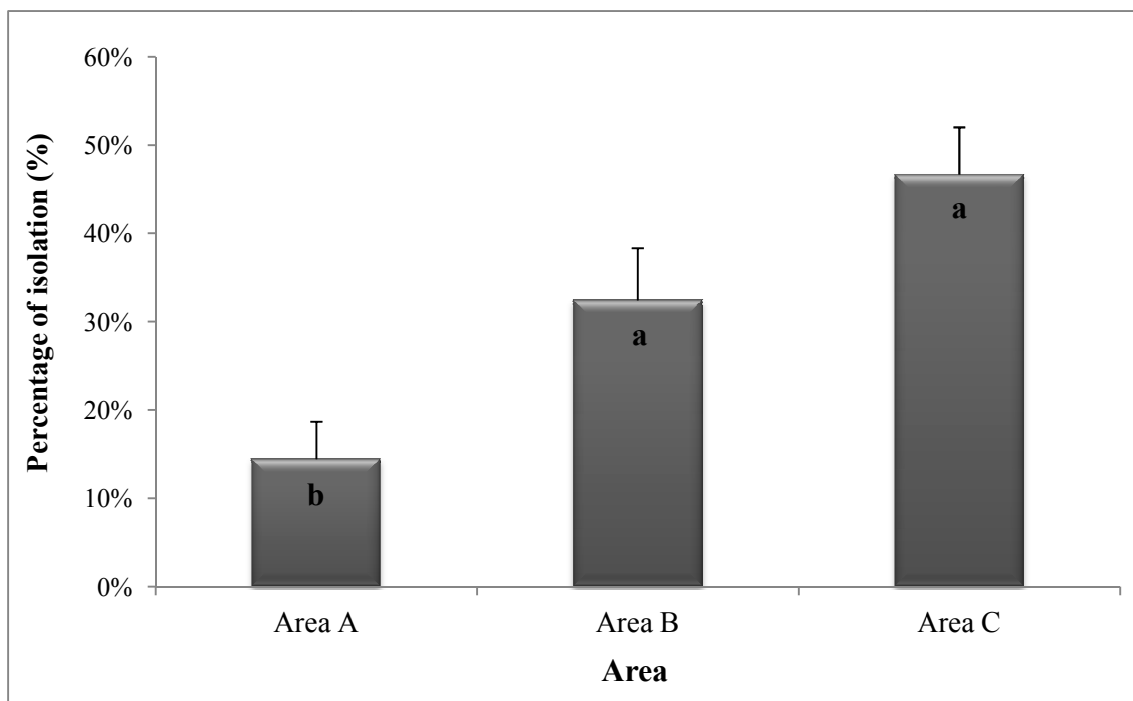


Figure 3.4. Comparison of the frequency of isolation of *A. carbonarius* isolated from directly plated grapes on MEA96, from the three areas of research, regardless of the farming system and the cultivar. The different letters indicate a significant difference of *A. carbonarius* percentage of isolation among the three areas (Duncan's multiple range tests, $p < 0.05$), while one-way analysis of variance (ANOVA) revealed statistically significant differences among the three areas ($F_{2,132} = 9.576$, $p = 0.000$). The error bars represent the standard errors of the means.

3.2.1. Incidence of *Aspergillus carbonarius* in Area A

The Figure 3.5 shows the incidence of *A. carbonarius* in Area A vineyards of Kotsifali cultivar, in relation to the farming system. Although the differences are not statistically significant, the incidence of *A. carbonarius* seems to be higher in integrated farming system (23.33%), followed by organic (14.67%) and conventional (5.33%) farming systems.

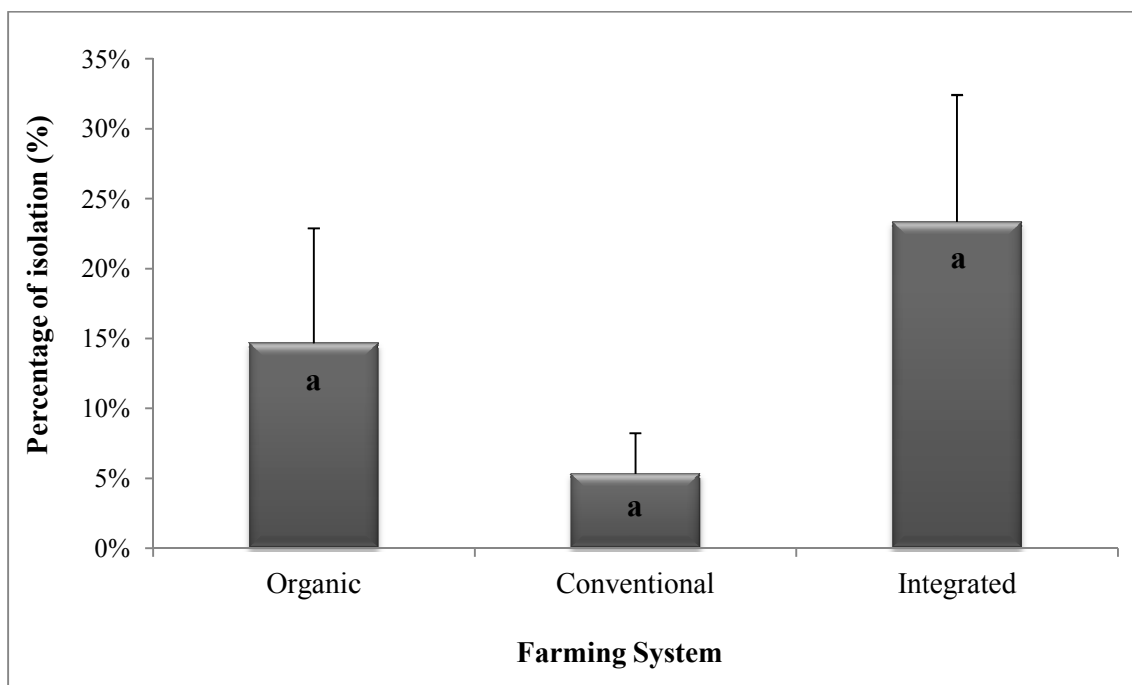


Figure 3.5. Comparison of the frequency of isolation of *A. carbonarius* isolated from directly plated grapes on MEA96, from Kotsifali vineyards of Area A. The same letters indicate no significant difference (Duncan's multiple range tests, $p < 0.05$) among the three farming systems, while one-way analysis of variance (ANOVA) revealed no statistically significant differences ($F_{2,42} = 1.534$, $p = 0.227$). The error bars represent the standard errors of the means.

3.2.2. Incidence of *Aspergillus carbonarius* in Area B

The incidence of *A. carbonarius* in Area B vineyards of Soultanina cultivar in relation to the three farming systems is shown in Figure 3.6. The incidence of *A. carbonarius* was significantly different among the three Farming Systems ($p = 0.001$). The isolation of *A. carbonarius* was more frequent in grape samples from the organic farming system (58.67%), followed by integrated (32%) and conventional (6.67%) systems.

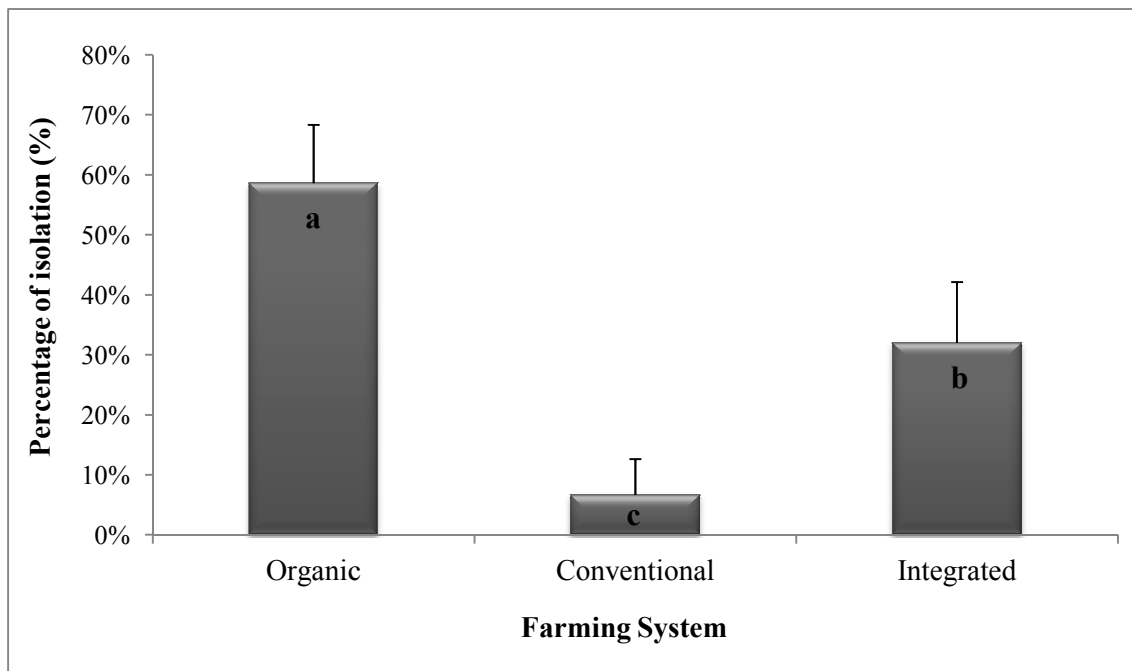


Figure 3.6. Comparison of the frequency of isolation of *A. carbonarius* from grapes directly plated on MEA96 from Soultanina vineyards of Area B grown in the three systems. The different letters indicate significant difference (Duncan's multiple range tests, $p < 0.05$) among the three farming systems, while one-way analysis of variance (ANOVA) revealed statistically significant differences ($F_{2,42} = 8.703$, $p = 0.001$). The error bars represent the standard errors of the means.

3.2.3. Incidence of *Aspergillus carbonarius* in Area C

Figure 3.7 compares the isolation frequency of *A. carbonarius* in Area C vineyards (Soultanina cultivar) grown under the three farming systems. Statistical analysis revealed no statistically significant differences in the incidence of *A. carbonarius* among the three farming systems ($p = 0.112$). However, there was a marked higher frequency of isolation from grape samples of the conventional farming system (60%), followed by organic farming system (47.33%) and the integrated farming system (32.67%).

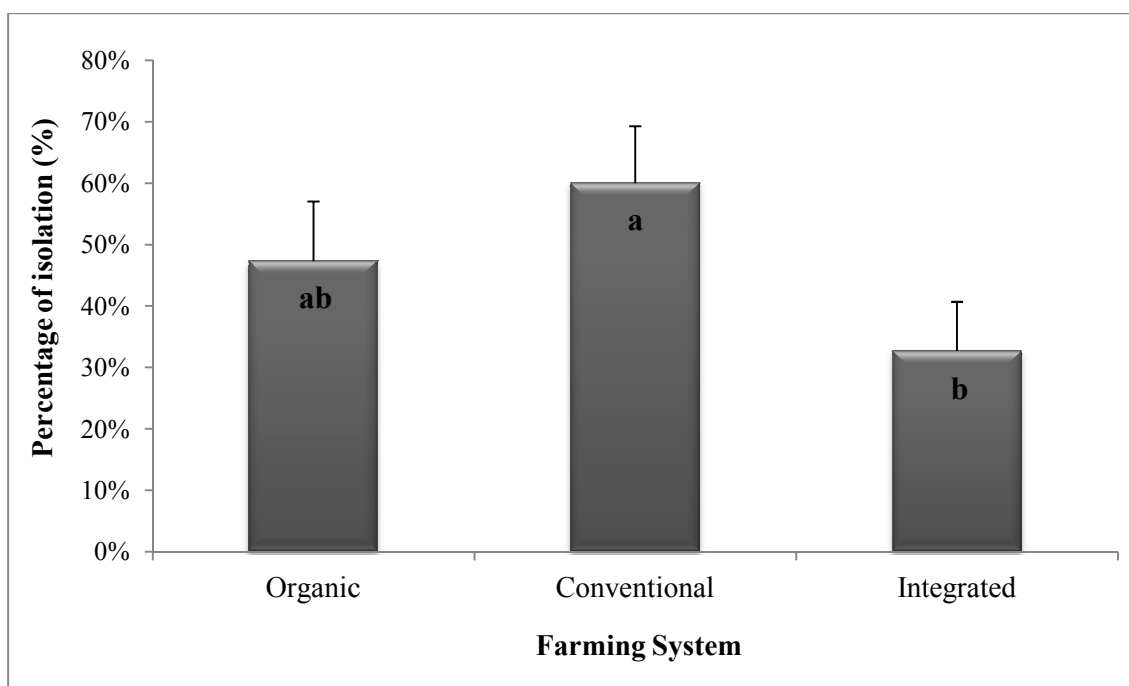


Figure 3.7. Comparison of the frequency of isolation of *A. carbonarius* from directly plated Soultanina grapes from the three farming systems in from Area C. The different letters indicate significant difference (Duncan's multiple range tests, $p < 0.05$) among the three farming systems, whereas one-way analysis of variance (ANOVA) revealed no statistically significant differences ($F_{2,42} = 2.303$, $p = 0.112$). The error bars represent the standard errors of the means.

3.3. FARMING SYSTEM AND AREA EFFECTS TO *ASPERGILLUS CARBONARIUS* INCIDENCE

The effects of farming system, area and their interaction, in the vineyards of the two areas of Soultanina cultivar were analyzed. Figure 3.8 shows the frequency of isolation (%) of *A. carbonarius* from Soultanina grape samples from Areas B and C.

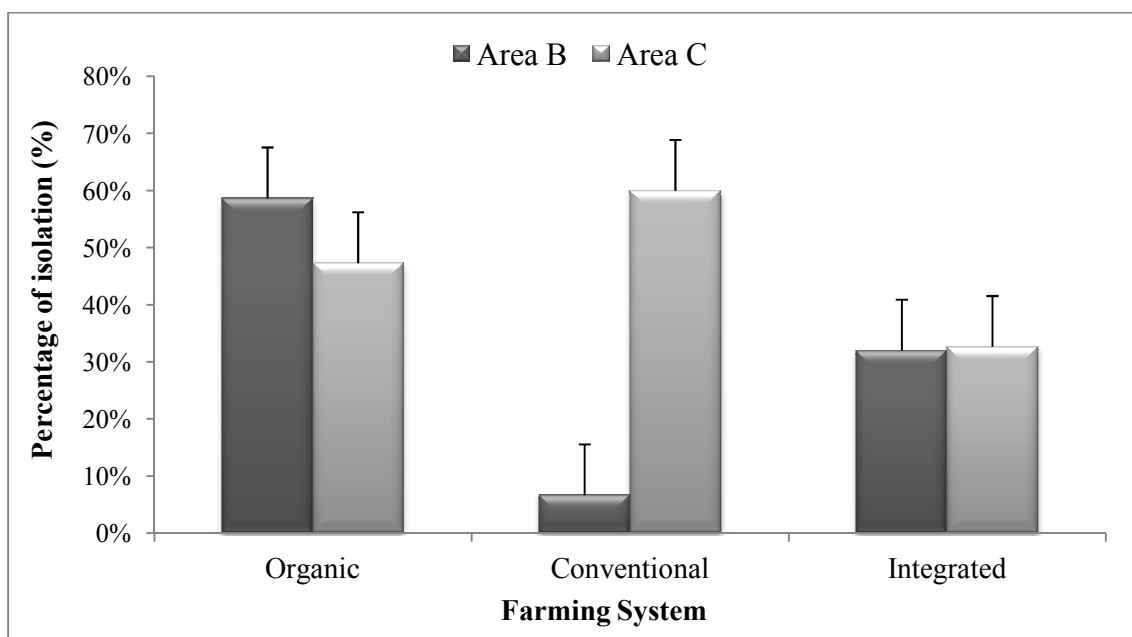


Figure 3.8. Frequency of isolation of *A. carbonarius* from directly plated Soultanina grapes on MEA96 grown in the three farming systems in two areas. The error bars represent the standard errors of the means.

Analysis of covariance (ANCOVA) showed that the influence of the farming systems ($F_{2,84} = 3.418, p = 0.037$), as well as the interaction of farming systems and areas ($F_{2,84} = 7.445, p = 0.001$) were statistically significant, whereas the influence of the areas of research was not significant ($F_{1,84} = 3.818, p = 0.054$) (Table 3.1).

Table 3.1. Tests of Between-Subjects Effects (ANCOVA) (Farming System x Area B and C).

Source	Sum of Squares	Degrees of Freedom	Mean Square	F	Sig.
Farming System	0.815	2	0.407	3.418	0.037
Area	0.455	1	0.455	3.818	0.054
Farming System x Area	1.775	2	0.887	7.445	0.001
Error	10.013	84	0.119		

As it is shown in the Figure 3.9, in both of Soultanina cultivar areas, *A. carbonarius* contamination was reduced in the Integrated Farming System, when compared to the Organic Farming System. In the Conventional Farming System the results were unclear as there was a big heterogeneity between areas B and C, as revealed from Levene's test for homogeneity of variances (Figure 3.10).

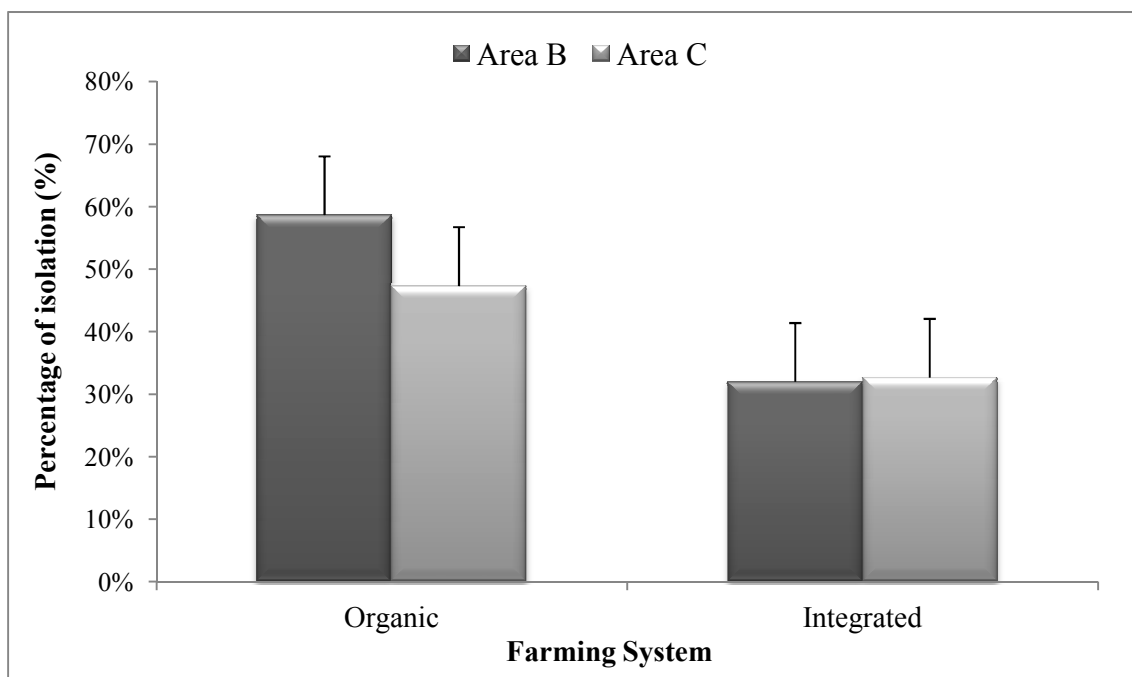


Figure 3.9. Frequency of isolation (%) of *A. carbonarius* on direct plating cultures (MEA96), from Soultanina grape samples, regarding the farming system (OFS and IFS) and the area of research (B and C). The error bars represent the standard errors of the means.

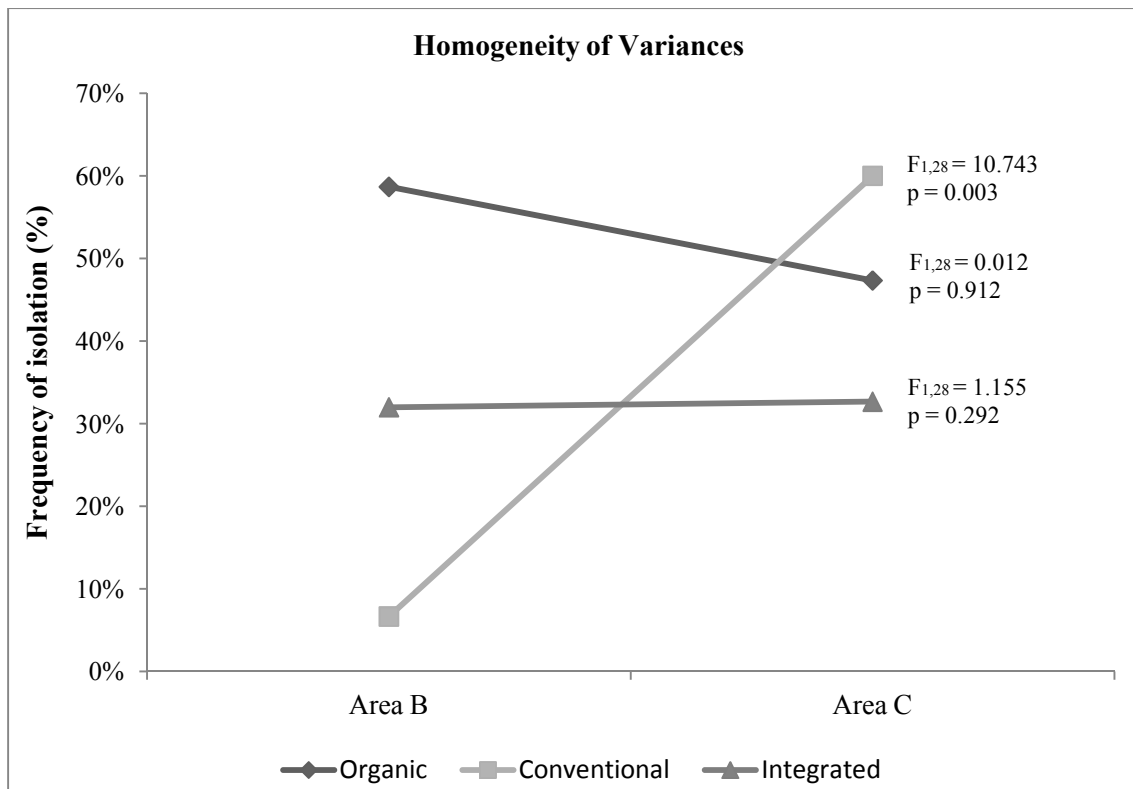


Figure 3.10. Homogeneity of variances of *A. carbonarius* frequency of isolation between areas B and C, in relation to the farming system (OFS, CFS and IFS).

MYCOLOGICAL ANALYSIS OF GRAPES

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DIRECT PLATING METHOD

3.4. EFFECT OF FARMING SYSTEM ON THE INCIDENCE OF THE MOST DOMINANT FUNGAL SPECIES AND GENERA

3.4.1. Farming system effects on Kotsifali cultivar vineyards of Area A

Figures 3.11 and 3.12 show the effect of the farming system on the incidence of the most dominant fungal species and genera on grape samples of the Kotsifali cultivar vineyards, in Area A, in 2011 and 2012. The results are presented as the frequency of isolation (%) of each of fungal species and genera, from grape samples.

One-way analysis of variance (ANOVA) of fungal species and genera frequency of isolation (%) from grapes of Kotsifali cultivar vineyards in Area A, in 2011, revealed statistically significant differences between the farming systems for *A. carbonarius* ($F_{1,38} = 7.734$, $p = 0.008$) and *Alternaria spp.* ($F_{1,38} = 8.738$, $p = 0.005$), while there were no significant differences for *Aspergillus* section *Nigri* ($F_{1,38} = 0.780$, $p = 0.383$), *Penicillium spp.* ($F_{1,38} = 1.643$, $p = 0.208$), Yeasts ($F_{1,38} = 1.787$, $p = 0.189$) and *Cladosporium spp.* ($F_{1,38} = 1.190$, $p = 0.282$).

In 2012, one-way analysis of variance (ANOVA) of fungal species and genera frequency of isolation (%) from grapes of Kotsifali cultivar vineyards in Area A, revealed statistically significant differences between the farming systems, for *Aspergillus* section *Nigri* ($F_{1,38} = 4.744$, $p = 0.036$) and *A. carbonarius* ($F_{1,38} = 5.630$, $p = 0.023$), but not significant differences for *Penicillium spp.* ($F_{1,38} = 0.016$, $p = 0.584$), Yeasts ($F_{1,38} = 1.900$, $p = 0.176$), *Cladosporium spp.* ($F_{1,38} = 0.950$, $p = 0.336$) and *Alternaria spp.* ($F_{1,38} = 1.686$, $p = 0.202$).

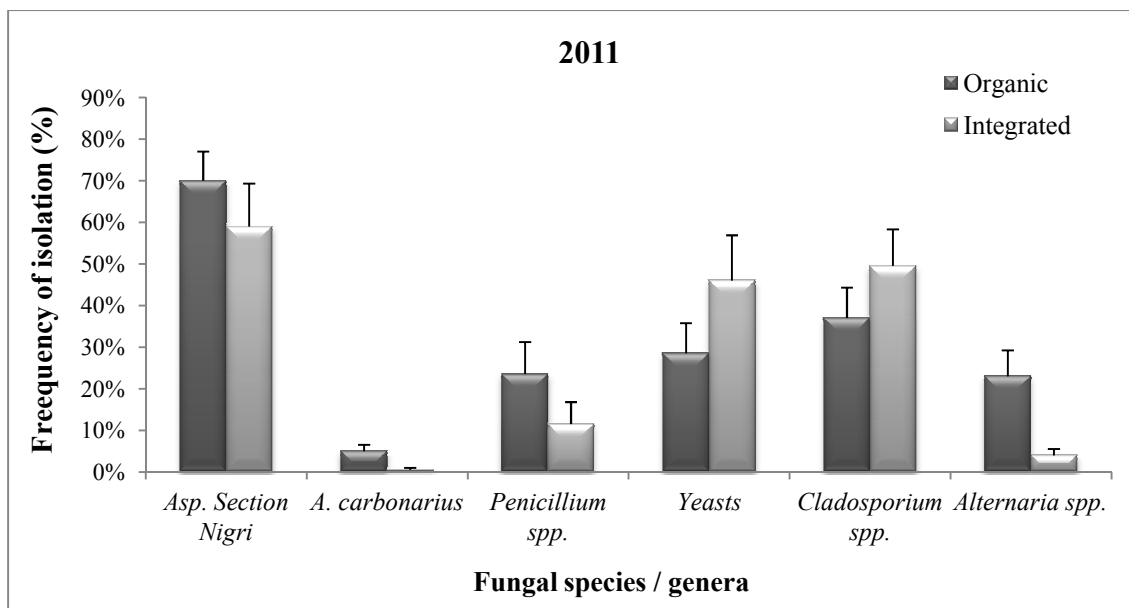


Figure 3.11. Average of frequency of isolation (%) of the most dominant fungal species / genera on direct plating cultures (MEA96), from Kotsifali cultivar grape samples of Area A vineyards, in 2011. The error bars represent the standard errors of the means.

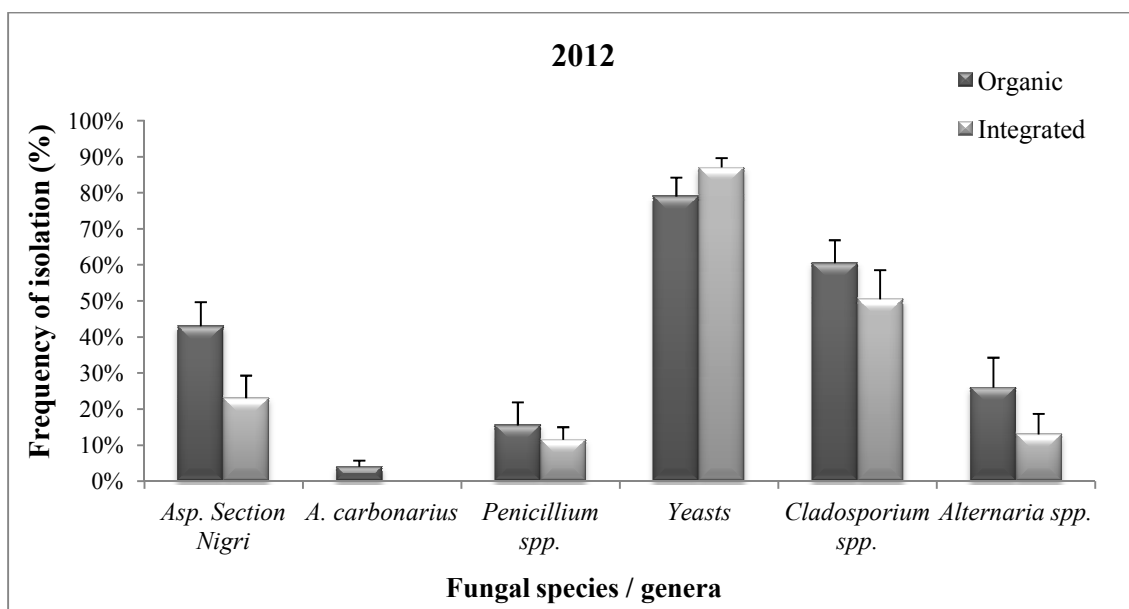


Figure 3.12. Average of frequency of isolation (%) of the most dominant fungal species / genera on direct plating cultures (MEA96), from Kotsifali cultivar grape samples of Area A vineyards, in 2012. The error bars represent the standard errors of the means.

3.4.2. Farming system effects on Soultanina cultivar vineyards of Area B

Figures 3.13 and 3.14 show the effect of farming system on the incidence of the most dominant fungal species and genera on grape cultivar Soultanina vineyards in Area B, in 2011 and 2012. The results are presented as the frequency of isolation (%) of each of fungal species and genera, from grape samples.

One-way analysis of variance (ANOVA) of fungal species and genera frequency of isolation (%) from grapes of Soultanina cultivar vineyards in Area B, for the year 2011, revealed statistically significant differences between the farming systems only for *A. carbonarius* ($F_{1,38} = 6.011$, $p = 0.019$), whereas there were not significant differences between the farming systems for *Aspergillus* section *Nigri* ($F_{1,38} = 0.040$, $p = 0.842$), *Penicillium* spp. ($F_{1,38} = 0.134$, $p = 0.716$), Yeasts ($F_{1,38} = 0.358$, $p = 0.553$), *Cladosporium* spp. ($F_{1,38} = 2.433$, $p = 0.127$) and *Alternaria* spp. ($F_{1,38} = 0.809$, $p = 0.374$).

In 2012, one-way analysis of variance (ANOVA) of fungal species and genera frequency of isolation (%) from grapes of Soultanina cultivar vineyards in Area B, revealed statistically significant differences between the farming systems for *Aspergillus* section *Nigri* ($F_{1,38} = 7.066$, $p = 0.011$), *A. carbonarius* ($F_{1,38} = 18.106$, $p = 0.000$) and for *Alternaria* spp. ($F_{1,38} = 5.264$, $p = 0.027$), while there were not significant differences between the farming systems for *Penicillium* spp. ($F_{1,38} = 0.446$, $p = 0.508$), Yeasts ($F_{1,38} = 0.608$, $p = 0.440$) and *Cladosporium* spp. ($F_{1,38} = 0.776$, $p = 0.384$).

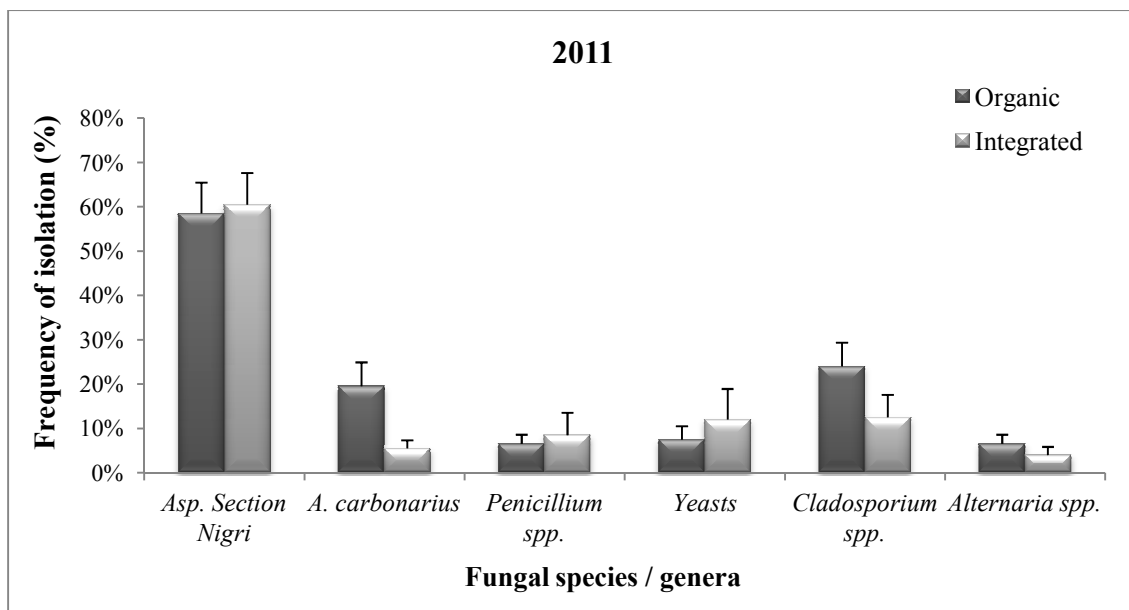


Figure 3.13. Average of frequency of isolation (%) of the most dominant fungal species / genera on direct plating cultures (MEA96), from Soultanina cultivar grape samples of Area B vineyards in 2011. The error bars represent the standard errors of the means.

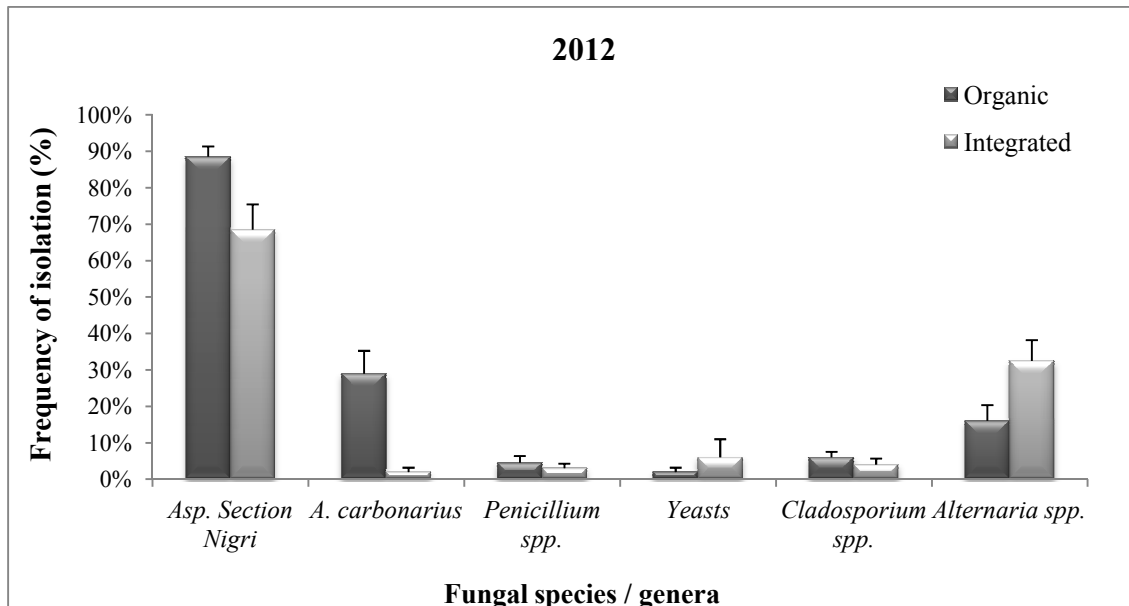


Figure 3.14. Average of frequency of isolation (%) of the most dominant fungal species / genera on direct plating cultures (MEA96), from Soultanina cultivar grape samples of Area B vineyards in 2012. The error bars represent the standard errors of the means.

3.4.3. Farming system effects on Soultanina cultivar vineyards in Area C

Figures 3.15 and 3.16 show the effect of the farming system to the incidence of the most dominant fungal species and genera on Soultanina cultivar grapes in Area C, in 2011 and 2012. The results are presented as the frequency of isolation (%) of each of fungal species and genera, from grape samples.

One-way analysis of variance (ANOVA) of fungal species and genera frequency of isolation (%) from grapes of Soultanina cultivar vineyards in Area C, for the year 2011, revealed statistically significant differences between the farming systems for *Aspergillus* section *Nigri* ($F_{1,38} = 21.296$, $p = 0.000$) and *Alternaria* spp. ($F_{1,38} = 8.393$, $p = 0.006$), while there were not significant differences between the farming systems for *Aspergillus carbonarius* ($F_{1,38} = 0.253$, $p = 0.618$), *Penicillium* spp. ($F_{1,38} = 1.667$, $p = 0.204$), Yeasts ($F_{1,38} = 1.000$, $p = 0.324$) and *Cladosporium* spp. ($F_{1,38} = 1.294$, $p = 0.262$).

In 2012, one-way analysis of variance (ANOVA) of fungal species and genera frequency of isolation (%) from grapes of Soultanina cultivar vineyards in Area C, revealed statistically significant differences between the farming systems almost for all fungal species and genera, with the exception of *Cladosporium* spp. More analytically, one-way analysis of variance (ANOVA) for year 2012, showed significant statistically differences for *Aspergillus* section *Nigri* ($F_{1,38} = 45.675$, $p = 0.000$), *A. carbonarius* ($F_{1,38} = 35.426$, $p = 0.000$), *Penicillium* spp. ($F_{1,38} = 5.911$, $p = 0.020$), Yeasts ($F_{1,38} = 20.859$, $p = 0.000$), and *Alternaria* spp. ($F_{1,38} = 6.400$, $p = 0.016$), while there were not significant differences between the farming systems for *Cladosporium* spp. ($F_{1,38} = 3.700$, $p = 0.062$).

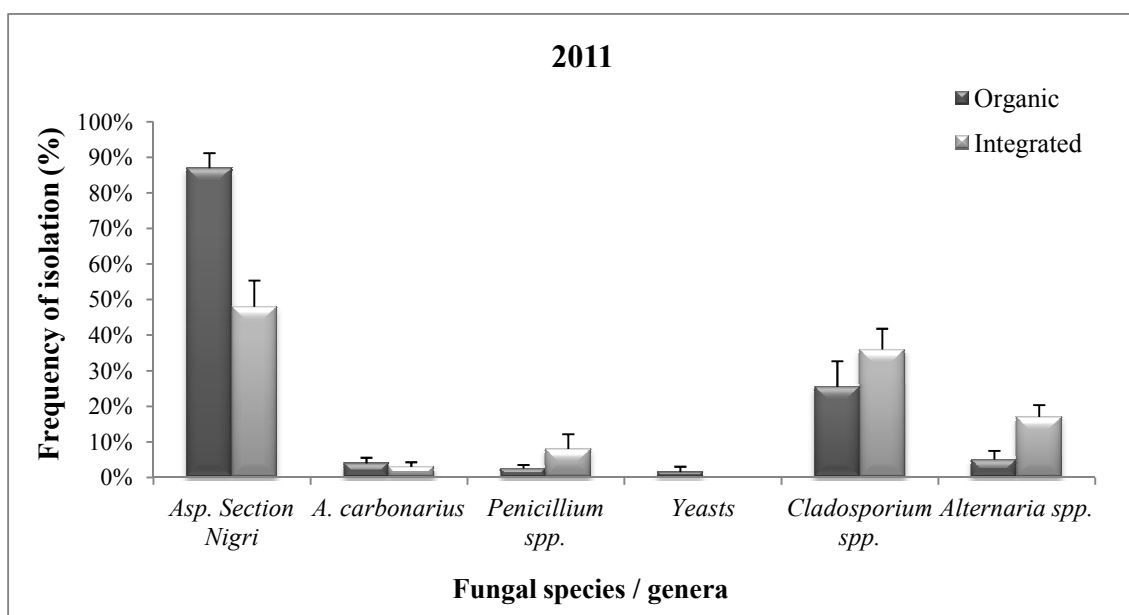


Figure 3.15. Average of frequency of isolation (%) of the most dominant fungal species / genera on direct plating cultures (MEA96), from Soultanina cultivar grape samples of Area C vineyards in 2011. The error bars represent the standard errors of the means.

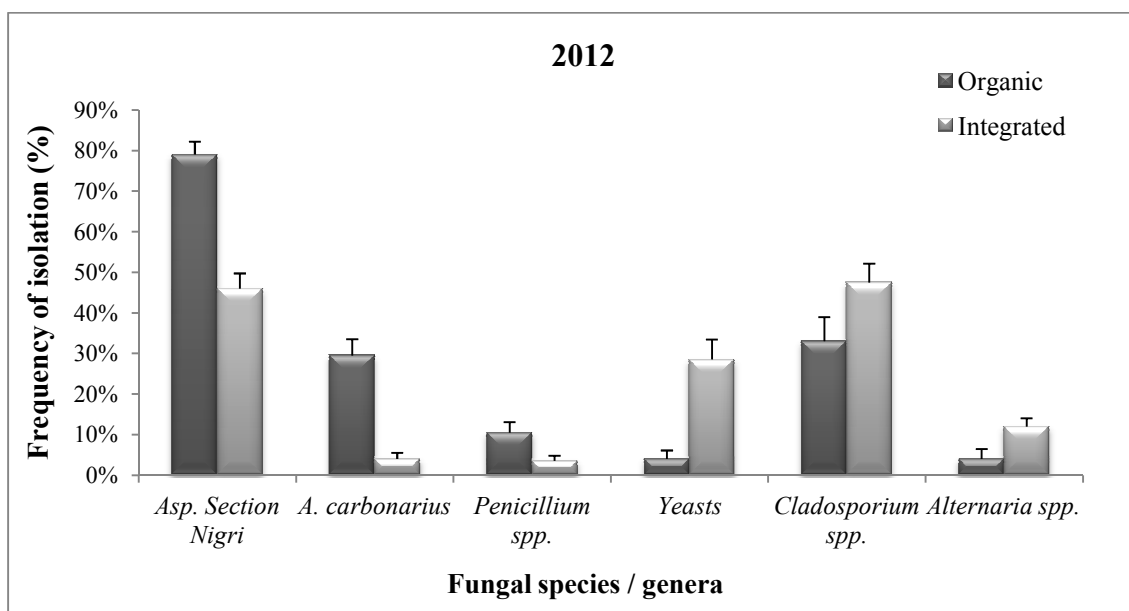


Figure 3.16. Average of frequency of isolation (%) of the most dominant fungal species / genera on direct plating cultures (MEA96), from Soultanina cultivar grape samples of Area C vineyards in 2012. The error bars represent the standard errors of the means.

3.5. INCIDENCE OF *ASPERGILLUS CARBONARIUS* IN THE THREE AREAS OF RESEARCH

Figure 3.17 shows the incidence of *A. carbonarius* in the three areas of research in 2011 and 2012 regardless of the farming system and the cultivar. The isolation of *A. carbonarius* was significantly lower in Area A, than in Area B in 2011, and both Areas B and C in 2012. In Areas A and B, the presence of *A. carbonarius* in grape samples, was similar for both years, while in Area C, the frequency of isolation of *A. carbonarius* was significantly lower in 2011 samples, when compared with 2012 samples.

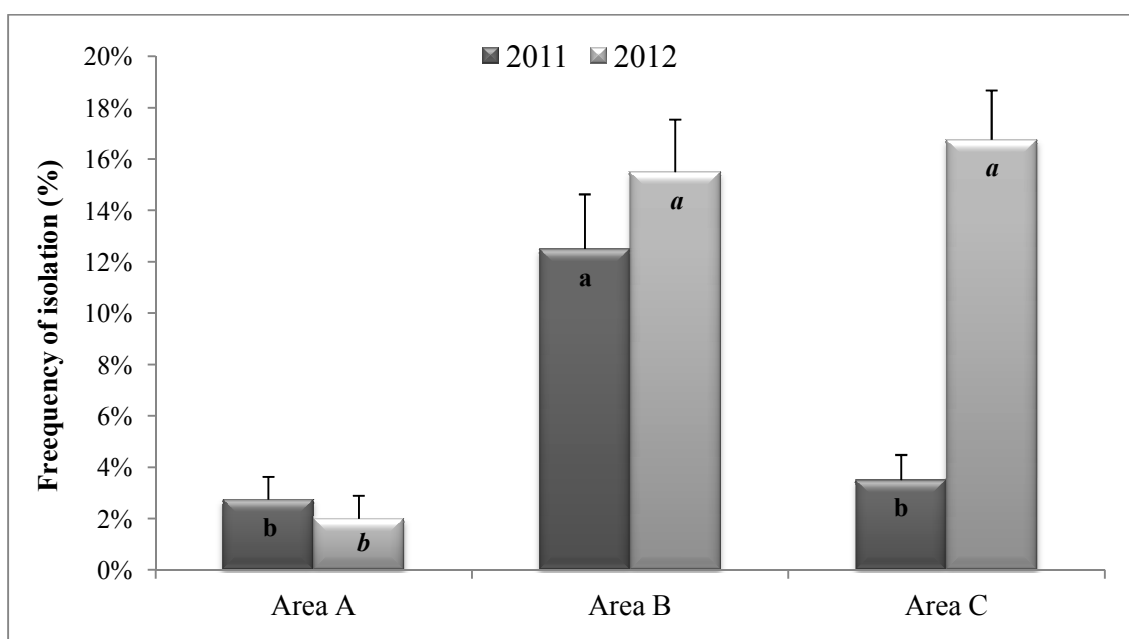


Figure 3.17. Frequency of isolation of *A. carbonarius* on direct plating cultures (MEA96), from overall grape samples from the three areas of research for 2011 and 2012, regardless of the farming system and the cultivar. The different letters indicate a significant difference of *A. carbonarius* percentage of isolation among the three areas (Duncan's multiple range tests, $p < 0.05$), while one-way analysis of variance (ANOVA) revealed statistically significant differences among the three areas for both of years. $F_{2,117} = 8.077$, $p = 0.001$ for 2011 and $F_{2,117} = 8.392$, $p = 0.000$ for 2012. The error bars represent the standard errors of the means.

3.5.1. Incidence of *Aspergillus carbonarius* in Area A

Figure 3.18 shows the incidence of *A. carbonarius* in Kotsifali cultivar vineyards in Area A for 2011 and 2012 years, in relation to the farming system. Although the frequency of isolation of *A. carbonarius* in Area A was low for both of farming systems, the incidence of *A. carbonarius* in integrated farming system vineyards was significantly lower from organic farming system vineyards for both of years 2011 and 2012 where the rates were zero.

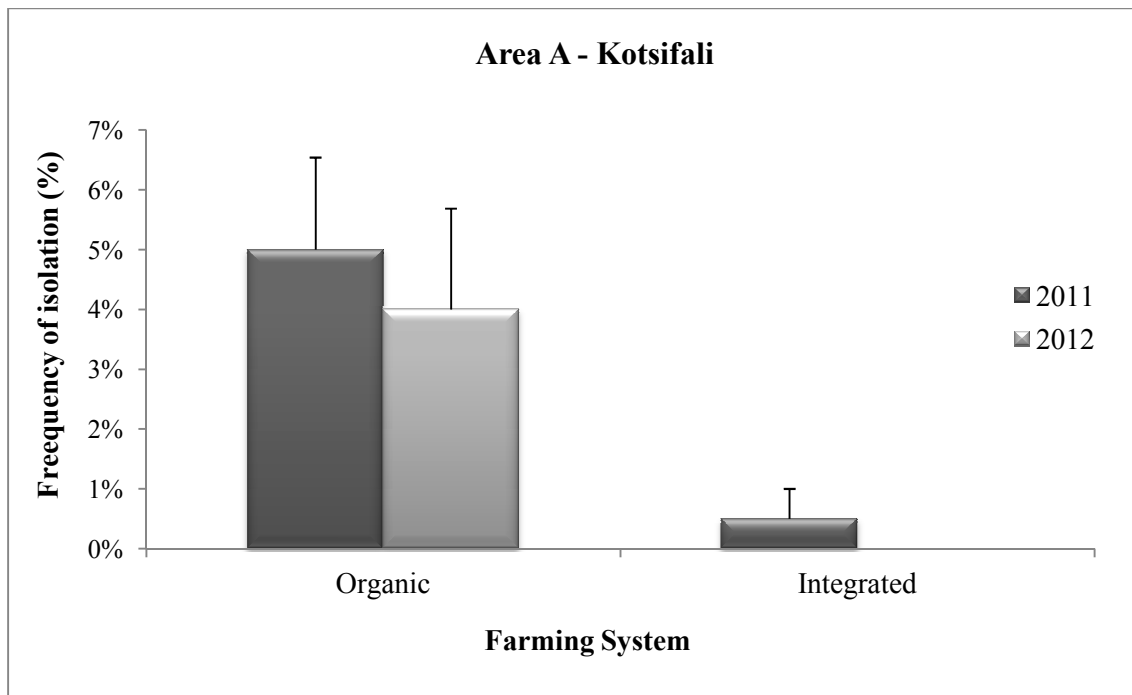


Figure 3.18. Frequency of isolation of *A. carbonarius* on direct plating cultures (MEA96), from Kotsifali grape samples of Area A for years 2011 and 2012, regarding the farming system, where one-way analysis of variance (ANOVA) revealed statistically significant differences for both years 2011 ($F_{1,38} = 7.734$, $p = 0.008$) and 2012 ($F_{1,38} = 5.630$, $p = 0.023$). The error bars represent the standard errors of the means.

3.5.2. Incidence of *Aspergillus carbonarius* in Area B

Figure 3.19 shows the incidence of *A. carbonarius* in Soultanina cultivar vineyards in Area B in 2011 and 2012 in the three farming system. The frequency of isolation of *A. carbonarius* was significantly higher in organic farming system vineyards than in integrated farming system vineyards in both years. More analytically, the percentage of isolation was 19.50% in OFS and 5.50% in IFS vineyards for 2011, and 29.00% in OFS and 2.00% in IFS vineyards for 2012.

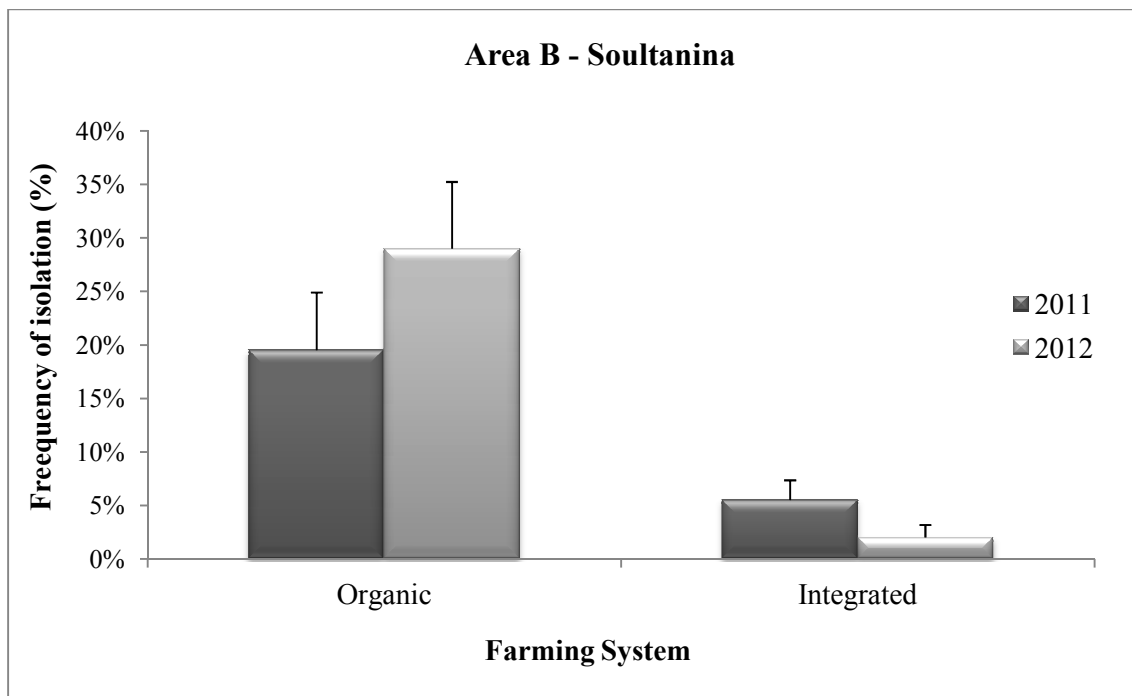


Figure 3.19. Frequency of isolation of *A. carbonarius* on direct plating cultures (MEA96), from Soultanina grape samples of Area B for years 2011 and 2012, regarding the farming system, where one-way analysis of variance (ANOVA) revealed statistically significant differences for both of years 2011 ($F_{1,38} = 6.011$, $p = 0.019$) and 2012 ($F_{1,38} = 18.106$, $p = 0.000$). The error bars represent the standard errors of the means.

3.5.3. Incidence of *Aspergillus carbonarius* in Area C

Figure 3.20 shows the incidence of *A. carbonarius* in Soultanina cultivar vineyards in Area C in 2011 and 2012. Interestingly, the frequency of isolation of *A. carbonarius* seems to be similar, with low isolation frequencies for both farming systems in 2011. The following year the presence of *A. carbonarius* seemed to be significantly higher in OFS vineyards rather than in IFS vineyards, with 29.50% and 4.00% frequency of isolation respectively.

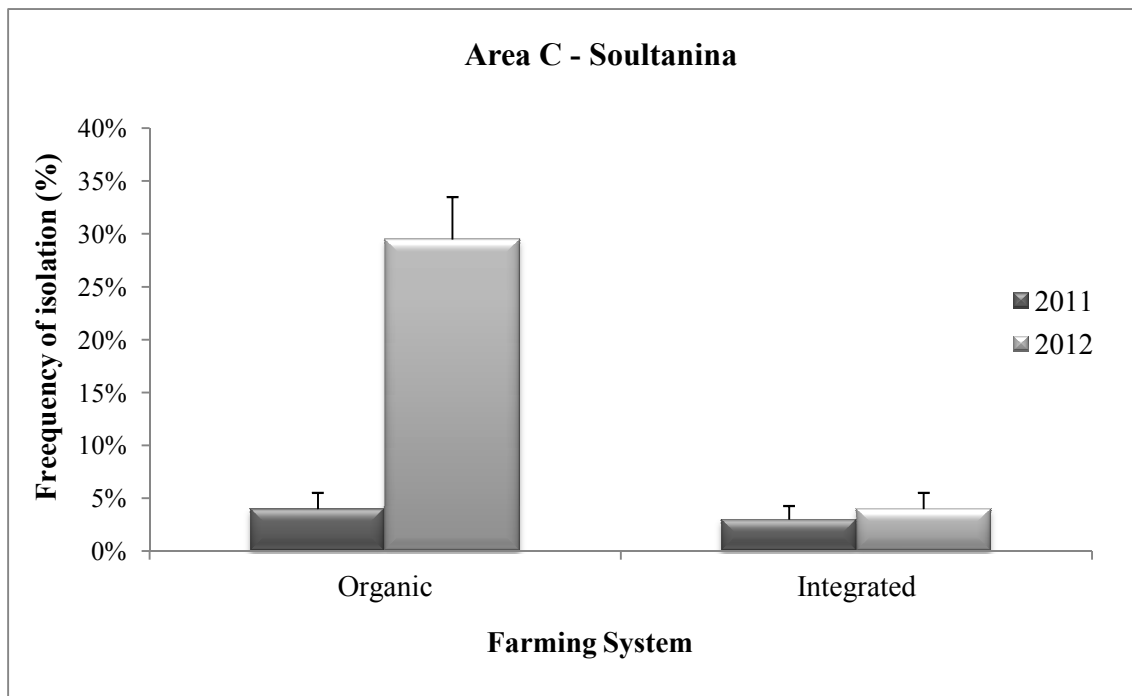


Figure 3.20. Frequency of isolation of *A. carbonarius* on direct plating cultures (MEA96), from Soultanina grape samples of Area C for years 2011 and 2012, regarding the farming system. One-way analysis of variance (ANOVA) revealed no statistically significant differences between the farming systems ($F_{1,38} = 0.253$, $p = 0.618$) for the year 2011, but statistically significant differences ($F_{1,38} = 35.426$, $p = 0.000$) for the year 2012. The error bars represent the standard errors of the means.

3.6. FARMING SYSTEM AND AREA EFFECTS TO *ASPERGILLUS CARBONARIUS* INCIDENCE

Figures 3.21 and 3.22 shows the effect of farming system and area of research, as well their interaction on the frequency of isolation (%) of *A. carbonarius*, for Soultanina cultivar vineyards of areas B and C, in 2011 and 2012.

Analysis of covariance (ANCOVA) showed that the influence of farming systems was statistically significant for both of years, while the influence of areas and the interaction between farming systems and areas was statistically significant for year 2011, but not significant for year 2012 (Tables 3.2 and 3.3).

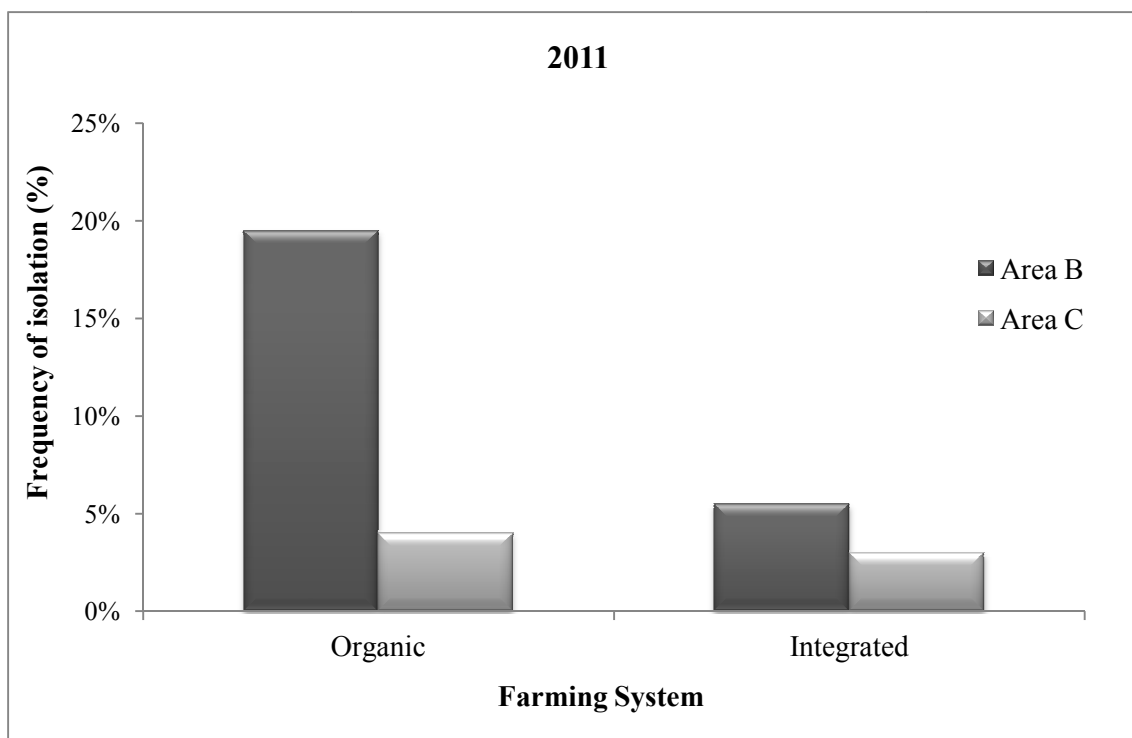


Figure 3.21. Frequency of isolation (%) of *A. carbonarius* on direct plating cultures (MEA96), of Soultanina cultivar grape samples, from areas B and C for year 2011, regarding the farming system and the area of research.

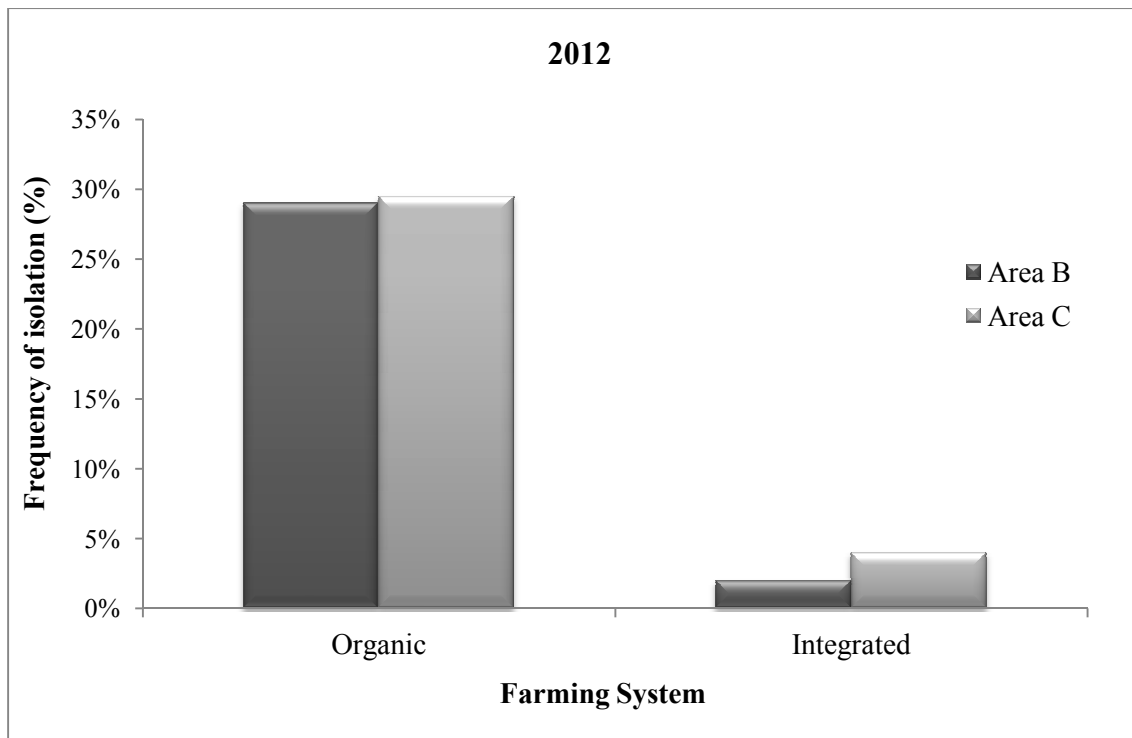


Figure 3.22. Frequency of isolation (%) of *A. carbonarius* on direct plating cultures (MEA96), of Soultanina cultivar grape samples, from areas B and C for year 2012, regarding the farming system and the area of research.

Table 3.2. Tests of Between-Subjects Effects (ANCOVA), for frequency of isolation of *A. carbonarius* on direct plating cultures (MEA96), of Soultanina grape samples from areas B and C, for year 2011, regarding the farming system and the area of research.

Source	Sum of Squares	Degrees of Freedom	Mean Square	F	Sig.
Farming System	0.113	1	0.113	6.156	0.015
Area	0.162	1	0.162	8.864	0.004
Farming System x Area	0.085	1	0.085	4.623	0.035
Error	1.389	76	0.018		

Table 3.3. Tests of Between-Subjects Effects (ANCOVA), for frequency of isolation of *A. carbonarius* on direct plating cultures (MEA96), of Soultanina grape samples from areas B and C, for year 2012, regarding the farming system and the area of research.

Source	Sum of Squares	Degrees of Freedom	Mean Square	F	Sig.
Farming System	1.378	1	1.378	47.020	0.000
Area	0.003	1	0.003	0.107	0.745
Farming System x Area	0.001	1	0.001	0.038	0.845
Error	2.227	76	0.029		

3.7. FARMING SYSTEM AND CULTIVAR EFFECTS TO *ASPERGILLUS CARBONARIUS* INCIDENCE

The effect of farming system and cultivar, as well their interaction with frequency of isolation (%) of *A. carbonarius* in 2011 and 2012 regardless of research area are shown in Figures 3.23 and 3.24.

Analysis of covariance (ANCOVA) showed that the influence of farming systems, as well the influence of cultivar, were statistically significant for both of years, while the interaction between farming systems and cultivar was not statistically significant for year 2011, but statistically significant for year 2012 (Tables 3.4 and 3.5).

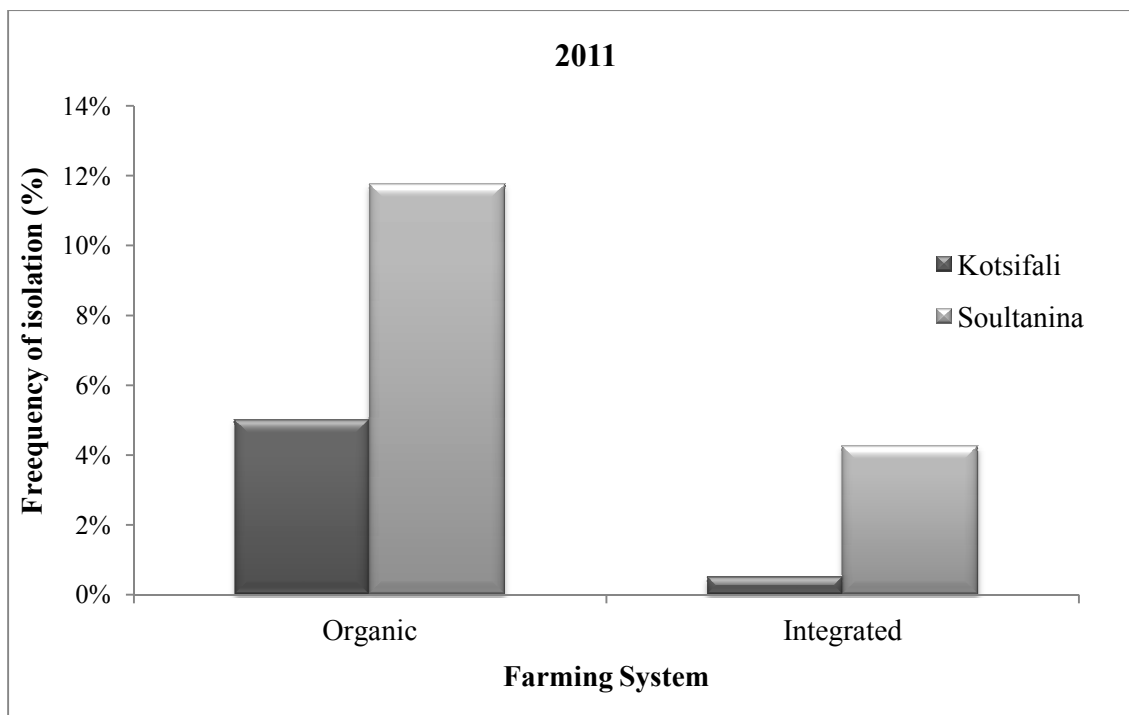


Figure 3.23. Average of frequency of isolation (%) of *A. carbonarius* on direct plating cultures (MEA96), of Kotsifali and Soultanina cultivar grape samples, from all areas of research for year 2011, regarding the farming system and the cultivar.

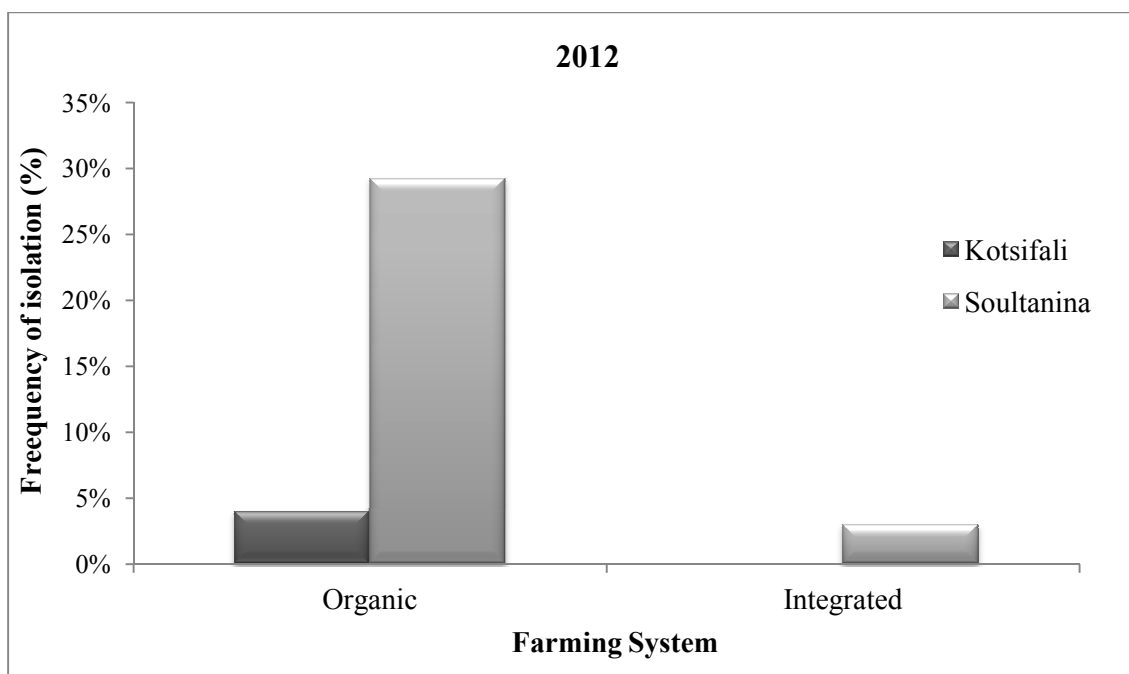


Figure 3.24. Average of frequency of isolation (%) of *A. carbonarius* on direct plating cultures (MEA96), of Kotsifali and Soultanina cultivar grape samples, from all areas of research for year 2012, regarding the farming system and the cultivar.

Table 3.4. Tests of Between-Subjects Effects (ANCOVA) for frequency of isolation (%) of *A. carbonarius* on direct plating cultures (MEA96), of Kotsifali and Soultanina cultivar grape samples, from all areas of research for year 2011, regarding the farming system and the cultivar.

Source	Sum of Squares	Degrees of Freedom	Mean Square	F	Sig.
Farming System	0.096	1	0.096	6.418	0.013
Cultivar	0.073	1	0.073	4.914	0.029
Farming System x Cultivar	0.006	1	0.006	0.401	0.528
Error	1.735	116	0.015		

Table 3.5. Tests of Between-Subjects Effects (ANCOVA) for frequency of isolation (%) of *A. carbonarius* on direct plating cultures (MEA96), of Kotsifali and Soultanina cultivar grape samples, from all areas of research for year 2012, regarding the farming system and the cultivar.

Source	Sum of Squares	Degrees of Freedom	Mean Square	F	Sig.
Farming System	0.610	1	0.610	30.245	0.000
Cultivar	0.532	1	0.532	26.378	0.000
Farming System x Cultivar	0.330	1	0.330	16.363	0.000
Error	2.340	116	0.020		

3.8. RELATIVE INCIDENCE OF *A. CARBONARIUS* IN RELATION TO OTHER *ASPERGILLUS* SECTION *NIGRI* SPECIES

Figure 3.25 shows the relative frequency of isolation between *A. carbonarius* and *Aspergillus* section *Nigri* species, on grape berries of Kotsifali cultivar, from Area A vineyards, in relation to the three farming systems in 2011 and 2012.

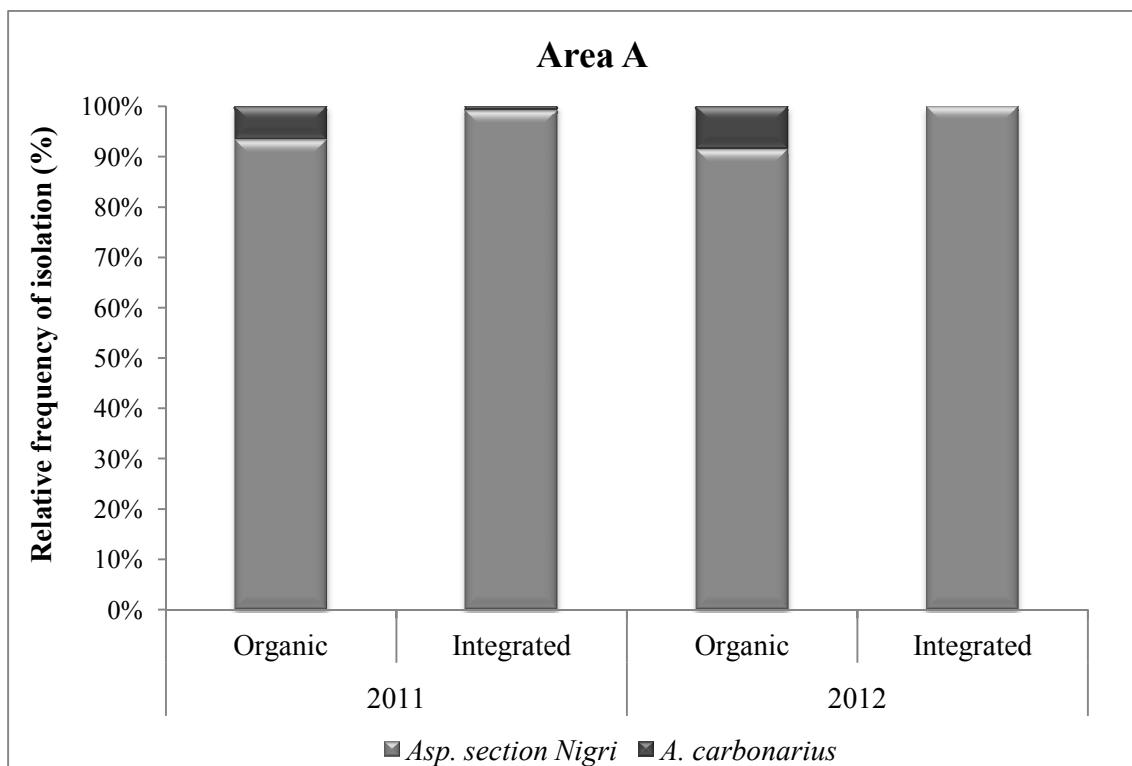


Figure 3.25. Relative frequency of isolation of *A. carbonarius* and *Aspergillus* section *Nigri* species, on grape berries from Area A, the years 2011 and 2012.

In 2011, *A. carbonarius* was isolated from the 5% of berries of the OFS and the 0.5% of the IFS vineyards, while the rest of *Aspergillus* section *Nigri* species were isolated from the 70% of berries of the OFS and the 59% of IFS vineyards.

Similarly in 2012, *A. carbonarius* was isolated from the 4% of berries of the OFS, and there was not any isolation from berries of the IFS vineyards, while the rest of

Aspergillus section *Nigri* species were isolated from the 43% of berries of the OFS and the 23% of IFS vineyards.

Figure 3.26 shows the relative frequency of isolation between *A. carbonarius* and *Aspergillus* section *Nigri* species, on grape berries of Soultanina cultivar, from Area B vineyards, in relation with the farming system in 2011 and 2012.

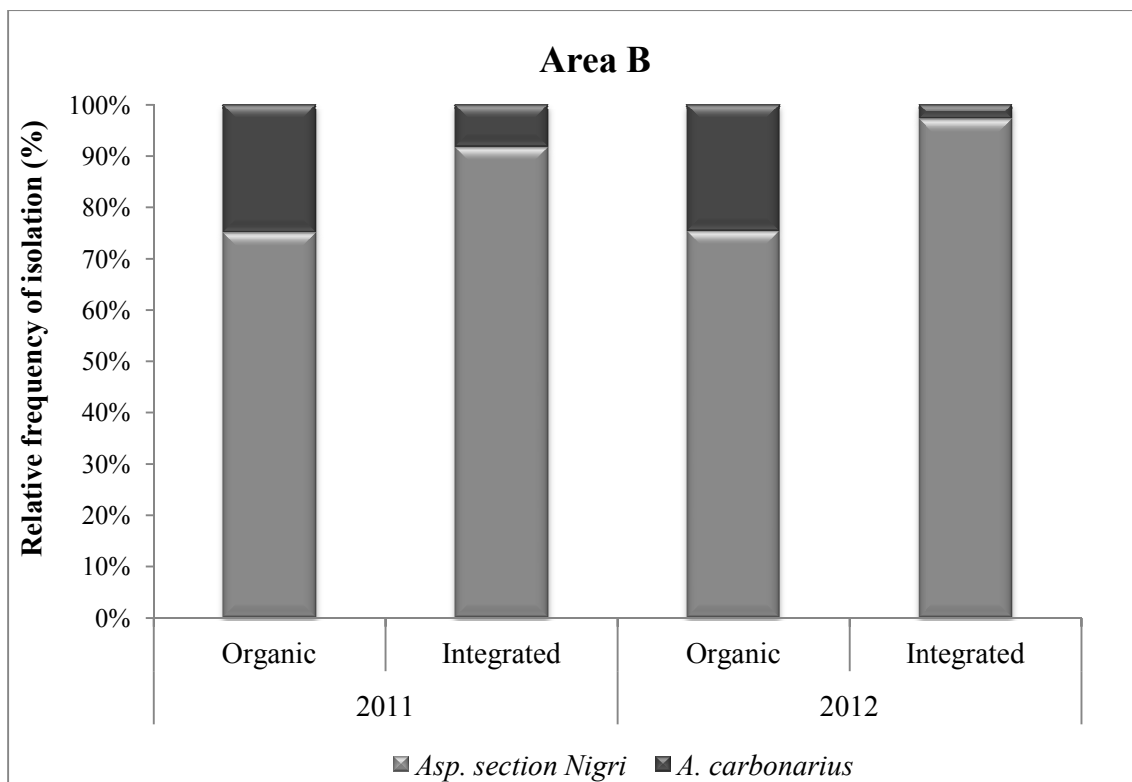


Figure 3.26. Relative frequency of isolation of *A. carbonarius* and *Aspergillus* section *Nigri* species, on grape berries from Area B, the years 2011 and 2012.

In 2011, *A. carbonarius* was isolated from the 19.5% of berries of the OFS and the 5.5% of the IFS vineyards, while the rest of *Aspergillus* section *Nigri* species were isolated from the 58.5% of berries of the OFS and the 60.5% of IFS vineyards.

In 2012, *A. carbonarius* was isolated from the 29% of berries of the OFS, and the 2% of the IFS vineyards, while the rest of *Aspergillus* section *Nigri* species were isolated from the 88.5% of berries of the OFS and the 68.5% of IFS vineyards.

Figure 3.27 is shown the relative frequency of isolation between *A. carbonarius* and *Aspergillus* section *Nigri* species, on grape berries of Soultanina cultivar, from Area C vineyards, in relation with the farming system in 2011 and 2012.

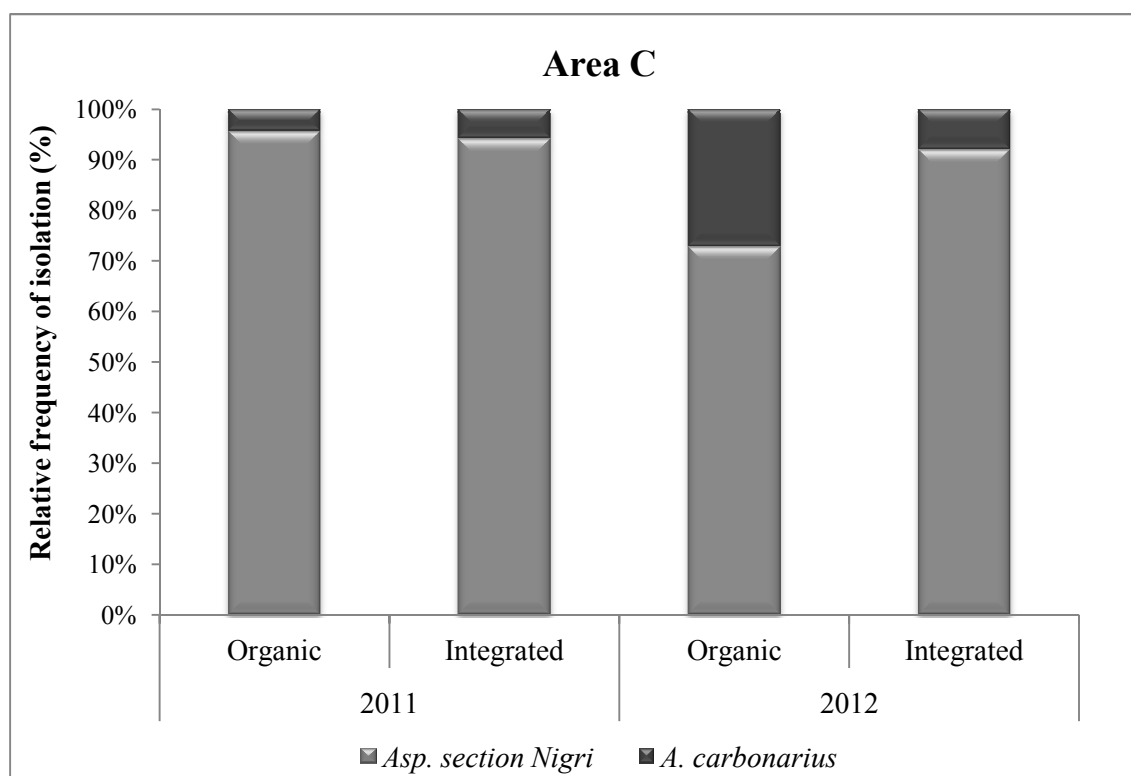


Figure 3.27. Relative frequency of isolation of *A. carbonarius* and *Aspergillus* section *Nigri* species, on grape berries from Area C, the years 2011 and 2012.

In 2011, *A. carbonarius* was isolated from the 4% of berries of the OFS and the 3% of the IFS vineyards, while the rest of *Aspergillus* section *Nigri* species were isolated from the 87% of berries of the OFS and the 48% of IFS vineyards.

In 2012, in contrast with the previous year, *A. carbonarius* was isolated from the 29.5% of berries of the OFS, and the 4% of the IFS vineyards, while the rest of *Aspergillus* section *Nigri* species were isolated from the 79% of berries of the OFS and the 46% of IFS vineyards.

MYCOLOGICAL ANALYSIS OF GRAPES

RESULTS (2011-2012)

FUNGAL POPULATION ISOLATION USING THE DILUTION PLATING METHOD

3.9. EFFECT OF FARMING SYSTEM ON THE INCIDENCE OF THE MOST DOMINANT FUNGAL SPECIES AND GENERA

3.9.1. Farming system effects on Kotsifali cultivar vineyards in Area A

Figures 3.28 and 3.29 show the effect of the farming system on the incidence of the most dominant fungal species and genera on grape samples of the Kotsifali cultivar vineyards, in Area A, in 2011 and 2012. The results are presented as the average of Log_{10} CFUs g^{-1} grapes, for each of fungal species and genera.

One-way analysis of variance (ANOVA) of fungal species and genera Log_{10} CFUs g^{-1} grapes of Kotsifali cultivar vineyards in Area A, in 2011, revealed statistically significant differences between the farming systems, only for *Aspergillus* section *Nigri* ($F_{1,118} = 16.313$, $p = 0.000$) and *Cladosporium spp.* ($F_{1,118} = 5.099$, $p = 0.026$), while there were no significant differences for *A. carbonarius* ($F_{1,118} = 0.319$, $p = 0.573$), *Penicillium spp.* ($F_{1,118} = 3.688$, $p = 0.057$), Yeasts ($F_{1,118} = 0.007$, $p = 0.935$) and *Alternaria spp.* ($F_{1,118} = 1.837$, $p = 0.178$).

In 2012, one-way analysis of variance (ANOVA) of fungal species and genera Log_{10} CFUs g^{-1} grapes of Kotsifali cultivar vineyards in Area A, revealed statistically significant differences between the farming systems, for *Aspergillus* section *Nigri* ($F_{1,118} = 64.625$, $p = 0.000$), *A. carbonarius* ($F_{1,118} = 7.278$, $p = 0.008$), *Cladosporium spp.* ($F_{1,118} = 18.122$, $p = 0.000$) and *Alternaria spp.* ($F_{1,118} = 9.177$, $p = 0.003$), but no significant differences for *Penicillium spp.* ($F_{1,118} = 2.337$, $p = 0.129$) and Yeasts ($F_{1,118} = 1.560$, $p = 0.214$).

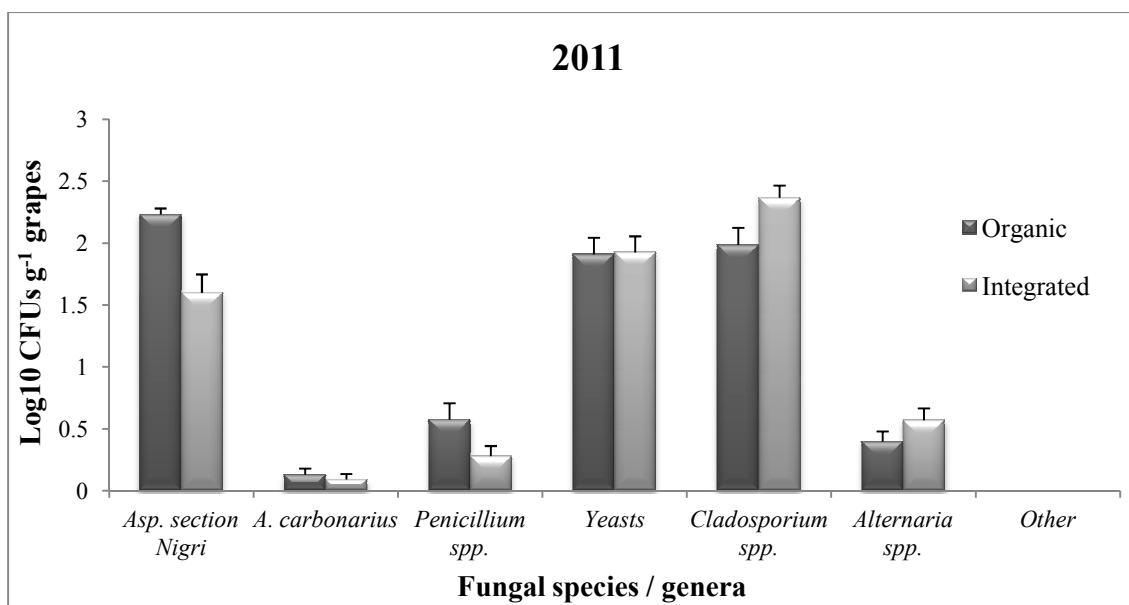


Figure 3.28. Average of Log₁₀ CFUs g⁻¹ grapes of the most dominant fungal species / genera on dilution plate isolations on MEA96, from Kotsifali cultivar grape samples of Area A vineyards in 2011. The error bars represent the standard errors of the means.

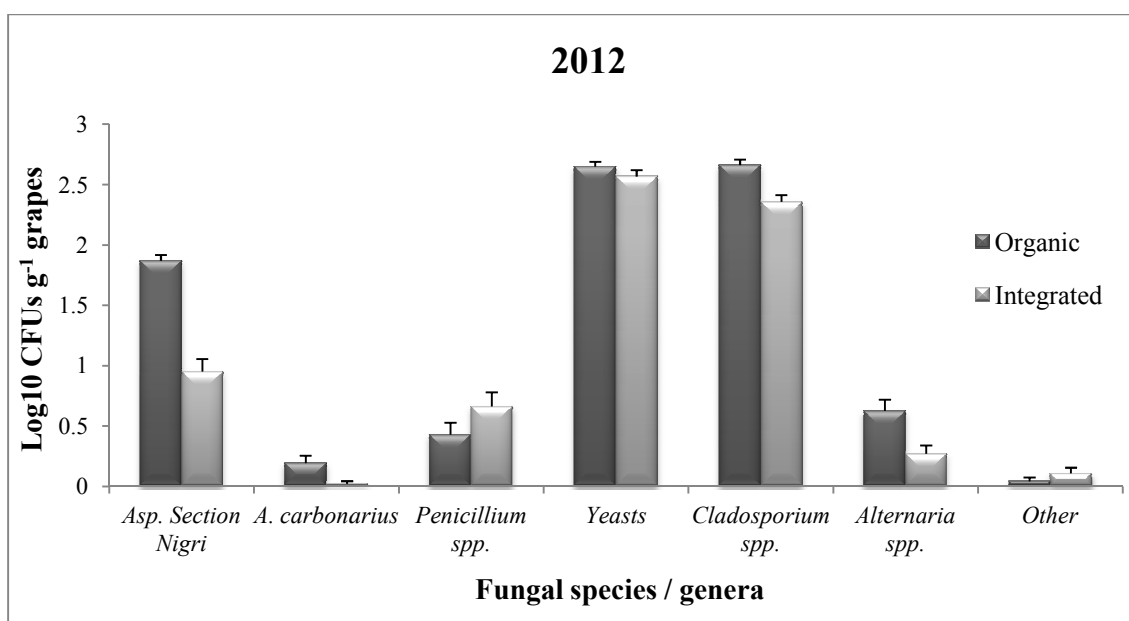


Figure 3.29. Average of Log₁₀ CFUs g⁻¹ grapes of the most dominant fungal species / genera on dilution plate isolations on MEA96, from Kotsifali cultivar grape samples of Area A vineyards, in 2012. The error bars represent the standard errors of the means.

3.9.2. Farming system effects on Soultanina cultivar vineyards in Area B

Figures 3.30 and 3.31 show the effect of the farming system on the incidence of the most dominant fungal species and genera on grape cultivar Soultanina vineyards, in Area B, in 2011 and 2012. The results are presented as the average of Log_{10} CFUs g^{-1} grapes, for each of fungal species and genera.

One-way analysis of variance (ANOVA) of fungal species and genera Log_{10} CFUs g^{-1} grapes of Soultanina cultivar vineyards in Area B, in 2011, revealed statistically significant differences between the farming systems for *Aspergillus* section *Nigri* ($F_{1,118} = 39.689, p = 0.000$), *A. carbonarius* ($F_{1,118} = 11.801, p = 0.001$), Yeasts ($F_{1,118} = 7.431, p = 0.007$), *Cladosporium* spp. ($F_{1,118} = 4.319, p = 0.040$) and *Alternaria* spp. ($F_{1,118} = 14.750, p = 0.000$), whereas there was no significant differences between the farming systems for *Penicillium* spp. ($F_{1,118} = 3.813, p = 0.053$).

Similarly, in 2012, one-way analysis of variance (ANOVA) of fungal species and genera Log_{10} CFUs g^{-1} grapes of Soultanina cultivar vineyards in Area B, revealed statistically significant differences between the farming systems for *Aspergillus* section *Nigri* ($F_{1,118} = 55.642, p = 0.000$), *A. carbonarius* ($F_{1,118} = 38.029, p = 0.000$), *Penicillium* spp. ($F_{1,118} = 7.581, p = 0.007$), Yeasts ($F_{1,118} = 23.769, p = 0.000$) and *Cladosporium* spp. ($F_{1,118} = 19.685, p = 0.000$), while there were no significant differences between the farming systems for *Alternaria* spp. ($F_{1,118} = 1.009, p = 0.317$).

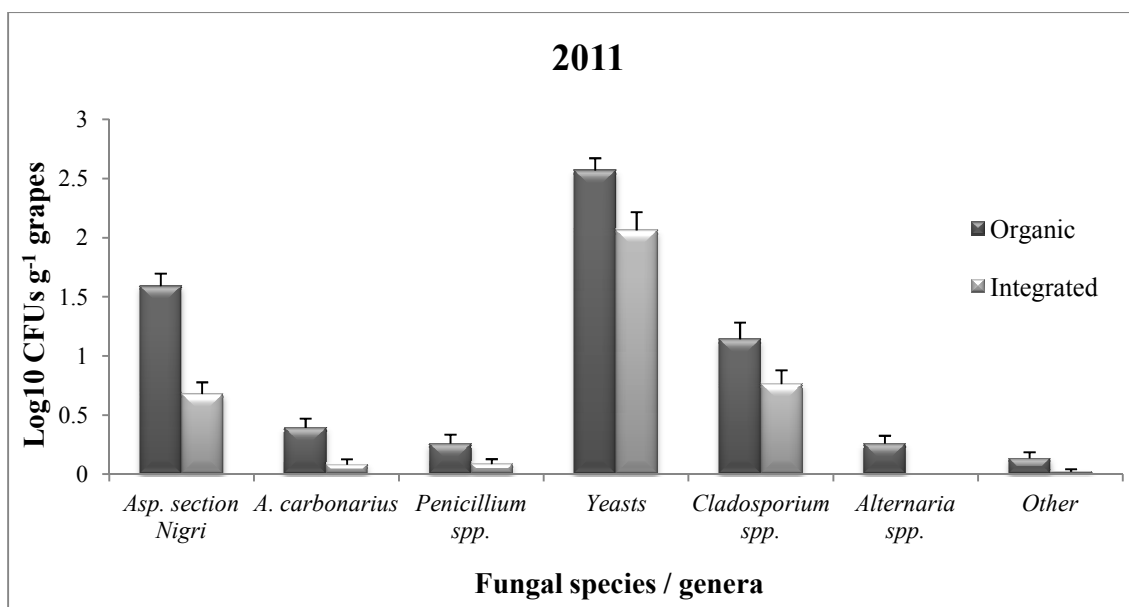


Figure 3.30. Average of Log₁₀ CFUs g⁻¹ grapes of the most dominant fungal species / genera on dilution plating cultures (MEA96), from Soultanina cultivar grape samples of Area B vineyards, in 2011. The error bars represent the standard errors of the means.

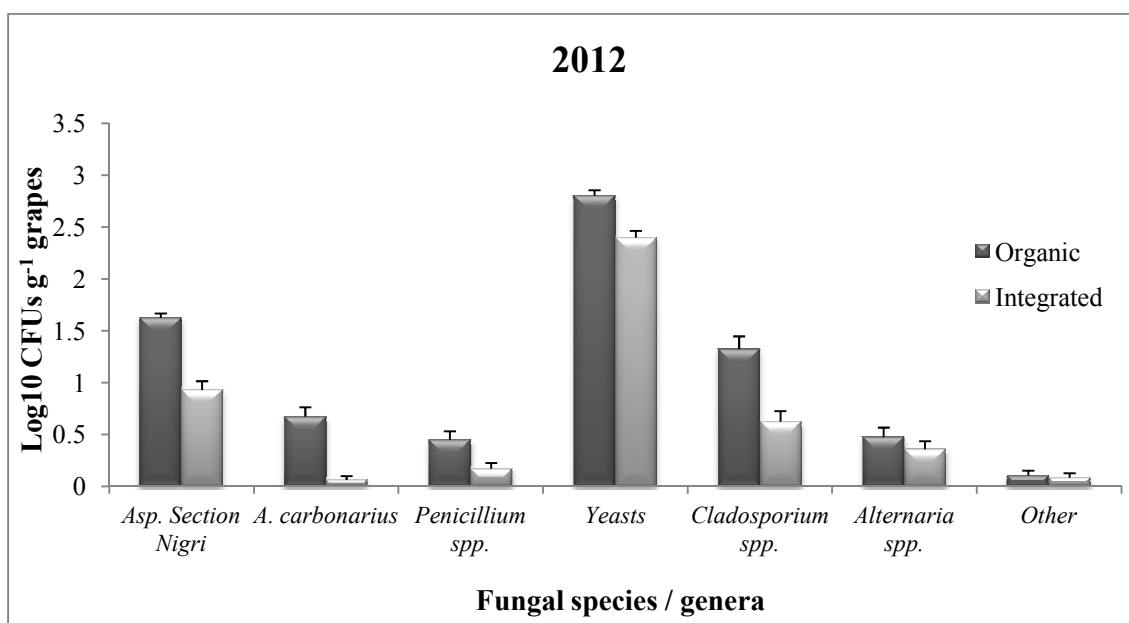


Figure 3.31. Average of Log₁₀ CFUs g⁻¹ grapes of the most dominant fungal species / genera on dilution plating cultures (MEA96), from Soultanina cultivar grape samples of Area B vineyards, in 2012. The error bars represent the standard errors of the means.

3.9.3. Farming system effects on Soultanina cultivar vineyards in Area C

Figures 3.32 and 3.33 show the effect of the farming system on the incidence of the most dominant fungal species and genera on grape samples of Soultanina cultivar vineyards, in Area C, in 2011 and 2012. The results are presented as the average of Log_{10} CFUs g^{-1} grapes, for each of fungal species and genera.

One-way analysis of variance (ANOVA) of fungal species and genera Log_{10} CFUs g^{-1} grapes of Soultanina cultivar vineyards in Area C, in 2011, revealed statistically significant differences between the farming systems for *Aspergillus* section *Nigri* ($F_{1,118} = 13.810, p = 0.000$), Yeasts ($F_{1,118} = 115.585, p = 0.000$) and *Cladosporium* spp. ($F_{1,118} = 63.184, p = 0.000$), whereas there were no significant differences between the farming systems for *Aspergillus carbonarius* ($F_{1,118} = 0.025, p = 0.874$), *Penicillium* spp. ($F_{1,118} = 1.807, p = 0.182$) and *Alternaria* spp. ($F_{1,118} = 0.293, p = 0.589$).

In 2012, one-way analysis of variance (ANOVA) of fungal species and genera Log_{10} CFUs g^{-1} grapes of Soultanina cultivar vineyards in Area C, revealed statistically significant differences between the farming systems almost for all fungal species and genera, with the exception of *Penicillium* spp. More analytically, one-way analysis of variance (ANOVA) for year 2012, showed significant statistically differences for *Aspergillus* section *Nigri* ($F_{1,118} = 108.608, p = 0.000$), *A. carbonarius* ($F_{1,118} = 94.668, p = 0.000$), Yeasts ($F_{1,118} = 74.965, p = 0.000$), *Cladosporium* spp. ($F_{1,118} = 14.716, p = 0.000$) and *Alternaria* spp. ($F_{1,118} = 8.459, p = 0.004$), while there were no significant differences between the farming systems for *Penicillium* spp. ($F_{1,118} = 2.934, p = 0.089$).

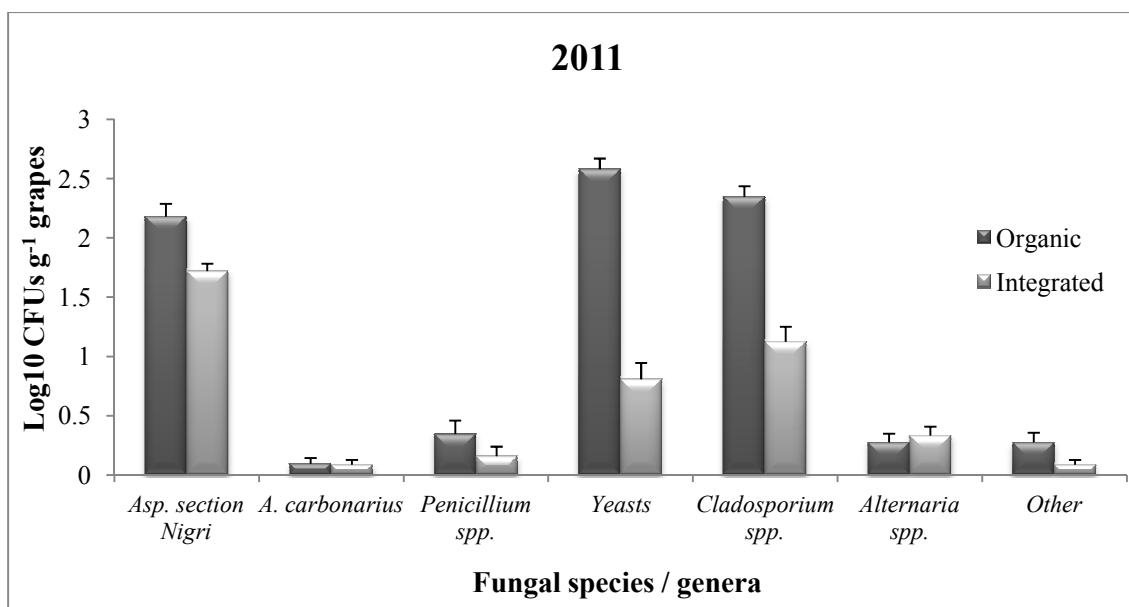


Figure 3.32. Average of Log₁₀ CFUs g⁻¹ grapes of the most dominant fungal species / genera on dilution plating cultures (MEA96), from Soultanina cultivar grape samples of Area C vineyards, in 2011. The error bars represent the standard errors of the means.

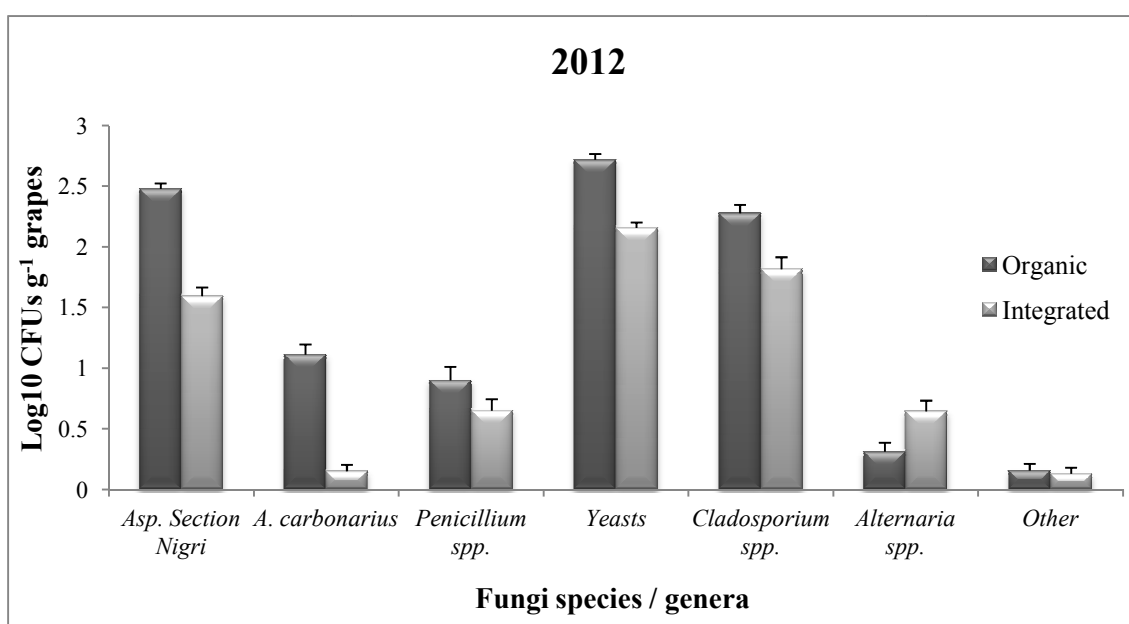


Figure 3.33. Average of Log₁₀ CFUs g⁻¹ grapes of the most dominant fungal species / genera on dilution plating cultures (MEA96), from Soultanina cultivar grape samples of Area C vineyards, in 2012. The error bars represent the standard errors of the means.

3.10. INCIDENCE OF *ASPERGILLUS CARBONARIUS* IN THE THREE AREAS OF RESEARCH

Figure 3.34 shows the overall incidence of *A. carbonarius* in the three areas of research in 2011 and 2012, regardless of the farming system and the cultivar.

As shown, *A. carbonarius* Log₁₀ CFUs g⁻¹ grapes was significantly higher in the Area B, in relation with the other two areas in 2011, while the next year *A. carbonarius* Log₁₀ CFUs g⁻¹ grapes were higher in the Area C, followed by the Area B and the Area A.

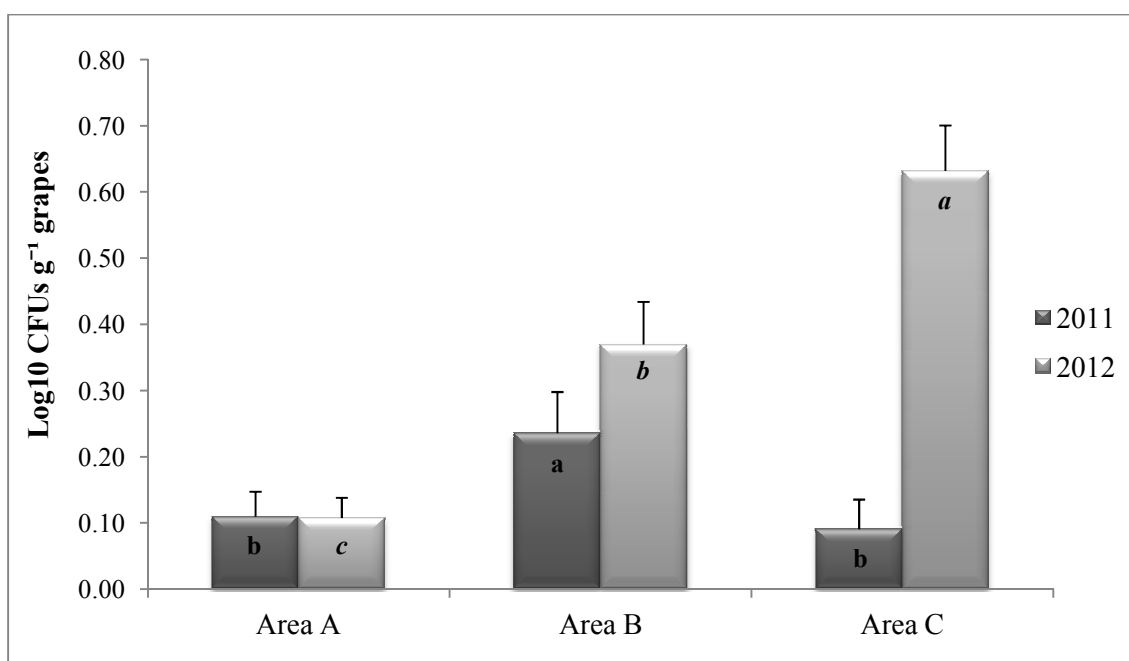


Figure 3.34. *A. carbonarius* Log₁₀ CFUs g⁻¹ grapes on dilution plating cultures (MEA96), from overall grape samples from the three areas of research for 2011 and 2012, regardless of the farming system and the cultivar. The different letters indicate a significant difference of *A. carbonarius* Log₁₀ CFUs g⁻¹ grapes among the three areas (Duncan's multiple range tests, $p < 0.05$) per year of research, while one-way analysis of variance (ANOVA) revealed statistically significant differences among the three areas for both of the years ($F_{2,357} = 4.281$, $p = 0.015$ in 2011 and $F_{2,357} = 23.807$, $p = 0.000$ for 2012). The error bars represent the standard errors of the means.

3.10.1. Incidence of *Aspergillus carbonarius* in Area A

Figure 3.35 shows the incidence of *A. carbonarius* in Kotsifali cultivar vineyards in Area A in 2011 and 2012, in relation to the farming system. *A. carbonarius* Log₁₀ CFUs g⁻¹ grapes were higher in organic farming system for both of the years, although one-way analysis of variance (ANOVA) revealed statistically significant differences between the two farming systems only for the year 2012.

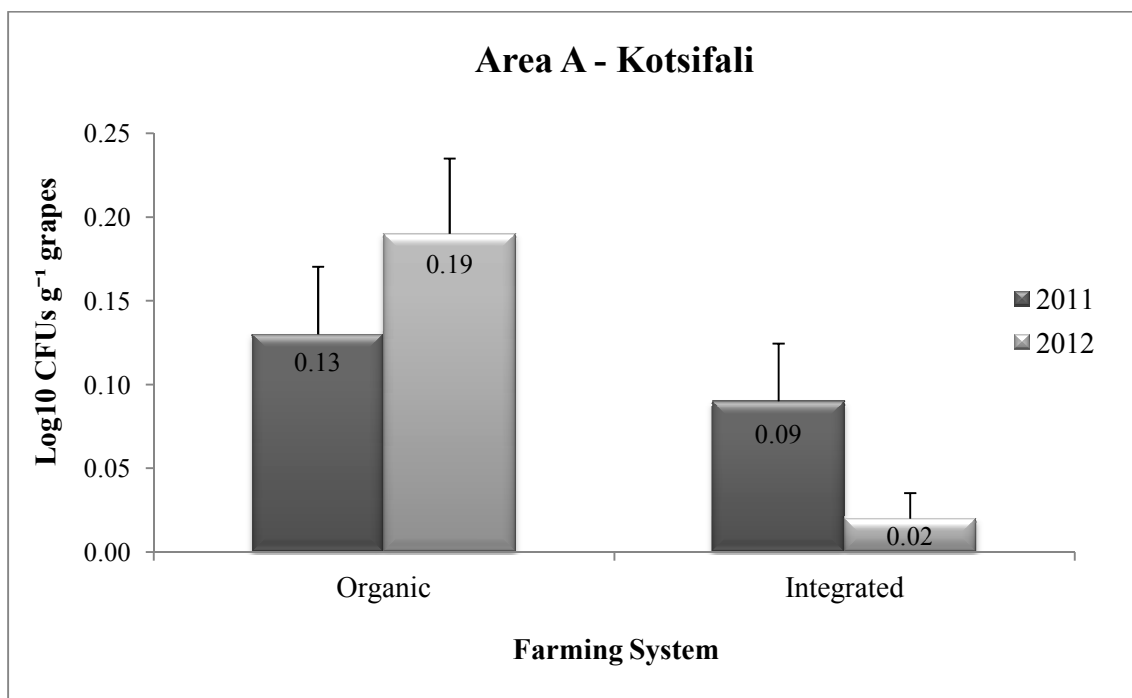


Figure 3.35. *A. carbonarius* Log₁₀ CFUs g⁻¹ grapes on dilution plating cultures (MEA96), from Kotsifali grape samples of Area A for years 2011 and 2012, regarding the farming system, where one-way analysis of variance (ANOVA) revealed no statistically significant differences in 2011 ($F_{1,118} = 0.319$, $p = 0.573$), but statistically significant differences in 2012 ($F_{1,118} = 7.278$, $p = 0.008$). The error bars represent the standard errors of the means.

3.10.2. Incidence of *Aspergillus carbonarius* in Area B

Figure 3.36 shows the incidence of *A. carbonarius* in Soultanina cultivar vineyards in Area B in 2011 and 2012, regarding the farming system. *A. carbonarius* Log₁₀ CFUs g⁻¹ grapes were statistically significant higher in organic farming system in relation to the integrated farming system for both of the years.

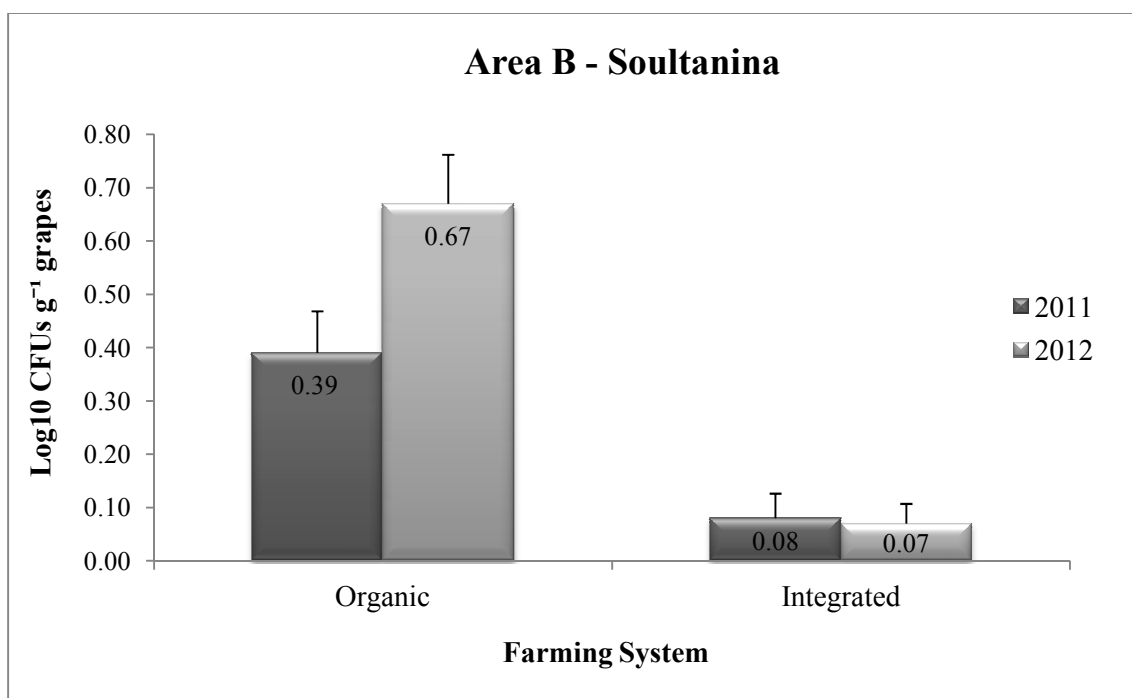


Figure 3.36. *A. carbonarius* Log₁₀ CFUs g⁻¹ grapes on dilution plating cultures (MEA96), from Soultanina grape samples of Area B for the years 2011 and 2012, regarding the farming system, where one-way analysis of variance (ANOVA) revealed statistically significant differences for both of the years 2011 ($F_{1,118} = 11.801$, $p = 0.001$) and 2012 ($F_{1,118} = 38.029$, $p = 0.000$). The error bars represent the standard errors of the means.

3.10.3. Incidence of *Aspergillus carbonarius* in Area C

Figure 3.37 shows the incidence of *A. carbonarius* in Soultanina cultivar vineyards in Area C in 2011 and 2012, regarding the farming system.

Interestingly, *A. carbonarius* Log₁₀ CFUs g⁻¹ grapes seems to be similar, with low rates for both of farming systems for 2011 year. The next year *A. carbonarius* Log₁₀ CFUs g⁻¹ grapes were statistically significant higher in organic farming system, rather than integrated farming system.

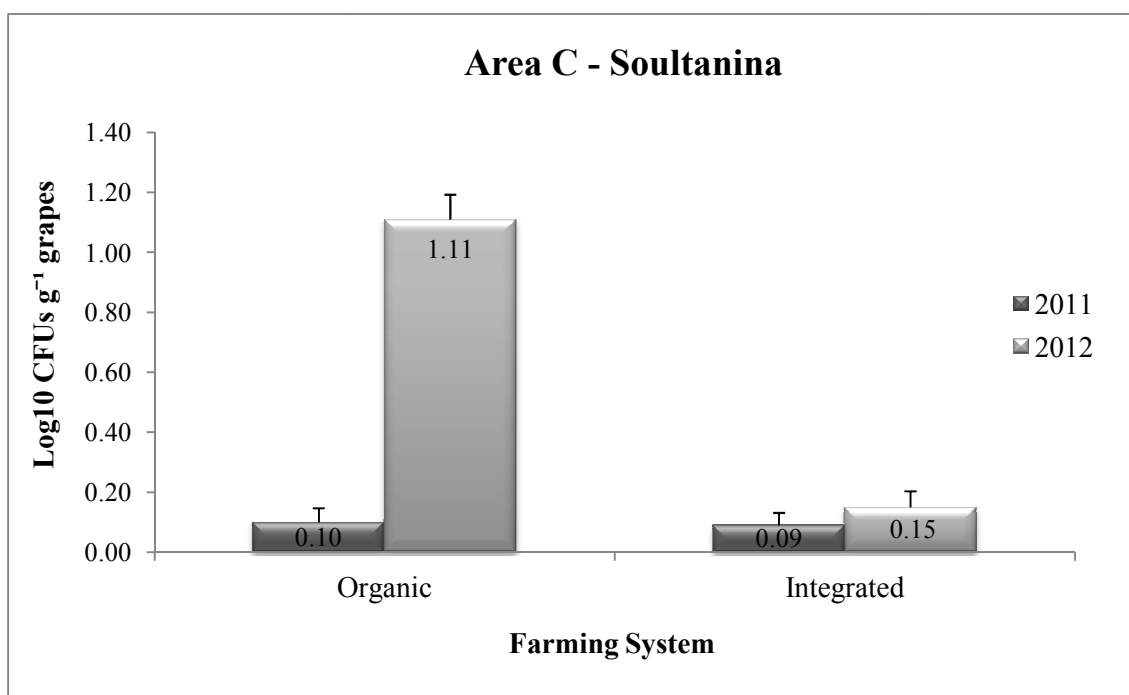


Figure 3.37. *A. carbonarius* Log₁₀ CFUs g⁻¹ grapes on dilution plating cultures (MEA96), from Soultanina grape samples of Area C for the years 2011 and 2012, regarding the farming system. One-way analysis of variance (ANOVA) revealed no statistically significant differences between the farming systems ($F_{1,118} = 0.025$, $p = 0.874$) for the year 2011, but statistically significant differences ($F_{1,118} = 94.668$, $p = 0.000$) for the year 2012. The error bars represent the standard errors of the means.

3.11. RELATIVE INCIDENCE OF *A. CARBONARIUS* IN RELATION WITH OTHER *ASPERGILLUS* SECTION *NIGRI* SPECIES.

In Area A, in 2011 the percentage of *A. carbonarius* in relation with the rest *Aspergillus* section *Nigri* species was about the same between the two farming systems, with *A. carbonarius* to be the 1.18% of the total *Aspergillus* section *Nigri* CFUs g⁻¹ grapes of OFS, and the 1.19% of the total *Aspergillus* section *Nigri* CFUs g⁻¹ grapes of IFS vineyards.

In the same area, in 2012, *A. carbonarius* accounted the 3.88% of the total *Aspergillus* section *Nigri* CFUs g⁻¹ grapes of OFS, and the 4.62% of the total *Aspergillus* section *Nigri* CFUs g⁻¹ grapes of IFS vineyards.

As shown in the Figure 3.38, which present the relative percentage of *A. carbonarius* CFUs g⁻¹ grapes, in relation to the rest of *Aspergillus* section *Nigri* species CFUs g⁻¹ grapes, there are no significant differences between the two farming systems in the case of Kotsifali cultivar, in Area A, for both of the years 2011 and 2012.

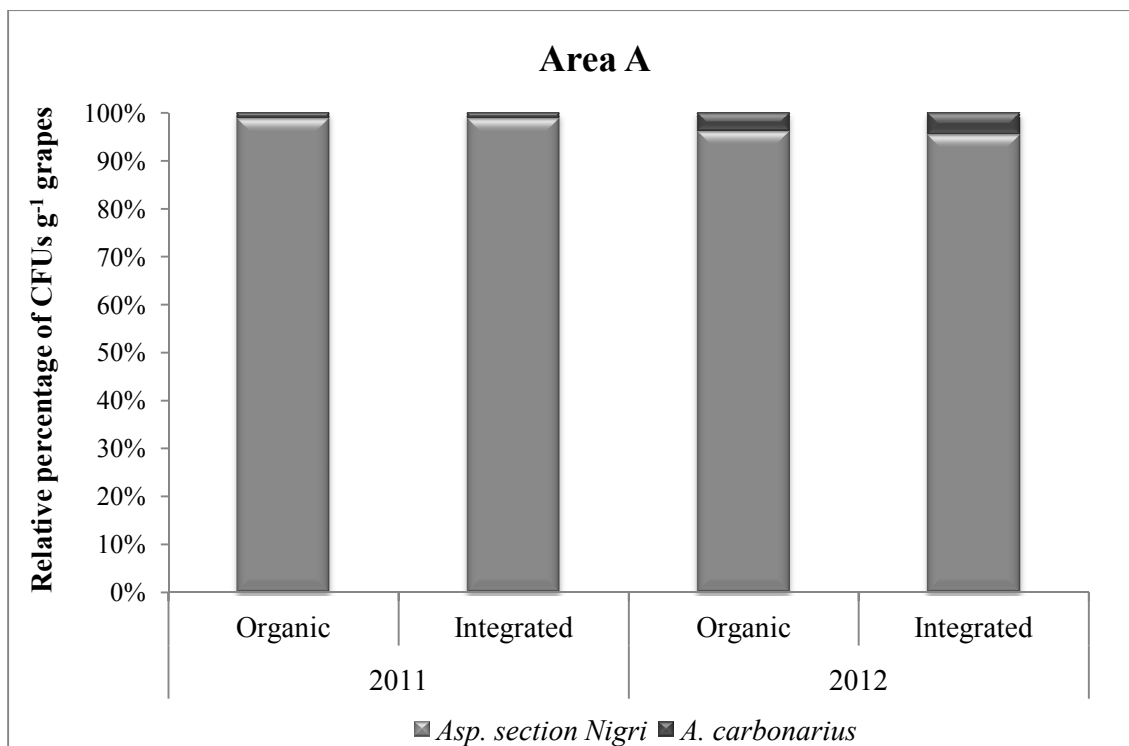


Figure 3.38. Relative percentage of *A. carbonarius* and *Aspergillus* section *Nigri* species CFUs g⁻¹ grapes, in Area A in 2011 and 2012.

Figure 3.39 shows the relative percentage of *A. carbonarius* in relation to the rest of *Aspergillus* section *Nigri* species CFUs g⁻¹ grapes of Soultanina cultivar vineyards in Area B, in relation with the farming system, in 2011 and 2012.

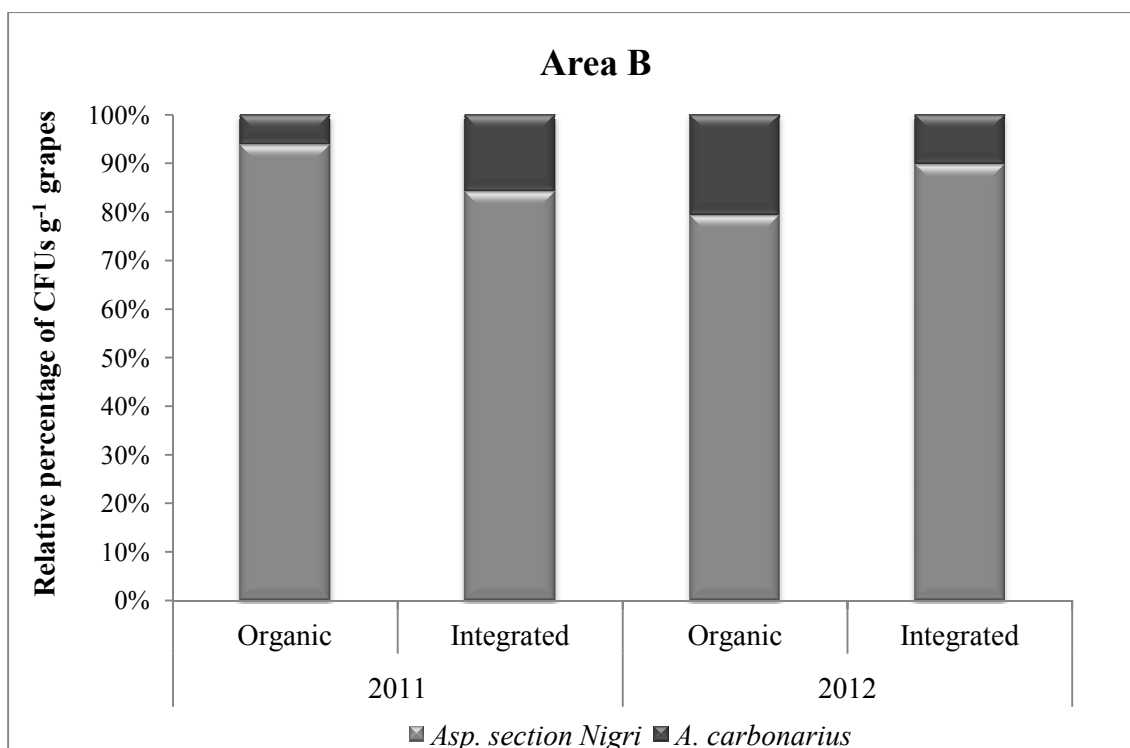


Figure 3.39. Relative percentage of *A. carbonarius* and *Aspergillus* section *Nigri* species CFUs g⁻¹ grapes, in Area B in 2011 and 2012.

In 2011, in contrast to the next year, the percentage of *A. carbonarius* in relation to the rest *Aspergillus* section *Nigri* species, seems to be lower in OFS than in IFS vineyards, although *A. carbonarius* CFUs g⁻¹ grapes were 2.13 times higher in OFS than in IFS vineyards, and that is because of the much less percentage of the rest *Aspergillus* section *Nigri* species at IFS vineyards, in relation with OFS vineyards.

More analytically, in 2011, *A. carbonarius* was the 6.21% of the totally *Aspergillus* section *Nigri* CFUs g⁻¹ grapes in OFS, and the 15.86% in IFS vineyards, while in 2012, *A. carbonarius* consisted the 20.73% in OFS and the 10.26% in IFS, of the total *Aspergillus* section *Nigri* species CFUs g⁻¹ grapes.

Figure 3.40, is shown the relative percentage between *A. carbonarius* and the rest *Aspergillus* section *Nigri* species CFUs g⁻¹ grapes of Soultanina cultivar vineyards in Area C, in relation to the farming system, in 2011 and 2012.

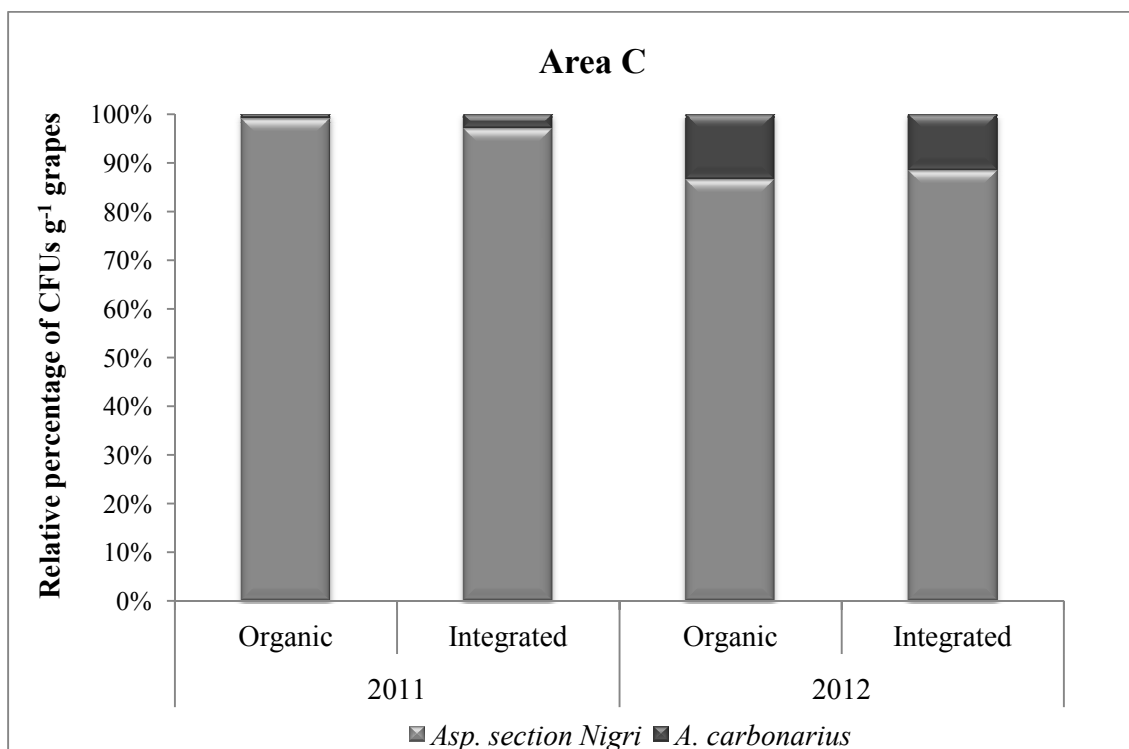


Figure 3.40. Relative percentage of *A. carbonarius* and *Aspergillus* section *Nigri* species CFUs g⁻¹ grapes, in Area C in 2011 and 2012.

Similarly to Area B vineyards, in 2011 the percentage of *A. carbonarius* in relation to the rest *Aspergillus* section *Nigri* species, seems to be lower in OFS than in IFS vineyards, although *A. carbonarius* CFUs g⁻¹ grapes were more in OFS than in IFS vineyards.

In 2011, *A. carbonarius* percentage of the total *Aspergillus* section *Nigri* species CFUs g⁻¹ grapes, was 0.91% in OFS and 2.95% in IFS vineyards, while in 2012 was 13.42% in OFS and 11.55% in IFS vineyards.

MYCOLOGICAL ANALYSIS OF SOIL RESULTS (2011-2012)

3.12. FARMING SYSTEM EFFECTS ON THE INCIDENCE OF THE MOST DOMINANT FUNGAL SPECIES AND GENERA IN SOIL

3.12.1. Farming system effects on soil fungal diversity in Area A in Kotsifali cultivar vineyards

Figures 3.41 and 3.42 show the effect of the farming system on the incidence of the most dominant fungal species and genera (found on grapes) in soil of Kotsifali cultivar vineyards in Area A, in 2011 and 2012. The results are presented as the average of Log_{10} CFUs g^{-1} soil, for each of the fungal species and genera.

One-way analysis of variance (ANOVA) of fungal species and genera Log_{10} CFUs g^{-1} soil of Kotsifali cultivar vineyards of Area A, in 2011, revealed statistically significant differences between the farming systems for *Aspergillus* section *Nigri* species ($F_{1,118} = 10.691$, $p = 0.001$) and *Penicillium spp.* ($F_{1,118} = 16.772$, $p = 0.000$), while there were not significant differences between the farming systems for *A. carbonarius* ($F_{1,118} = 0.892$, $p = 0.347$), Yeasts ($F_{1,118} = 0.016$, $p = 0.901$), *Cladosporium spp.* ($F_{1,118} = 0.918$, $p = 0.340$) and *Alternaria spp.* ($F_{1,118} = 0.439$, $p = 0.509$).

In 2012, one-way analysis of variance (ANOVA) of fungal species and genera Log_{10} CFUs g^{-1} soil of Kotsifali cultivar vineyards of Area A, revealed statistically significant differences between the farming systems for *Aspergillus* section *Nigri* species ($F_{1,118} = 4.406$, $p = 0.038$), *Penicillium spp.* ($F_{1,118} = 27.972$, $p = 0.000$) and *Cladosporium spp.* ($F_{1,118} = 33.918$, $p = 0.000$), but no statistically significant differences between the farming systems for *A. carbonarius* ($F_{1,118} = 0.784$, $p = 0.378$), Yeasts ($F_{1,118} = 2.061$, $p = 0.154$) and *Alternaria spp.* ($F_{1,118} = 3.717$, $p = 0.056$).

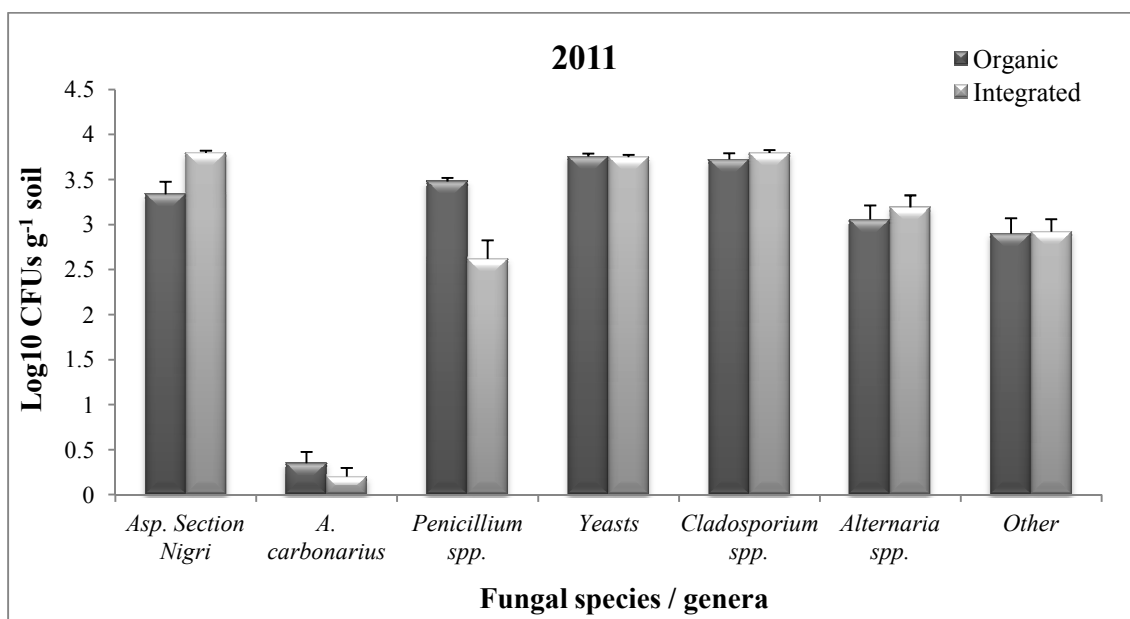


Figure 3.41. Average of Log₁₀ CFUs g⁻¹ soil of the most dominant fungal species / genera on soil dilution plating cultures (DRBC), from Kotsifali cultivar vineyards of Area A soil samples, in 2011. The error bars represent the standard errors of the means.

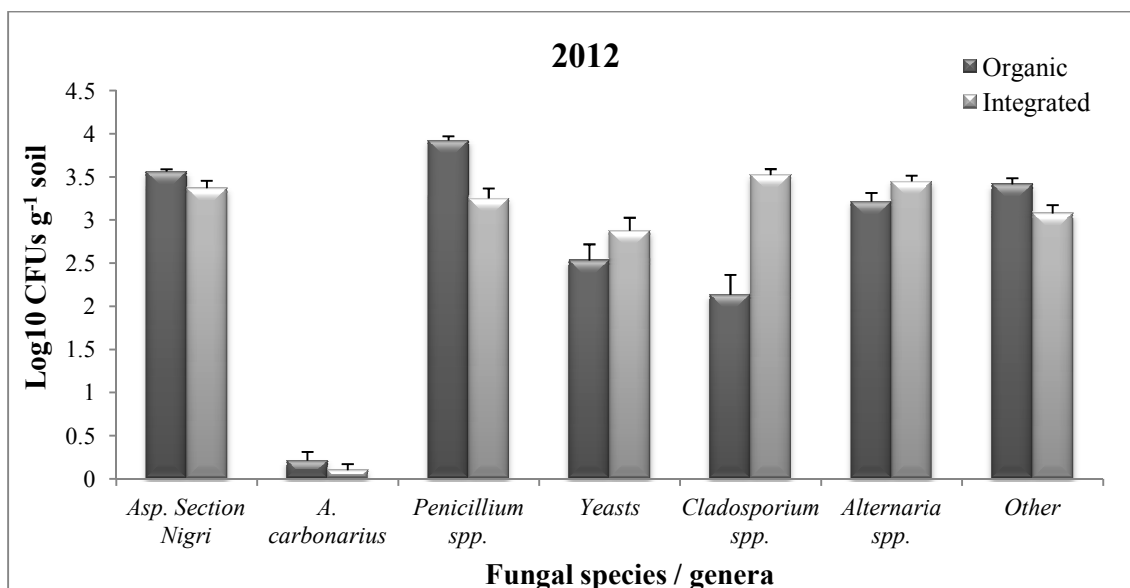


Figure 3.42. Average of Log₁₀ CFUs g⁻¹ soil of the most dominant fungal species / genera on soil dilution plating cultures (DRBC), from Kotsifali cultivar vineyards of Area A soil samples, in 2012. The error bars represent the standard errors of the means.

3.12.2. Farming system effects on soil fungal diversity in Soultanina cultivar vineyards in Area B

Figures 3.43 and 3.44 show the effect of the farming system on the incidence of the most dominant fungal species and genera in soil of Soultanina cultivar vineyards, in Area B, in 2011 and 2012. The results are presented as the average of Log_{10} CFUs g^{-1} soil, for each of fungal species and genera.

One-way analysis of variance (ANOVA) of fungal species and genera Log_{10} CFUs g^{-1} soil of Soultanina cultivar vineyards in Area B, in 2011, revealed statistically significant differences between the farming systems for *A. carbonarius* ($F_{1,118} = 43.818$, $p = 0.000$), Yeasts ($F_{1,118} = 15.141$, $p = 0.000$) and *Cladosporium spp.* ($F_{1,118} = 6.963$, $p = 0.009$), while there were not significant differences between the farming systems for *Aspergillus* section *Nigri* species ($F_{1,118} = 0.420$, $p = 0.518$), *Penicillium spp.* ($F_{1,118} = 3.259$, $p = 0.074$) and *Alternaria spp.* ($F_{1,118} = 2.163$, $p = 0.144$).

In 2012, one-way analysis of variance (ANOVA) of fungal species and genera Log_{10} CFUs g^{-1} soil of Soultanina cultivar vineyards of Area B, revealed statistically significant differences between the farming systems for *A. carbonarius* ($F_{1,118} = 56.727$, $p = 0.000$), *Penicillium spp.* ($F_{1,118} = 7.858$, $p = 0.006$), Yeasts ($F_{1,118} = 26.459$, $p = 0.000$) and *Cladosporium spp.* ($F_{1,118} = 27.545$, $p = 0.000$), but no statistically significant differences between the farming systems for *Aspergillus* section *Nigri* species ($F_{1,118} = 0.277$, $p = 0.599$) and *Alternaria spp.* ($F_{1,118} = 1.162$, $p = 0.283$).

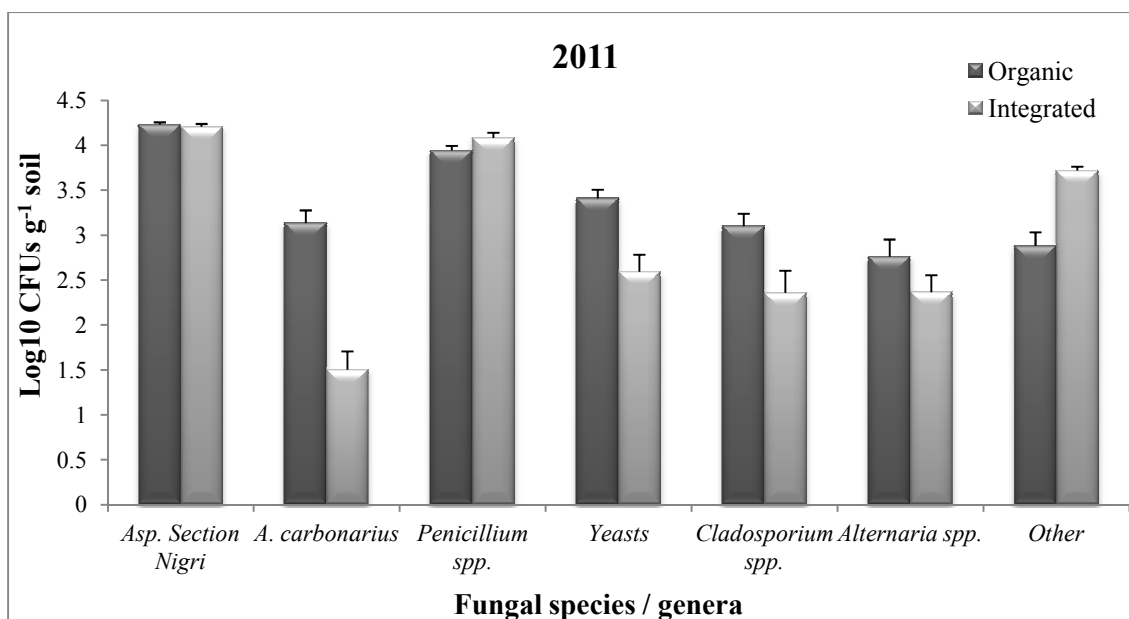


Figure 3.43. Average of Log₁₀ CFUs g⁻¹ soil of the most dominant fungal species / genera on soil dilution plating cultures (DRBC), from Soultanina cultivar vineyards of Area B soil samples, in 2011. The error bars represent the standard errors of the means.

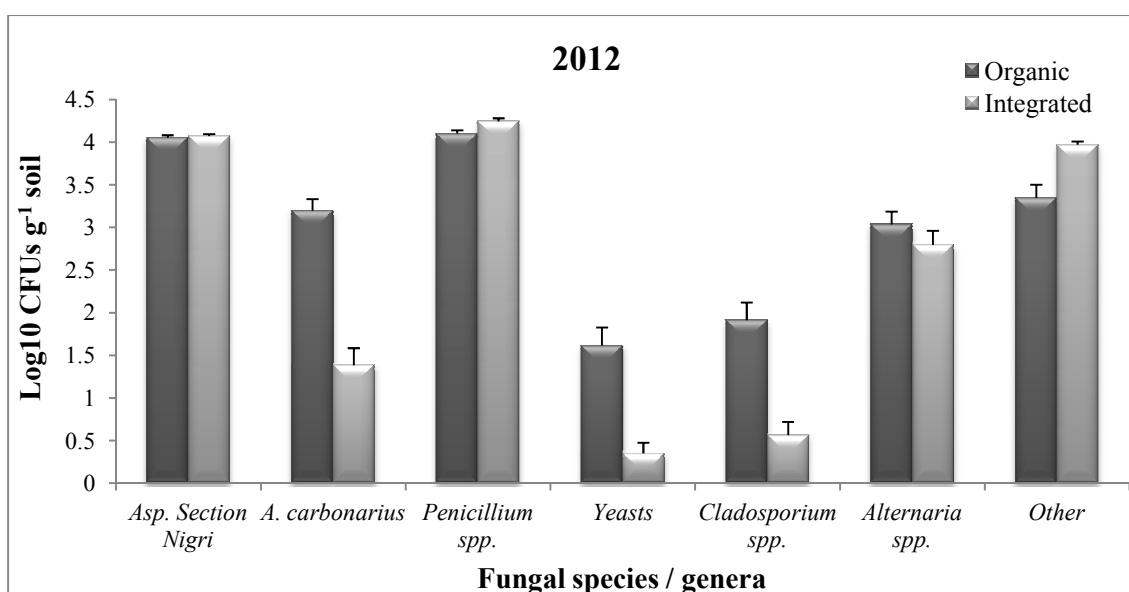


Figure 3.44. Average of Log₁₀ CFUs g⁻¹ soil of the most dominant fungal species / genera on soil dilution plating cultures (DRBC), from Soultanina cultivar vineyards of Area B soil samples, in 2012. The error bars represent the standard errors of the means.

3.12.3. Farming system effects on soil fungal diversity in Soultanina cultivar vineyards in Area C

Figures 3.45 and 3.46 show the effect of the farming system to the incidence of the most dominant fungal species and genera in soil of Soultanina cultivar vineyards, in Area C, in 2011 and 2012. The results are presented as the average of Log_{10} CFUs g^{-1} soil, for each of fungal species and genera.

One-way analysis of variance (ANOVA) of fungal species and genera Log_{10} CFUs g^{-1} soil of Soultanina cultivar vineyards in Area C, in 2011, revealed statistically significant differences between the farming systems for *Aspergillus* section *Nigri* species ($F_{1,118} = 80.393$, $p = 0.000$), *Penicillium* spp. ($F_{1,118} = 20.422$, $p = 0.000$) and *Cladosporium* spp. ($F_{1,118} = 23.136$, $p = 0.000$), while there were not significant differences between the farming systems for *A. carbonarius* ($F_{1,118} = 2.134$, $p = 0.147$), Yeasts ($F_{1,118} = 2.834$, $p = 0.095$) and *Alternaria* spp. ($F_{1,118} = 0.050$, $p = 0.824$).

In 2012, one-way analysis of variance (ANOVA) of fungal species and genera Log_{10} CFUs g^{-1} soil of Soultanina cultivar vineyards in Area C, revealed statistically significant differences between the farming systems for *A. carbonarius* ($F_{1,118} = 77.370$, $p = 0.000$), Yeasts ($F_{1,118} = 4.793$, $p = 0.031$) and *Alternaria* spp. ($F_{1,118} = 22.455$, $p = 0.000$), whereas there were not statistically significant differences between the farming systems for *Aspergillus* section *Nigri* species ($F_{1,118} = 3.490$, $p = 0.064$), *Penicillium* spp. ($F_{1,118} = 1.937$, $p = 0.167$) and *Cladosporium* spp. ($F_{1,118} = 1.422$, $p = 0.235$).

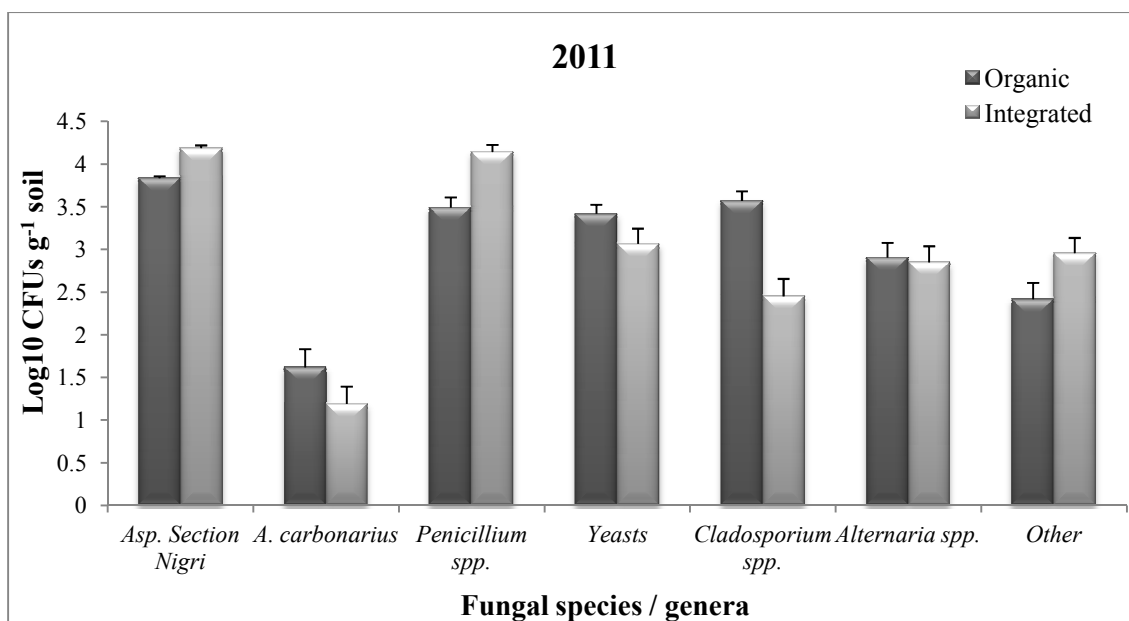


Figure 3.45. Average of Log₁₀ CFUs g⁻¹ soil of the most dominant fungal species / genera on soil dilution plating cultures (DRBC), from Soultanina cultivar vineyards of Area C soil samples, in 2011. The error bars represent the standard errors of the means.

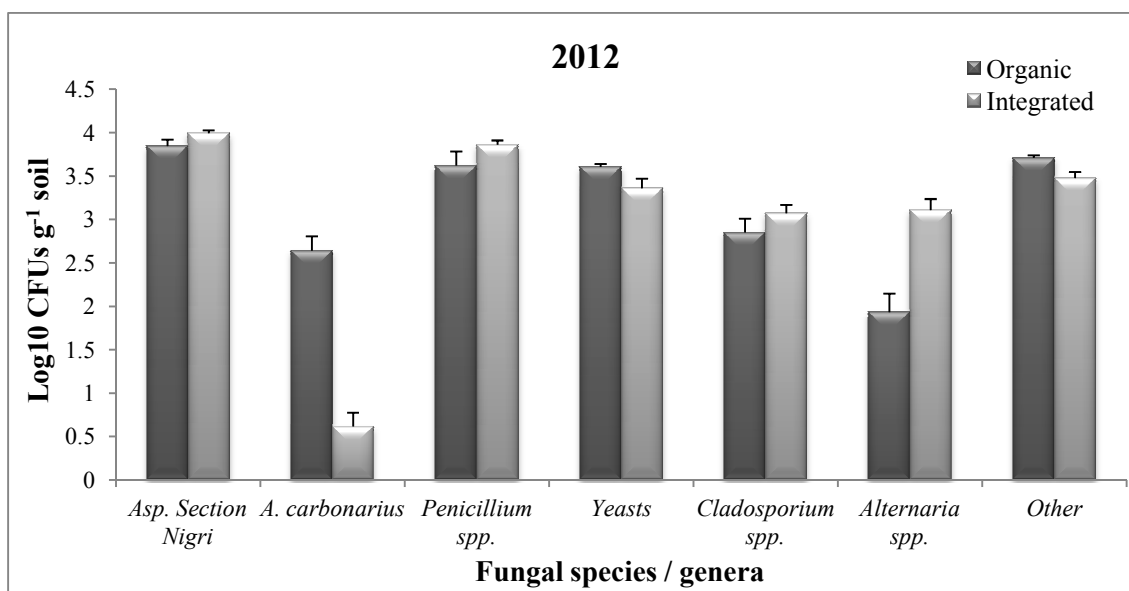


Figure 3.46. Average of Log₁₀ CFUs g⁻¹ soil of the most dominant fungal species / genera on soil dilution plating cultures (DRBC), from Soultanina cultivar vineyards of Area C soil samples, in 2012. The error bars represent the standard errors of the means.

3.13. INCIDENCE OF *ASPERGILLUS CARBONARIUS* IN SOIL OF THE THREE AREAS OF RESEARCH

Figure 3.47 shows the overall incidence of *A. carbonarius* in the soil of the three areas of research in 2011 and 2012, regardless of the farming system and the cultivar of the vineyards. As shown, *A. carbonarius* Log₁₀ CFUs g⁻¹ soil was significantly higher in the Area B, followed by the Area C and the Area A for both of the years of research.

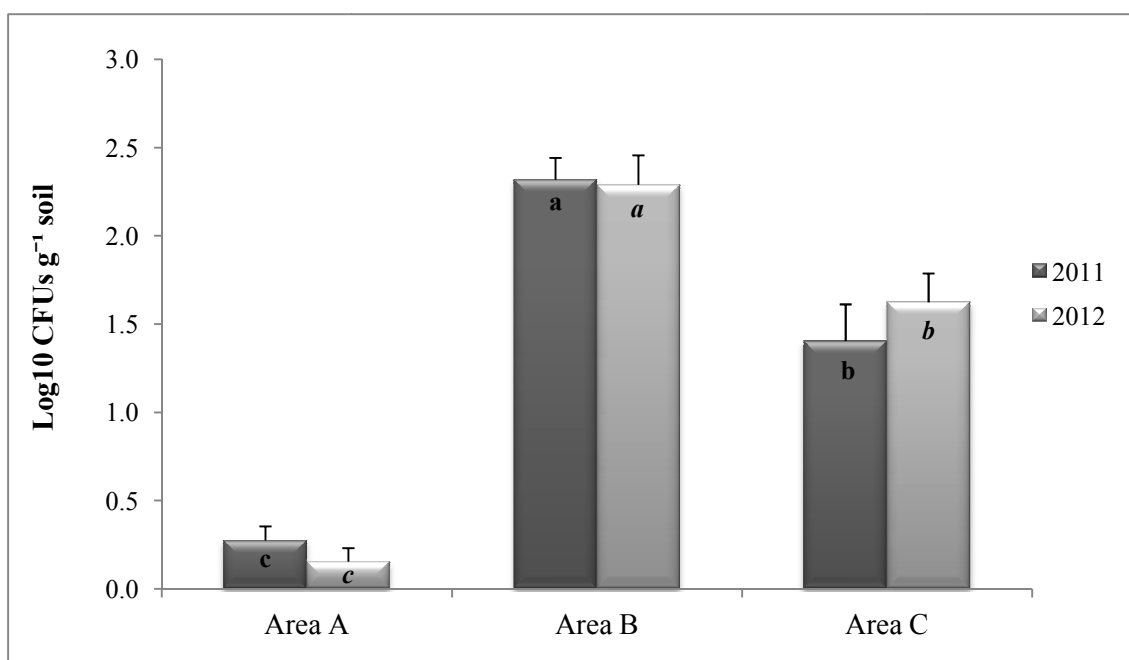


Figure 3.47. *A. carbonarius* Log₁₀ CFUs g⁻¹ soil on dilution plating cultures (DRBC), from overall soil samples from the three areas of research for 2011 and 2012, regardless of the farming system and the cultivar of the vineyards. The different letters indicate a significant difference of *A. carbonarius* Log₁₀ CFUs g⁻¹ soil among the three areas (Duncan's multiple range tests, $p < 0.05$) per year of research, while one-way analysis of variance (ANOVA) revealed statistically significant differences among the three areas for both of the years ($F_{2,357} = 64.399$, $p = 0.000$ for 2011 and $F_{2,357} = 76.408$, $p = 0.000$ for 2012). The error bars represent the standard errors of the means.

3.13.1. Incidence of *A. carbonarius* in the soil of Area A vineyards

Figure 3.48 shows the incidence of *A. carbonarius* in the soil of Kotsifali cultivar vineyards in Area A in 2011 and 2012, in relation to the farming system. *A. carbonarius* Log₁₀ CFUs g⁻¹ soil were higher in organic farming system vineyards soil for both of the years, although one-way analysis of variance (ANOVA) revealed no statistically significant differences between the two farming systems for both of the years.

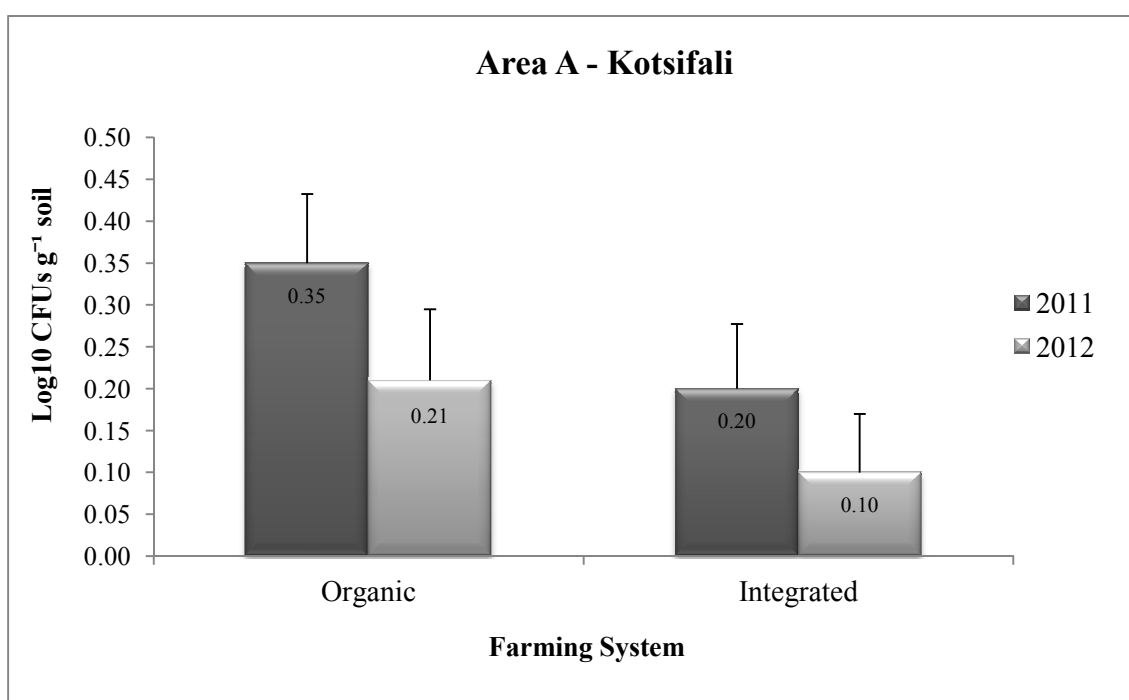


Figure 3.48. *A. carbonarius* Log₁₀ CFUs g⁻¹ soil on dilution plating cultures (DRBC), from soil samples of Area A vineyards for the years 2011 and 2012, regarding the farming system, where one-way analysis of variance (ANOVA) revealed no statistically significant differences for both of the years ($F_{1,118} = 0.892$, $p = 0.347$ for 2011 and $F_{1,118} = 0.784$, $p = 0.378$ for 2012). The error bars represent the standard errors of the means.

3.13.2. Incidence of *A. carbonarius* in the soil of Area B vineyards

Figure 3.49 shows the incidence of *A. carbonarius* in the soil of Soultanina cultivar vineyards in Area B in 2011 and 2012, regarding the farming system. *A. carbonarius* Log₁₀ CFUs g⁻¹ soil were statistically significant higher in organic farming system vineyards soil in relation to the integrated farming system vineyards for both of the years.

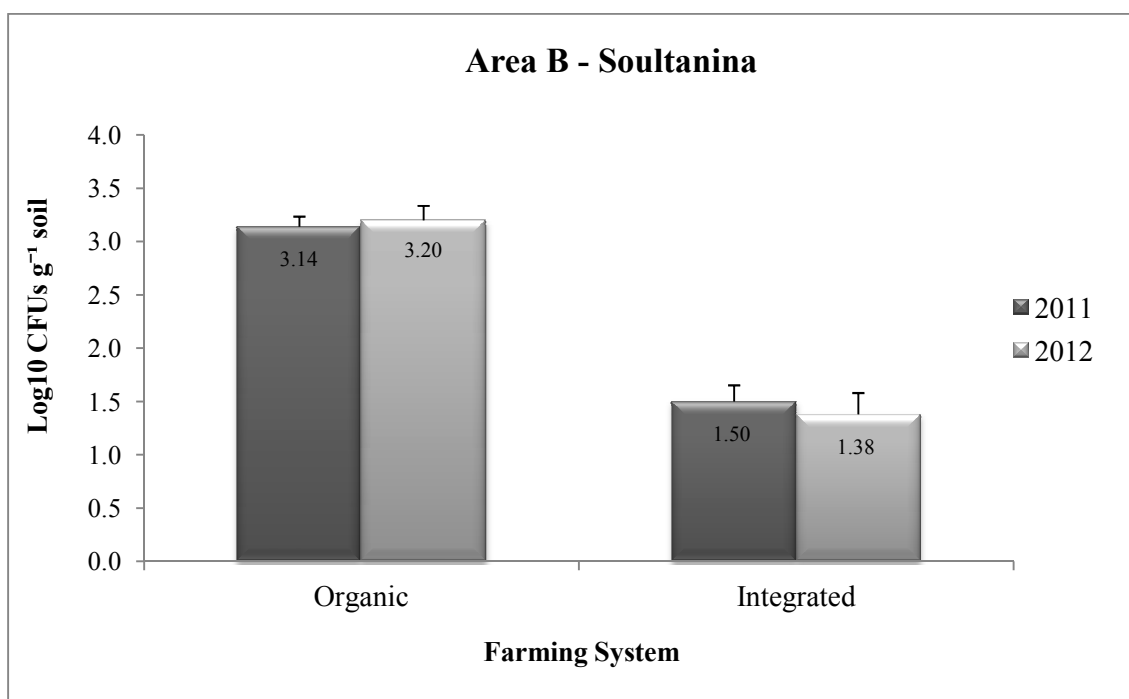


Figure 3.49. *A. carbonarius* Log₁₀ CFUs g⁻¹ soil on dilution plating cultures (DRBC), from soil samples of Area B vineyards for the years 2011 and 2012, regarding the farming system, where one-way analysis of variance (ANOVA) revealed statistically significant differences for both of the years ($F_{1,118} = 43.818$, $p = 0.000$ and $F_{1,118} = 56.727$, $p = 0.000$ for 2011 and 2012, respectively). The error bars represent the standard errors of the means.

3.13.3. Incidence of *A. carbonarius* in the soil of Area C vineyards

Figure 3.50 shows the incidence of *A. carbonarius* in Soultanina cultivar vineyards in Area C in 2011 and 2012, regarding the farming system.

Interestingly, *A. carbonarius* Log₁₀ CFUs g⁻¹ grapes seems to be similar, with low rates for both of farming systems for 2011 year. The next year *A. carbonarius* Log₁₀ CFUs g⁻¹ grapes were statistically significant higher in organic farming system, rather than integrated farming system.

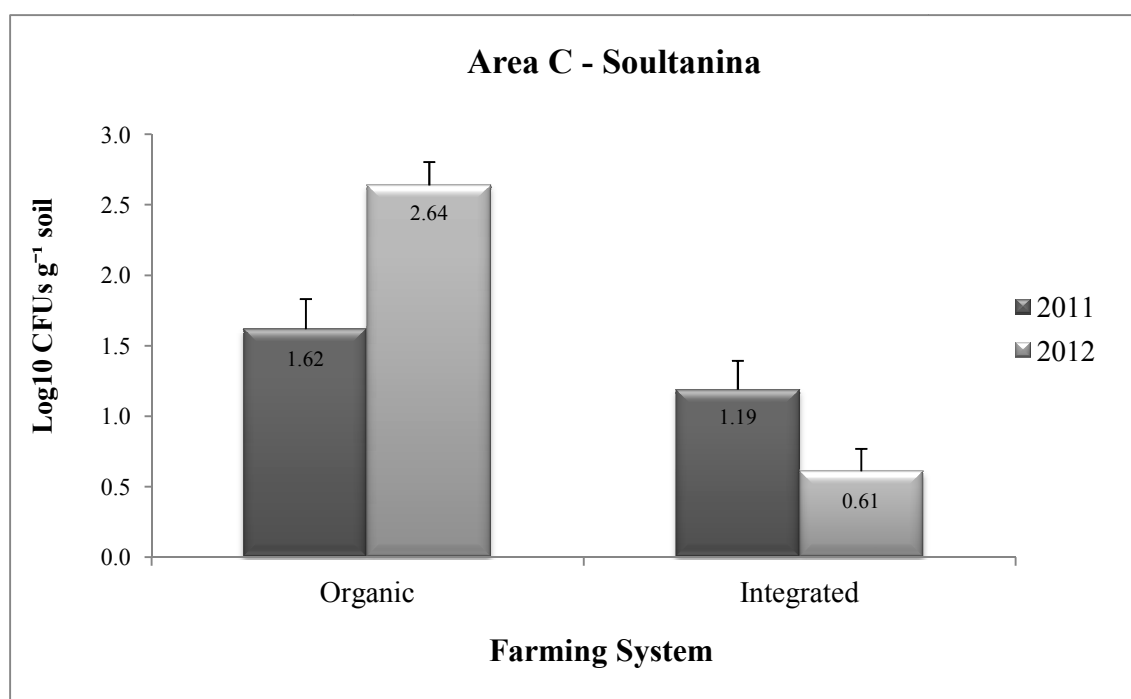


Figure 3.50. *A. carbonarius* Log₁₀ CFUs g⁻¹ soil on dilution plating cultures (DRBC), from soil samples of Area C vineyards for the years 2011 and 2012, regarding the farming system, where one-way analysis of variance (ANOVA) revealed no statistically significant differences for 2011 ($F_{1,118} = 2.134$, $p = 0.147$) but statistically significant differences for 2012 ($F_{1,118} = 77.370$, $p = 0.000$). The error bars represent the standard errors of the means.

3.14. RELATIVE INCIDENCE OF *ASPERGILLUS* SPECIES IN SOIL

Figure 3.51 shows the relative percentage of *Aspergillus* species CFUs g⁻¹ soil of Kotsifali cultivar vineyards in Area A, in relation with the farming systems in 2011 and 2012.

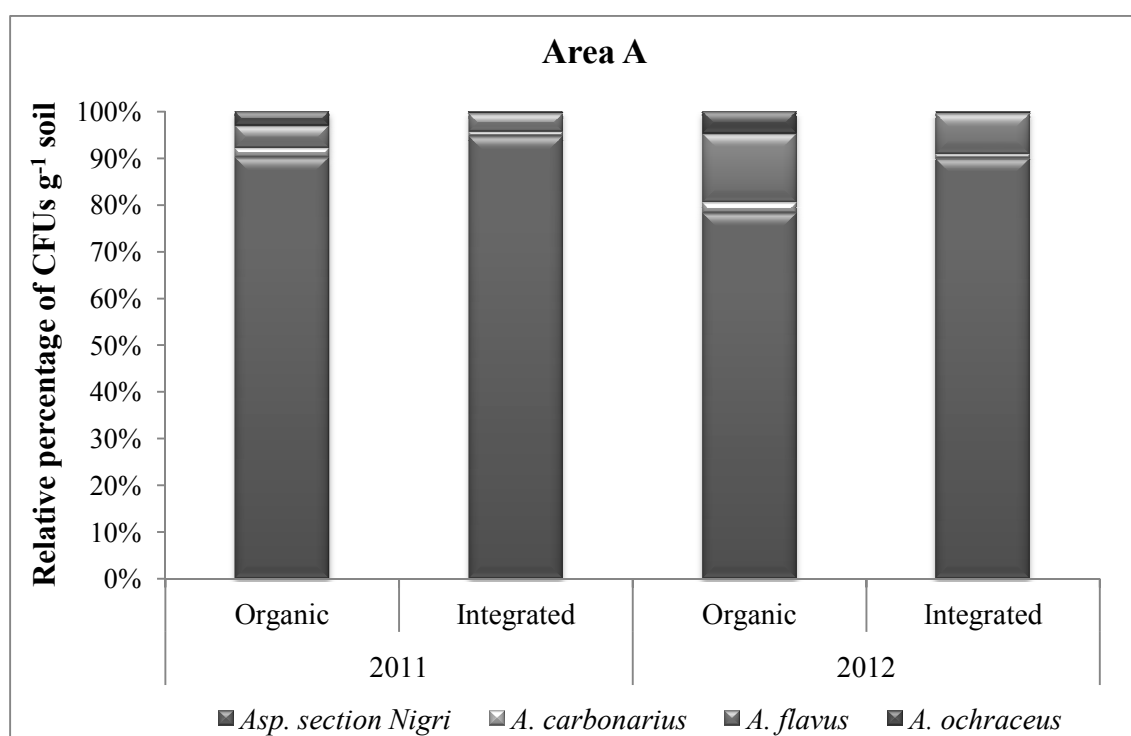


Figure 3.51. Relative percentage of *A. carbonarius*, *A. flavus*, *A. ochraceus* and *Aspergillus* section *Nigri* species CFUs g⁻¹ soil, in Area A in 2011 and 2012.

In 2011, the total of *Aspergillus* species CFUs g⁻¹ soil, was consisted of 90.12% of *Aspergillus* section *Nigri* species, followed by 4.78% of *A. flavus*, 3.11% of *A. ochraceus* and 1.99% of *A. carbonarius* in OFS vineyards, while in IFS vineyards, the total of *Aspergillus* species CFUs g⁻¹ soil was consisted of 94.65% of *Aspergillus* section *Nigri* species, 3.94% of *A. flavus*, 0.94% of *A. carbonarius* and 0.48% of *A. ochraceus*.

In 2012, the percentage of *Aspergillus* species CFUs g^{-1} soil was 78.24% for *Aspergillus* section *Nigri* species, 14.70% for *A. flavus*, 4.81% for *A. ochraceus* and 2.25% for *A. carbonarius*, in OFS vineyards, while in IFS vineyards the percentage of *Aspergillus* species was 89.89% for *Aspergillus* section *Nigri* species, 8.75% for *A. flavus*, 0.90% for *A. carbonarius* and 0.46% for *A. ochraceus*.

Figure 3.52 shows the relative percentage of *Aspergillus* species CFUs g^{-1} soil of Soultanina cultivar vineyards in Area B, in relation with the farming system, for the years 2011 and 2012.

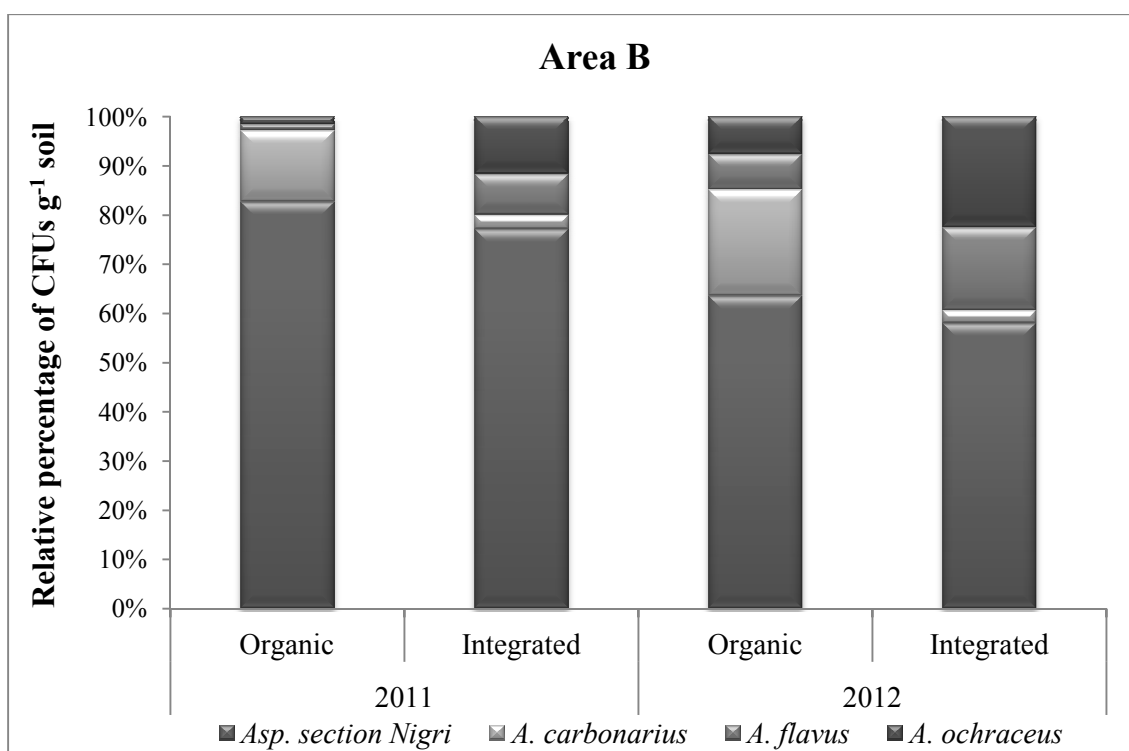


Figure 3.52. Relative percentage of *A. carbonarius*, *A. flavus*, *A. ochraceus* and *Aspergillus* section *Nigri* species CFUs g^{-1} soil, in Area B in 2011 and 2012.

The percentage of *Aspergillus* species CFUs g^{-1} soil, at the year 2011, was 82.60% for *Aspergillus* section *Nigri* species, 14.50% for *A. carbonarius*, 1.64% for *A. ochraceus* and 1.26% for *A. flavus* in OFS vineyards, while in IFS vineyards the

percentage of *Aspergillus* species was 77.09% for *Aspergillus* section *Nigri* species, 11.71% for *A. ochraceus*, 8.36% for *A. carbonarius* and 2.84% for *A. flavus*.

In 2012, the relative percentage of *Aspergillus* species CFUs g⁻¹ soil, was 63.49% for *Aspergillus* section *Nigri* species, 21.58% for *A. carbonarius*, 7.68% for *A. ochraceus* and 7.26% for *A. flavus* in OFS vineyards, while in IFS vineyards the relative percentage of *Aspergillus* species was 57.94% for *Aspergillus* section *Nigri* species, 22.62% for *A. ochraceus*, 16.86% for *A. flavus* and 2.58% for *A. carbonarius*.

Figure 3.53 shows the relative percentage of *Aspergillus* species CFUs g⁻¹ soil of Soultanina cultivar vineyards in Area C, in relation with the farming system, for the years 2011 and 2012.

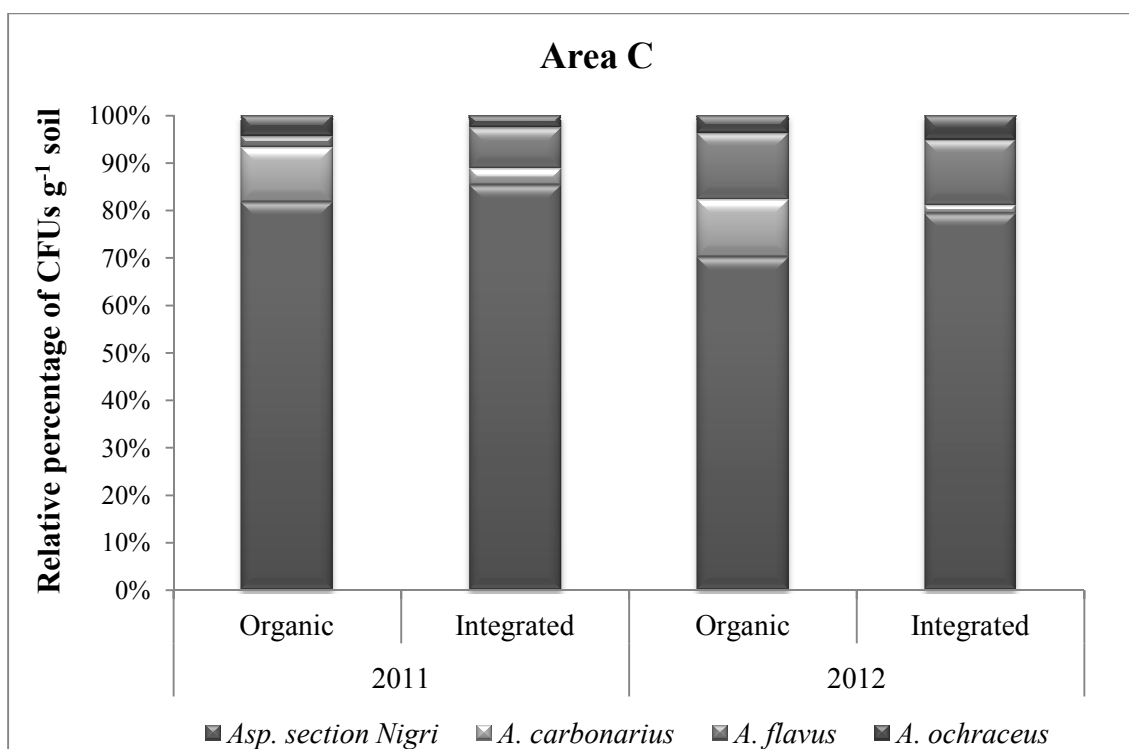


Figure 3.53. Relative percentage of *A. carbonarius*, *A. flavus*, *A. ochraceus* and *Aspergillus* section *Nigri* species CFUs g⁻¹ soil, in Area C in 2011 and 2012.

The relative percentage of the total of *Aspergillus* species CFUs g⁻¹ soil for 2011, was 81.67% for *Aspergillus* section *Nigri* species, 11.54% for *A. carbonarius*, 4.40% for *A. ochraceus* and 2.39% for *A. flavus* in OFS vineyards, while in IFS vineyards the relative percentage was 85.21% for *Aspergillus* section *Nigri* species, 8.68% for *A. flavus*, 3.52% for *A. carbonarius* and 2.59% for *A. ochraceus*.

In 2012, the relative percentage of *Aspergillus* species CFUs g⁻¹ soil, was 70.03% for *Aspergillus* section *Nigri* species, 14.00% for *A. flavus*, 12.19% for *A. carbonarius* and 3.78% for *A. ochraceus* in OFS vineyards, while in IFS vineyards, the relative percentage of *Aspergillus* species CFUs g⁻¹ soil, was 79.22% for *Aspergillus* section *Nigri* species, 13.85% for *A. flavus*, 5.17% for *A. ochraceus* and 1.77% for *A. carbonarius*.

3.15. CORRELATION OF *A. CARBONARIUS* OCCURRENCE IN GRAPES AND SOIL

Pearson product-moment correlation coefficient calculation of *A. carbonarius* Log₁₀ CFUs g⁻¹ grapes and soil, of the mycological analysis results of 2011, revealed a significant correlation (at the <0.01 level) for Area A ($r = +0.336$, $DF = 118$, $p = 0.000$) and Area B ($r = +0.259$, $DF = 118$, $p = 0.004$), but no significant correlation was found for Area C ($r = +0.069$, $DF = 118$, $p = 0.453$).

Regarding the mycological analysis results of 2012, there was a significant correlation (at the <0.01 level) between the *A. carbonarius* Log₁₀ CFUs g⁻¹ grapes and soil, in the Area B ($r = +0.264$, $DF = 118$, $p = 0.004$) and the Area C ($r = +0.341$, $DF = 118$, $p = 0.000$), while there was not significant correlation for the Area A ($r = +0.064$, $DF = 118$, $p = 0.485$).

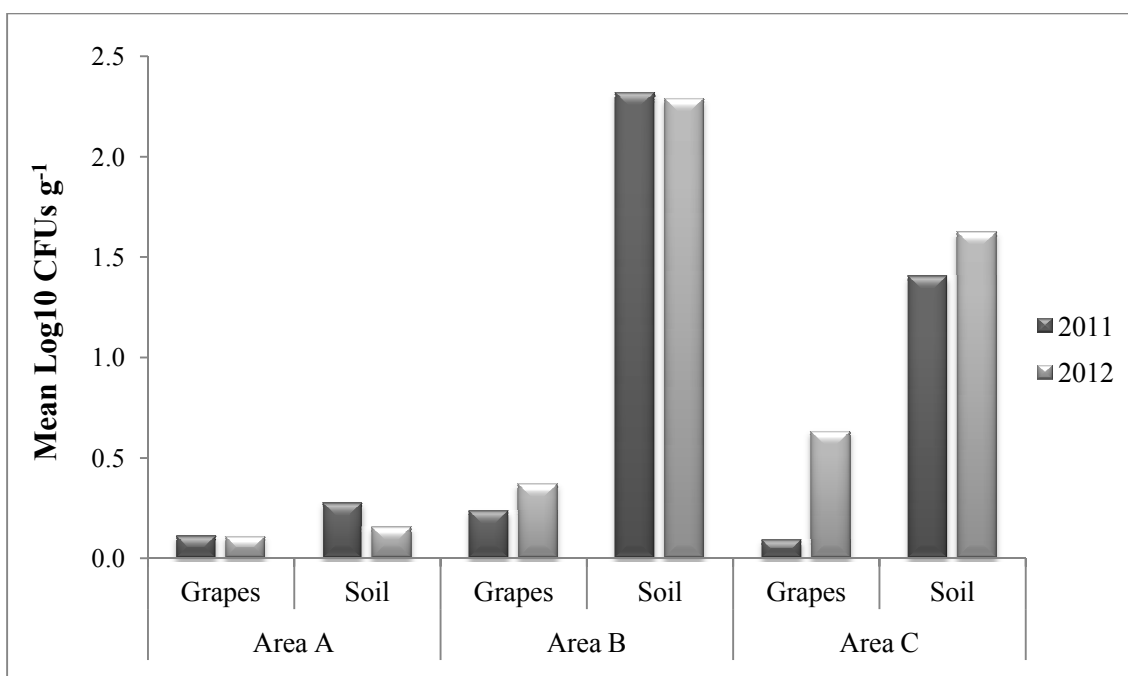


Figure 3.54. Mean values of *A. carbonarius* Log₁₀ CFUs g⁻¹ grapes and soil, per area and year of the research.

ADDITIONAL RESEARCH

3.16. OCHRATOXIN A PRODUCTION ABILITY OF *ASPERGILLUS CARBONARIUS* ISOLATES

Coconut Cream Agar (CCA 50%) cultures of *A. carbonarius* isolates, from the mycological analysis of grapes and soil, of the years 2011 and 2012, examined under UV-A light (365 nm) for blue-fluorescence, as an indication for their ability to produce Ochratoxin A.

In Area A, the 89.9% of the year 2011 and the 93.7% of the year 2012 *A. carbonarius* isolates were ochratoxigenic. In Area B, the 72.8% of the year 2011 and the 71.2% of the year 2012 isolates had the ability of OTA production. Finally, in Area C, the 78.9% of the year 2011 and the 75.3% of the year 2012 were also ochratoxigenic (Figure 3.55).

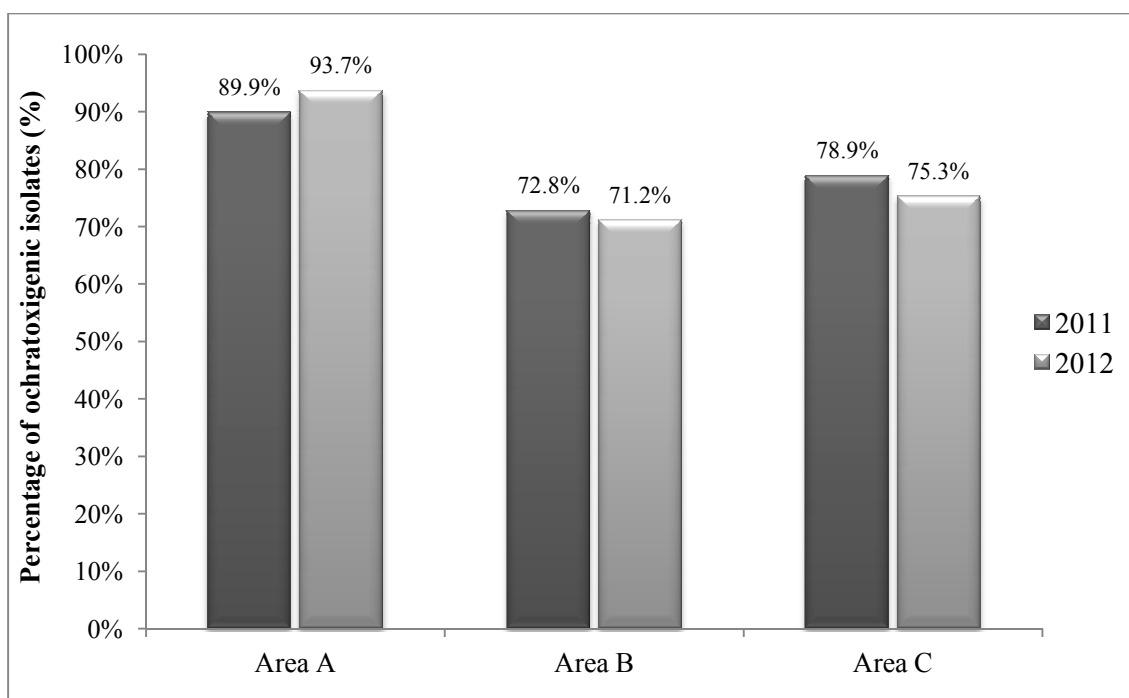


Figure 3.55. Percentage of *A. carbonarius* isolates from the three areas of research in 2011 and 2012 which had the ability to produce Ochratoxin A.

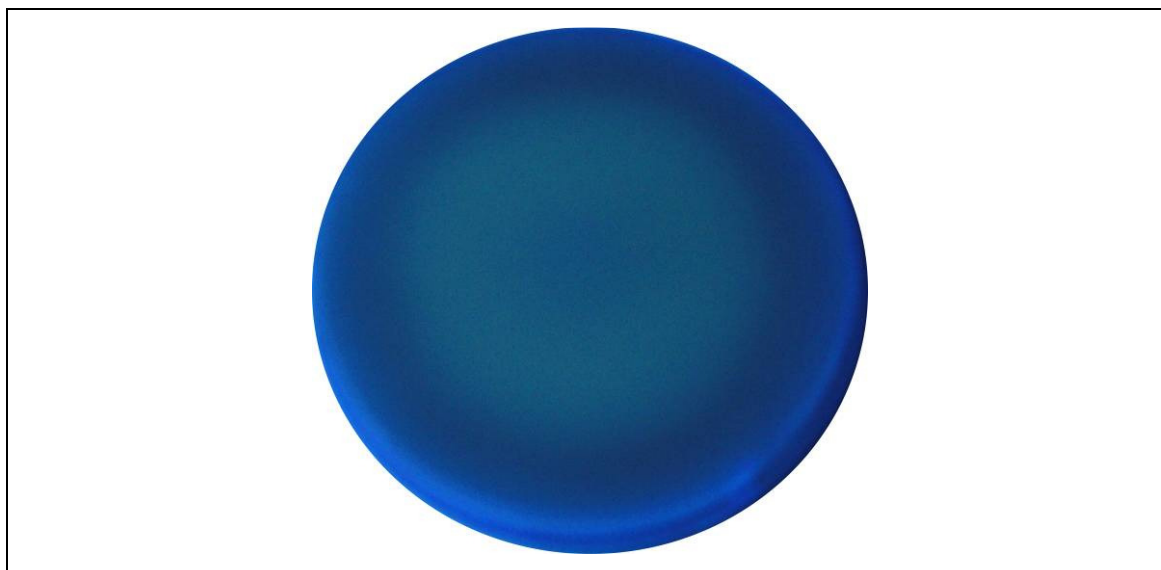


Plate 3.1. Blue-fluorescence under UV-A light (365nm) of reverse side culture of an ochratoxigenic strain of *A. carbonarius* on CCA 50% medium.

3.17. PHYSICOCHEMICAL ANALYSIS OF GRAPE SAMPLES

Besides the mycological analysis, all berry samples were analyzed for the determination of sugar content, the total acidity (as tartaric acid (H_2Ta) g L^{-1}), and pH, while the maturation index was calculated [sugars (g/Kg) / tartaric acids (g/l)], in order to examine any correlation with the mycological analysis results. However, Pearson product-moment correlation coefficient measure (SPSS) showed that there was not any effect of sugars content, total acidity, and pH to the *A. carbonarius* incidence. The results of berries juice physicochemical analysis are reported to the following Tables 3.6 and 3.7.

Table 3.6. Physicochemical characteristics of berries juice of the first year samples (2010) for each experimental vineyard.

	Sugar Content (%)	Total Acidity (H₂Ta g l⁻¹)	pH	Maturation Index
KA01	24.74	4.25	3.51	58.28
KA02	21.46	6.81	3.27	31.51
KA03	20.70	6.24	3.16	33.17
KAC1	24.68	4.83	3.42	51.10
KAC2	27.72	3.66	3.93	75.74
KAC3	25.52	4.02	3.39	63.48
KAI1	22.24	5.30	3.34	42.00
KAI2	25.78	3.27	3.86	78.84
KAI3	28.14	3.78	3.72	74.44
SBO1	19.38	5.00	3.48	38.80
SBO2	21.98	4.41	3.51	49.84
SBO3	20.12	4.85	3.47	41.48
SBC1	22.65	3.92	3.50	57.78
SBC2	26.00	4.44	3.56	58.56
SBC3	23.41	3.72	3.44	62.91
SBI1	20.36	5.55	3.48	36.68
SBI2	18.80	5.69	3.36	33.07
SBI3	18.10	5.79	3.41	31.26
SCO1	22.74	4.46	3.46	51.04
SCO2	22.10	4.26	3.41	51.88
SCO3	23.52	4.38	3.47	53.70
SCC1	20.84	3.56	3.52	58.62
SCC2	23.12	4.38	3.46	52.79
SCC3	23.52	3.60	3.72	65.33
SCI1	22.14	4.70	3.51	47.16
SCI2	18.24	4.23	3.52	43.12
SCI3	21.18	4.05	3.59	52.30

Table 3.7. Physicochemical characteristics of berries juice of the second and third year samples (2011 - 2012) for each experimental vineyard.

	Sugar Content (%)		Total Acidity (H ₂ Ta g l ⁻¹)		pH		Maturation Index	
	2011	2012	2011	2012	2011	2012	2011	2012
KA01	22.58	23.24	5.95	5.98	3.28	3.35	37.93	38.89
KA02	17.24	20.74	9.81	6.65	3.06	3.23	17.57	31.20
KA03	23.84	23.02	6.08	6.07	3.46	3.28	39.24	37.91
KA04	22.78	22.66	5.92	5.83	3.18	3.30	38.48	38.89
KAI1	21.06	22.30	6.26	6.19	3.38	3.27	33.63	36.01
KAI2	18.34	19.88	9.44	6.71	3.26	3.30	19.42	29.61
KAI3	19.66	21.10	8.57	6.56	3.15	3.29	22.93	32.16
KAI4	23.38	23.38	7.01	6.05	3.27	3.38	33.36	38.66
SBO1	18.76	15.78	7.83	8.14	3.12	3.23	23.96	19.39
SBO2	17.42	17.6	7.33	7.23	3.24	3.36	23.76	24.33
SBO3	21.16	18.08	6.90	6.95	3.28	3.26	30.65	26.00
SBO4	17.04	17.40	6.87	7.24	3.39	3.28	24.79	24.03
SBI1	16.98	18.58	7.35	6.60	3.30	3.30	23.10	28.14
SBI2	20.14	18.28	6.59	7.02	3.46	3.28	30.55	26.02
SBI3	16.76	15.48	6.44	7.97	3.35	3.24	26.01	19.42
SBI4	17.46	21.60	6.55	6.53	3.36	3.25	26.67	33.1
SCO1	21.00	22.54	6.71	6.21	3.2	3.29	31.29	36.32
SCO2	19.46	23.00	6.98	6.20	3.10	3.43	27.89	37.10
SCO3	18.40	21.66	5.88	6.11	3.10	3.31	31.29	35.47
SCO4	17.84	19.22	6.21	6.99	3.01	3.35	28.73	27.49
SCI1	16.64	15.44	7.47	8.68	3.13	3.24	22.27	17.78
SCI2	17.66	18.88	6.25	7.29	3.10	3.29	28.25	25.91
SCI3	17.32	16.68	6.61	7.39	3.39	3.22	26.22	22.56
SCI4	18.20	19.14	5.81	7.07	2.99	3.17	31.32	27.08

3.18. EFFECT OF CROP PROTECTION PRODUCTS ON *ASPERGILLUS CARBONARIUS* GROWTH

The effect of the 30 different crop protection products and gibberellic acid on the growth of two strains of *A. carbonarius*, in cultures of MEA medium, after seven days of incubation, in the dark, at 25 °C is shown in Table 3.8.

No growth of *A. carbonarius* was observed in 15 of the 31 treatments, while one-way analysis of variance (ANOVA) revealed statistically significant differences among the rest of the treatments, with *A. carbonarius* growth. Regarding the treatment with Gibberellic acid, a slight enhance (no significant) on *A. carbonarius* growth compared to the control was observed. The percentage of the final growth of *A. carbonarius* after seven days, in relation with control, is presented in the Figure 3.56.

Fourteen of the 23 chemical fungicides (60.87%) fully prevented the growth of *A. carbonarius*, while from the seven compounds approved for use in organic farming system that were tested, only Copper oxychloride (14.29%), prevented *A. carbonarius* growth.

In Figure 3.57, the mean daily growth of *A. carbonarius*, in treatments between the products that approved and not approved in organic farming system are presented. One way analysis of variance (ANOVA) revealed a statistically significant difference ($F_{1,88} = 15.951$, $p = 0.000$) between the two groups of products. As it is shown, the tested products that allowed in organic farming system were less effective.

Table 3.8. Mean daily growth, and percentage of growth in relation with control, of two strains of *A. carbonarius* in cultures of MEA medium, in dark, at 25 °C, containing different crop protection product substances.

Code	Treatment	Mean Daily Growth (mm day ⁻¹)
S0	CONTROL	13.85 ± 0.20 ^a
S1	ACROBAT 7,5/66,7 WG	n.g.
S2	ALIETTE 80 WG	n.g.
S3	ALPER 4/64 WP	n.g.
S4	ANTRACOL 70 WP	n.g.
S5	ANTRACOL COMBI X 65/2 WP	0.22 ± 0.40 ^g
S6	BAYFIDAN 250 EC	8.47 ± 0.44 ^c
S7	BORDEAUX MIXTURE 50 WP	3.10 ± 0.07 ^f
S8	COLLIS 20/10 SC	0.24 ± 0.12 ^g
S9	COPPER OXYCHLORIDE 50 WP	n.g.
S10	DITHANE M-45 80 WP	n.g.
S11	DOURO 10 EC	n.g.
S12	EQUATION PRO 22,5/30 WG	0.34 ± 0.25 ^g
S13	FLINT 50 WG	6.64 ± 0.96 ^{d,e}
S14	FOLICUR 250 EW	n.g.
S15	KARATHANE STAR 35 EC	10.39 ± 0.41 ^b
S16	KUMULUS 80 WG	8.42 ± 1.04 ^c
S17	KU-RAME	13.71 ± 0.15 ^a
S18	MILDEX 4,44/66,7 WG	3.12 ± 0.12 ^f
S19	NIMROD 25 EC	5.81 ± 0.37 ^e
S20	POLYRAM 80 WG	n.g.
S21	PROFILER 71.1 WG	n.g.
S22	PROSPER 500 EC	n.g.
S23	RENOVATION LADA	7.59 ± 1.48 ^{c,d}
S24	RIDOMIL GOLD MZ 68 WG	n.g.
S25	ROVRAL 75 WG	0.30 ± 0.73 ^g
S26	SCALA 40 SC	n.g.
S27	SODIUM BICARBONATE	0.11 ± 0.09 ^g
S28	SWITCH 25/37.5 WG	n.g.
S29	SYSTHANE 24 EC	n.g.
S30	TALOCUPER	13.18 ± 0.35 ^a
S31	VIO-GIBB 5 SL	14.06 ± 0.21 ^a

*The different letters indicate a significant difference of *A. carbonarius* growth among the treatments (Duncan's multiple range tests, $p < 0.001$).

±, Standard Deviation, n.g., No growth

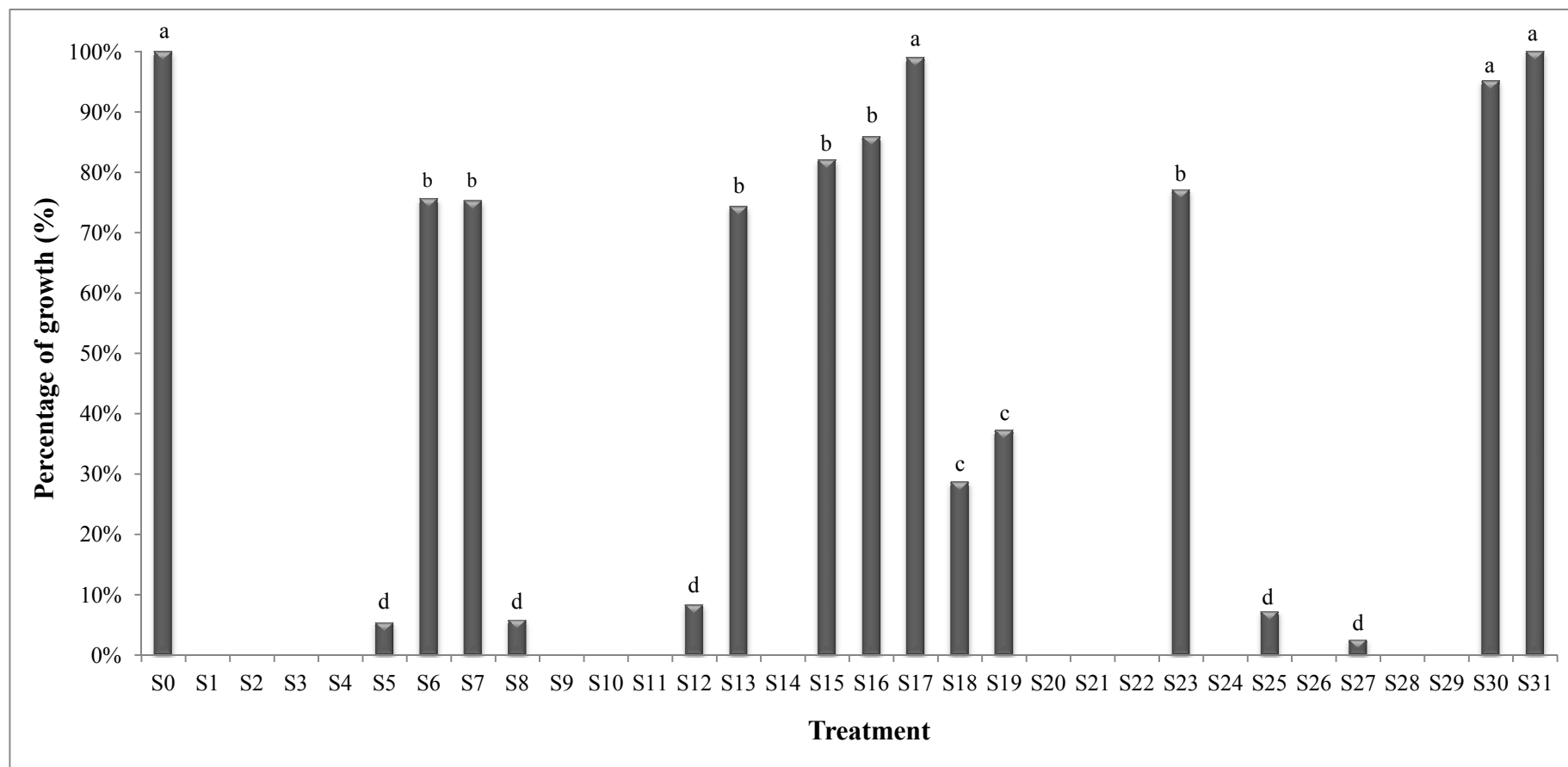


Figure 3.56. Percentage of growth in relation to the control of two strains of *A. carbonarius* in cultures of MEA medium, in the dark, at 25°C, for seven days, containing different crop protection product substances. The different letters indicate a significant difference of *A. carbonarius* growth among the treatments (Duncan's multiple range tests, $p < 0.001$).

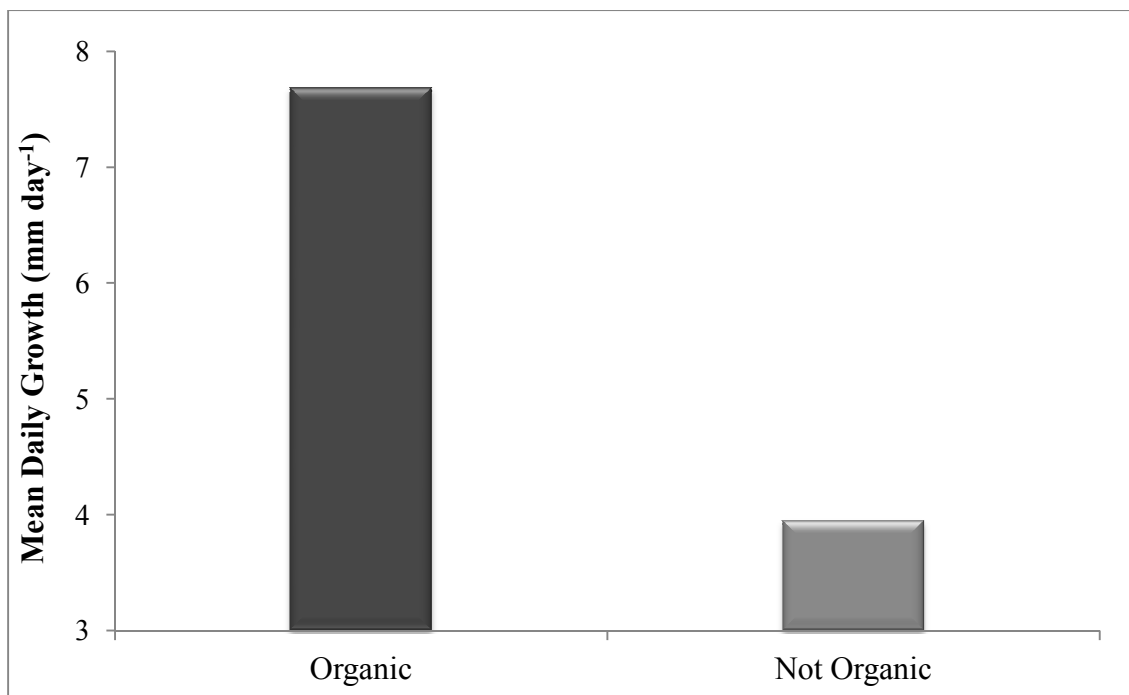


Figure 3.57. Mean daily growth of two strains of *A. carbonarius* in cultures of MEA medium, in the dark, at 25 °C, containing substances, approved and not approved for use in organic farming system vineyards.

From the total of the fungicides tested, including chemicals and organic compounds, only the 40% of those that are used against *Uncinula necator*, prevented totally the *A. carbonarius* growth, while the 75% of those that are used against *Plasmopara viticola* and two of the three *Botrytis cinerea* fungicides that tested, had the same result. Antracol Combi X 65/2 WP was not included, as it contains active substances against two of the fungus categories.

Regarding the fungicides that allowed *A. carbonarius* growth, there was a statistical significant difference among the three fungicides categories ($F_{2,57} = 25.041$, $p = 0.000$), with *Botrytis cinerea* fungicides to be the most effective against *A. carbonarius*, followed by *Plasmopara viticola* fungicides, while the fungicides that have as target the *Uncinula necator*, were the less effective (Figure 3.58).

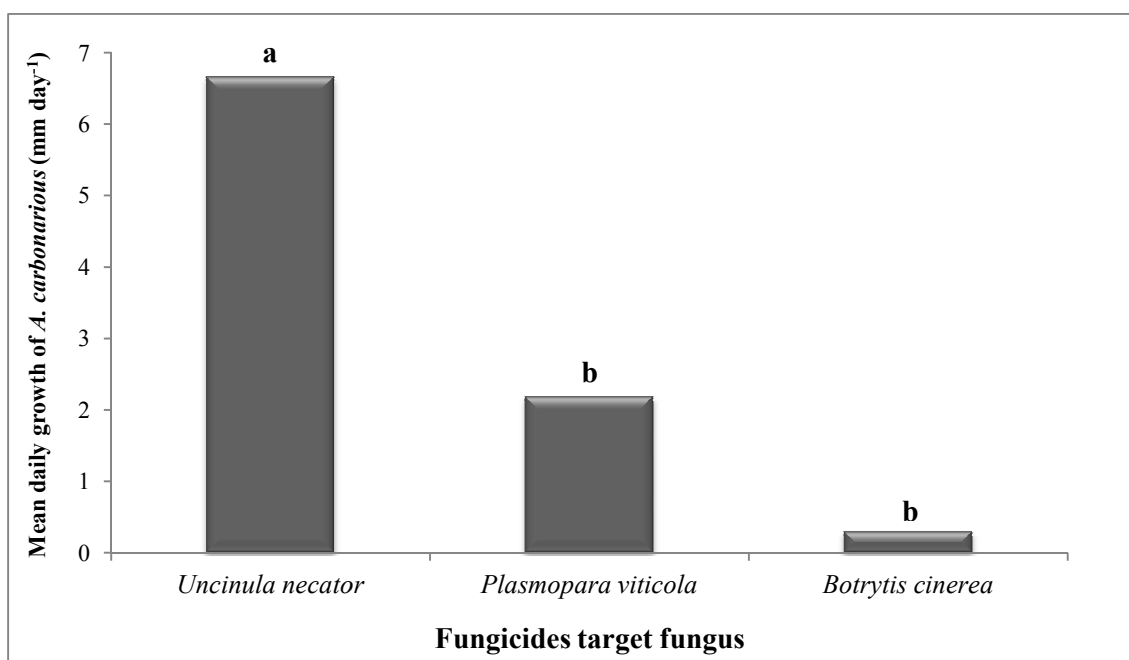


Figure 3.58. Mean daily growth of *A. carbonarius*, as it is affected by the three fungicide categories that are used against *Uncinula necator*, *Plasmopara viticola* and *Botrytis cinerea*. Different letters indicate a significant difference of *A. carbonarius* growth among the fungicide categories (Duncan's multiple range tests, $p < 0.001$).

3.19. EXAMINATION OF ESSENTIAL OILS INFLUENCE ON *A. CARBONARIUS* GROWTH

Of the five essential oils tested, only two reduced the growth of *A. carbonarius*. The different doses of essential oils of *Origanum dictamnus* ($F_{4,25} = 1775.986$, $p = 0.000$) and *Origanum vulgare* ($F_{4,25} = 2086.158$, $p = 0.000$) had a significant influence on *A. carbonarius* growth, while both of essential oils prevented totally the *A. carbonarius* growth in the concentration of 250 ppm.

In contrast, the essential oils of *Laurus nobilis* ($F_{4,25} = 2.781$, $p = 0.049$) and *Salvia officinalis* ($F_{4,25} = 2.845$, $p = 0.045$), seem to had a slightly enhance on the growth of *A. carbonarius*, with a statistical significance in the level of <0.05 . Finally, the essential oil of *Eucalyptus globulus* ($F_{4,25} = 0.028$, $p = 0.998$) had no influence on *A. carbonarius* growth. In the Table 3.9 and the Figure 3.59 is presented the mean daily growth per essential oil and concentration.

Table 3.9. Mean daily growth, of two strains of *A. carbonarius* in cultures of MEA medium, in the dark, at 25 °C, per their concentration of five essential oils.

	<i>Eucalyptus globulus</i> (mm day ⁻¹)	<i>Laurus nobilis</i> (mm day ⁻¹)	<i>Origanum dictamnus</i> (mm day ⁻¹)	<i>Origanum vulgare</i> (mm day ⁻¹)	<i>Salvia officinalis</i> (mm day ⁻¹)
Control	13.09 ± 0.23	13.09 ± 0.23	13.09 ± 0.23	13.09 ± 0.23	13.09 ± 0.23
25ppm	13.13 ± 0.72	13.30 ± 0.71	11.25 ± 0.55	12.22 ± 0.46	13.13 ± 0.38
75ppm	13.20 ± 0.60	13.58 ± 0.65	8.69 ± 0.22	6.88 ± 0.36	13.37 ± 0.21
150ppm	13.15 ± 0.63	13.70 ± 0.32	5.48 ± 0.23	4.52 ± 0.19	13.50 ± 0.66
250ppm	13.14 ± 0.58	14.03 ± 0.58	n.g.	n.g.	13.71 ± 0.12

±, Standard deviation

n.g., No Growth

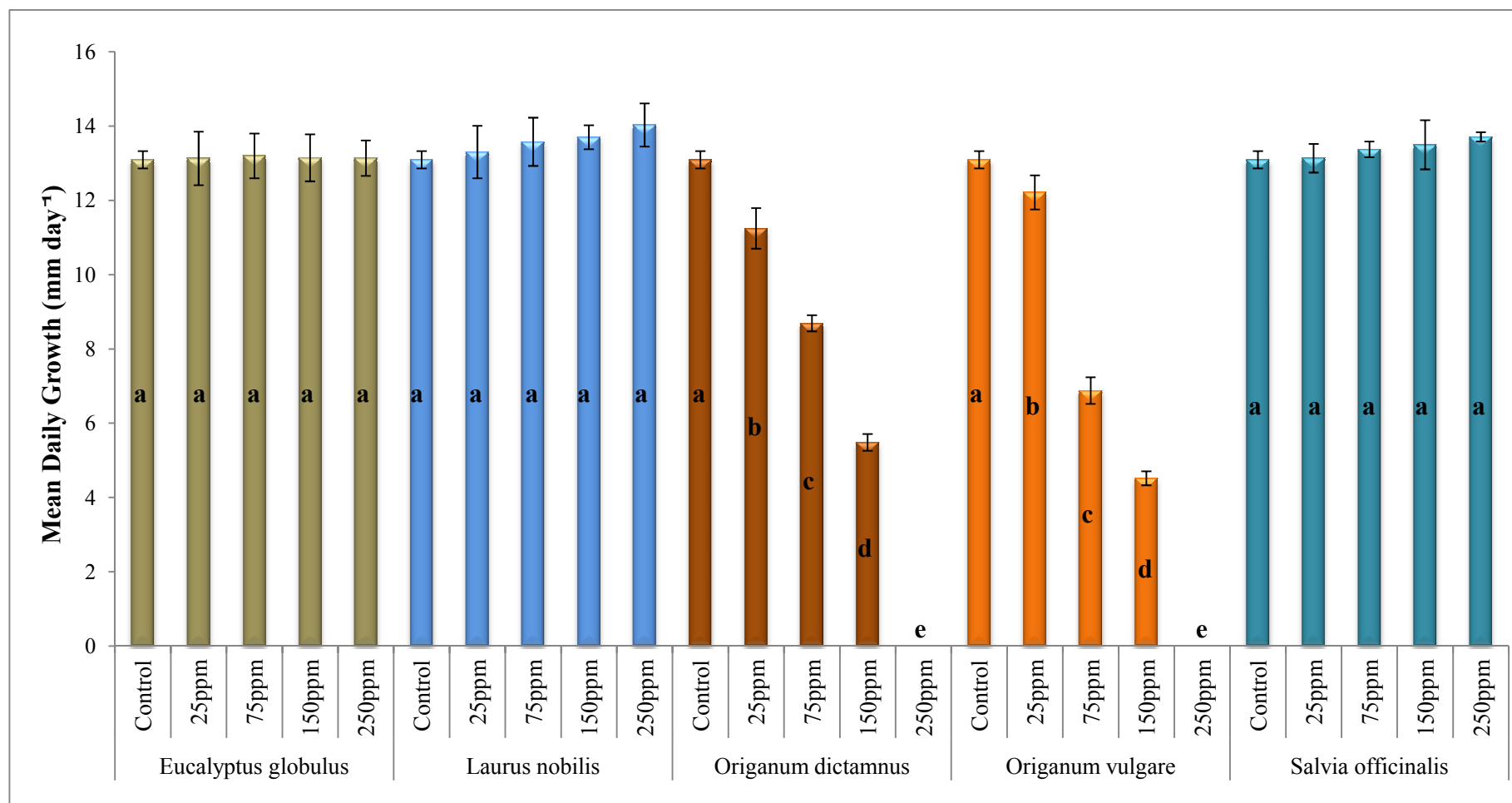


Figure 3.59. Mean daily growth, of two strains of *A. carbonarius* in cultures of MEA medium, in the dark, at 25 °C, per their concentrations of five essential oils. The different letters indicate a significant difference of *A. carbonarius* growth among the different concentrations of each essential oil (Duncan's multiple range tests, $p < 0.001$). The error bars represent the standard deviation of the means.

3.20. FARMING SYSTEM INFLUENCE ON OCHRATOXIN A OCCURRENCE IN WINE

The results from the 40 wines analysis are summarized in the Table 3.10. Ochratoxin A (OTA) concentrations in wines from Organic Farming System (OFS) grapes ranged from <0.05 to $0.10 \mu\text{g l}^{-1}$, with mean = $0.067 \mu\text{g l}^{-1}$ and median = $0.07 \mu\text{g l}^{-1}$, while in wines from Integrated Farming System (IFS) grapes OTA ranged from <0.05 to $0.07 \mu\text{g l}^{-1}$, with mean = $0.050 \mu\text{g l}^{-1}$ and median = $0.06 \mu\text{g l}^{-1}$. OTA concentration was not detected in seven from the 20 wines from IFS grapes, and one from the 20 wines from OFS grapes.

One-way analysis of variance (ANOVA) revealed a statistically significant difference in OTA concentration between the wines from the two farming systems ($F_{1,38} = 8.615$, $p = 0.006$) at <0.05 level of significance. A slight increase but not statistically significant was observed in wines of the year 2011 compared with the wines of the year 2012.

Table 3.10. Ochratoxin A concentration in wines from Organic and Integrated farming systems grapes, from the region of Peza (Area A) in Heraklion Prefecture.

Farming System	Positive / Total samples	Mean ^{a,b}	Median ^{a,b}	Range ^a
OFS ^c	19 / 20	0.067	0.07	$<0.05 - 0.10$
IFS ^d	13 / 20	0.050	0.06	$<0.05 - 0.07$

^a OTA $\mu\text{g l}^{-1}$ wine

^b $0.025 \mu\text{g l}^{-1}$ (LOD divided by 2) was considered as the OTA concentration for the samples with OTA under the limit of detection ($<0.05 \mu\text{g l}^{-1}$).

^c Wines from organic farming system grapes

^d Wines from integrated farming system grapes

CHAPTER FOUR

DISCUSSION, CONCLUSIONS AND FURTHER CONSIDERATIONS

4.1. DISCUSSION

4.1.1. INTRODUCTION

The results of the present study were presented in Chapter three and are discussed in this chapter in two sections (a) the results of the experimental year 2010 and (b) the results of the experimental years 2011-2012. In 2010 the effect of the three farming systems (Organic, Conventional and Integrated) were considered. In 2011-2012 two farming systems (Organic and Integrated) on the incidence of the most dominant fungal species and genera, and especially of *A. carbonarius*, were examined by mycological analysis of the grapes, using the direct and the dilution plating method. The direct plating method was used for the determination of the isolation frequency of fungi directly growing from grapes. The higher percentage of fungal incidence is assumed to be related to a higher potential for infection and risk of mycotoxin occurrence (Pitt and Hocking, 2009). The dilution plating method was used in order to estimate the population size of different fungi and the relative population diversity. Although the results of the two methods are not directly comparable, the combination of both methods, gives a more complete picture of the sample fungal status (Pitt and Hocking, 2009).

Additionally in the second experimental period, a mycological analysis of experimental vineyards soil was conducted. The effectiveness of the crop protection products most commonly used by the producers of the experimental vineyards (chemical fungicides and organic compounds), as well as five essential oils, against *A. carbonarius* was tested *in vitro*. Finally, OTA concentrations in wine samples produced

from grapes of Organic and Integrated farming systems vineyards of the experimental Area A were determined.

4.1.2. EXPERIMENTAL YEAR 2010

From the mycological analysis of the grapes, carried out in 2010 and presented in Figures 3.1, 3.2 and 3.3, it was found that the *Aspergillus spp.* were the most frequently isolated among the dominant fungal species/genera (*Penicillium spp.*, *Cladosporium spp.*, *Alternaria spp.*, *Botrytis spp.*, *Rhizopus spp.* and Yeasts). This is in accordance with the findings of similar studies of (Lasram *et al.*, 2007; Pateraki, 2008).

Based on the statistical analysis it is concluded that the farming systems affect the frequency of the different fungal species/genera in both cultivars and all the areas of research. However there are not always statistically significant differences in all the species and areas of research.

Regarding the incidence of *A. carbonarius*, the percentage of isolation was statistically significantly higher in the Soultanina cultivar vineyards compared to the Kotsifali cultivar vineyards, independent of the farming system (Figure 3.4). However, there was no significant difference between the two experimental areas of the Soultanina cultivar vineyards.

The differences in the incidence of *A. carbonarius* between the two cultivars can be explained by the different characteristics of the grapes *per se*, as well the differences in the cultivation methods of the two cultivars.

Soultanina is a typical white table grape cultivar, while Kotsifali is a red wine cultivar that gives quality wines in the experimental area A. Irrespective of the farming

system, the mean yield of the Kotsifali vineyards must be lower than 1000 Kg grapes str^{-1} (as it is laid down of the legislation for the VQPRD wines Peza). In Soultanina cultivar vineyards, the mean yield is from 2000 to 3000 Kg grapes str^{-1} . For such a high productions in Soultanina cultivar, the different inputs (fertilizers, growth regulators etc.) are about double compared to the Kotsifali cultivar vineyards. This intensive growing system leads to the increased size of berries (quality requirement), which are more susceptible to berry splitting, given the low thickness of the berry skin (Winkler, et al., 1974). On the contrary, the Kotsifali cultivar is characterized by thicker berry skin, more resistant to berry splitting, while in case of damage by insects or birds, tends to form cork in the attacked area (suberification) (Plate A.1 - appendix A). These differences in berry skin characteristics have a significant input on the susceptibility to *Aspergillus* spp. between the two cultivars according to Leong, et al. (2006a) and Belli, et al. (2007).

Another factor affecting the *Aspergillus* spp. incidence is the trellis system, which is different between the two cultivars. The V-type trellis system that is used in Soultanina cultivar vineyards (Plate A.3 – appendix A), do not allow the grapes and soil exposure to the sun, in order to protect the grapes from sun burns, but also to retain the yellow-green colour which is desired for Soultanina table grapes. In contrast, the vertical-type trellis system that is used in Kotsifali cultivar vineyards (Figure A.4 – appendix A), allow the exposure of the grapes to the sun, a necessary condition for the colouring of berries. As results from previous studies, *A. carbonarius* is susceptible to prolonged sun exposure (Leong, et al. 2006a), while UV radiation may be an inhibitory factor of the germination of *A. carbonarius* spores on berries (Leong, et al., 2006e; Hocking, et al., 2007), despite the black colour which seems to provide a kind of protection of sun light

and UV radiation (Esteban, et al., 2006a; Esteban et al., 2006b). It is therefore concluded that the difference between the two cultivars in *A. carbonarius* incidence, found in the present study, is also affected by the differences in trellis system. The greater sun exposure in Kotsifali cultivar vineyards results in lower incidence of *A. carbonarius*.

Regarding the influence of the farming systems, the frequency of isolation of *A. carbonarius* from Kotsifali grapes, was not statistically significantly different among the three farming systems (Figure 3.5). This is probably due to the fact that, in a normal growing year, the differences between the farming systems, as results from the producers' records, are minimal. Normally the pest management is limited to the control of the fungus *Uncinula necator* and the grape berry moth (*Lobesia botrana*).

In Soultanina vineyards, the frequency of *A. carbonarius* isolation tends to be lower in the integrated farming system compared to the organic farming system, in both areas, with a statistically significant difference in area B (Figures 3.6 and 3.7; Kontaxakis *et al.*, 2011).

In contrast to the organic and integrated farming systems, the results of conventional farming system were not clear, and despite that the factor of the area of research had no effect on the other two farming systems in Soultanina cultivar vineyards (Table 3.1 and Figure 3.9), the conventional farming system was characterized by a big heterogeneity in the same and between the areas of research (Figure 3.10).

It is suspected that this heterogeneity may be due to the lack of a specific growing protocol in the conventional farming system. In these vineyards the cultivation is based only on the volition of the producers. Despite the definition of the conventional farming

system (FAO, 2007), which is characterized by the intense use of synthetic inputs, such as chemical fertilizers and pesticides, the cultivation of the research vineyards, according to the producers' records, is not characterized by pre-determined but instead by miscellaneous inputs, cultivation practices and pest management. After all these points, and furthermore, this farming system tends to be abandoned, and is being replaced by the organic and integrated farming systems, as the certification of viticultural products according to a growing protocol (i.e. Globalgap) is nowadays a precondition for the sale of the products in international (Jaffee and Henson, 2005), as well as in national markets (Goumenaki and Kapetanakis, 2008). Therefore, the initial experimental plan was modified for the next two years of research. In the new experimental plan, only organic and integrated farming systems were studied for their influence on *A. carbonarius* incidence.

4.1.3. EXPERIMENTAL YEARS 2011-2012

The experimental year 2011, was characterized by a downy mildew (*Plasmopara viticola*) epidemic in South Greece and especially in Cretan vineyards, due to the extraordinary climatic conditions, highly favourable for the fungus, causing severe damages to vineyards mainly in northern and central Crete (N.AG.RE.F. – PPI of Heraklion, 2011). The frequent, low-intensity rainfall (Figures B.3, B.6 and B.9 - appendix B), the high relative humidity (Figures B.2, B.5 and B.8 – appendix B), the favourable temperatures (Figures B.1, B.4 and B.7 – appendix B), but also the long time humidity on vines leaves (Figures B.10, B.11 and B.12 – appendix B) in the first half of April, resulted in a high *Plasmopara viticola* inoculum potential and a rapid spread through the vineyards. That in combination with the ineffective treatments in some

vineyards of the early generations of the pathogen resulted in the very difficult or even impossible control of the disease (Stavarakakis *et al.*, 2012).

Given the severity of *Plasmopara viticola* infections, the Plant Protection Institute of Heraklion launched a high number of agricultural alerts, suggesting repetitive applications of appropriate fungicides. Indeed, as results from the producers' records, the usage of fungicides used to control *Plasmopara viticola* was extremely higher in comparison to the former years, for both farming systems. Especially in the integrated farming system, a wide range of fungicides were available and the most effective (translaminar and systemic) were used against the disease. On the contrary, in the organic farming system a restricted range of fungicides, which were approved for use and which were mainly copper compounds were available. The lower effectiveness of those compounds resulted in extremely higher levels in organic vineyards. Due to the severity of the disease, in three out of four organic experimental vineyards in area C, in order to protect the vines from a potential total destruction, the organic farming system was abandoned and systemic chemical fungicides were used. These experimental vineyards were therefore substituted the following experimental year (2012) with other organic farming system vineyards in the same area.

The next year (2012) despite the *Plasmopara viticola* high inoculum potential of the previous year, the unfavourable climatic conditions (Figures B.1 to B.9 – appendix B) and the preventive frequent fungicide applications against *Plasmopara viticola*, resulted in the reduced incidence of the fungus, and the absence of symptoms in vineyards (N.AG.RE.F. – PPI of Heraklion, 2012).

4.1.3.1. Mycological Analysis of grapes

From the mycological analysis of the grapes, using the direct plating method, the years 2011 and 2012, similar to the year 2010, *Aspergillus* section *Nigri* species, were the most frequently isolated among the dominant fungal species and genera (Figures 3.11 to 3.16).

The percentage of isolation of *Aspergillus* section *Nigri* species was lower compared to the experimental year 2010 (Figures 3.1, 3.2 and 3.3), while it was in general significantly higher in the organic farming system compared to the integrated farming system. On the contrast, the remaining dominant fungal species/genera were not affected to the same extent by the farming system.

Regarding the frequency of *A. carbonarius* isolation from grape samples, as is presented in Figure 3.17, it is also significantly lower than the respective frequency of isolation of the experimental year 2010 (Figure 3.1). This decrease in *A. carbonarius* incidence, as in the rest of *Aspergillus* section *Nigri* species, can be explained by the intensive use of fungicides against *Plasmopara viticola* as has been mentioned above.

More analytically, independently of the farming system, in area A the *A. carbonarius* frequency of isolation was 81% lower in the year 2011 compared to the previous year, while it was similarly low in the next year 2012. In area B, a decrease in *A. carbonarius* incidence of 61% between the years 2010 and 2011 was observed, and remained at approximately the same levels for the year 2012. In area C, an extreme decrease in *A. carbonarius* incidence was observed (92.5%) in the year 2011 in relation to the previous year. This decrease could be related to the abandoning of the organic farming system and to the intensive use of systemic chemical fungicides, of most of the “organic”

farming system experimental vineyards as mentioned above. This is supported by the results of the next year 2012 when, after the substitution of the “organic” vineyards, the *A. carbonarius* incidence was similar with the incidence of the Area B.

Apart from the year 2011 in area C, the frequency of isolation of *A. carbonarius* from Soultanina cultivar grapes was significantly higher compared to Kotsifali cultivar grapes (Figure 3.17) for both of the years 2011 and 2012. This is in accordance with the results found in the first experimental year 2010 (Figure 3.4).

Regarding the effect of the farming systems, the frequency of *A. carbonarius* isolation from grapes was statistically significantly higher in the organic farming system compared to the integrated farming system vineyards, in all the cultivars, experimental years and areas of research, apart from the Soultanina cultivar vineyards in area C the year 2011 (Figures 3.18, 3.19 and 3.20). Practically, in that area in 2011, the organic farming system protocol was not applied to organic farming system vineyards, as mentioned above.

From the analysis of covariance between the farming systems and the area of research, it results that, similarly to the year 2010 (Table 3.1), in 2012 (Table 3.3), the different areas of Soultanina cultivar were not affected the incidence of *A. carbonarius*. On the contrary, in 2011 the area seems to have an influence on the incidence of *A. carbonarius* (Table 3.2). However, this effect is more probably related to the irregular results in the area C that year.

Other studies refer to the incidence of *A. carbonarius* being affected by climatic and geographical differences among different regions (Battilani *et al.*, 2006d; Varga and Kozakiewicz, 2006; Valero *et al.*, 2008) with a positive gradient from West to East and

from North to South (Battilani *et al.*, 2006a). Pateraki (2008) in a study conducted in the same Prefecture, as the present study, found different incidences of *Aspergillus* section *Nigri* species among vineyards grown at different altitudes. Nevertheless, the similar incidence of *A. carbonarius* in the present study, between the two areas of Soultanina cultivar, can be explained by the fact that the areas of research of the present study were located in the same geographical region (Heraklion Prefecture), while the experimental vineyards in both areas were at a similar altitude with an average of 230 m.

Comparing the incidence of *A. carbonarius* in area A (Kotsifali vineyards) and areas B and C (Soultanina vineyards), a statistically significant lower incidence of *A. carbonarius* in area A, was found in all the experimental years. This difference can be attributed, among other factors, to the higher altitude of the area A (average altitude 430 m). Similarly, Natskoulis (2009) found a significantly lower incidence of *Aspergillus* section *Nigri* species in vineyards located in Arcadia (Peloponissos, Greece) with an altitude of 650 m, compared with vineyards located in Achaia (average altitude 120 m).

Apart from the different altitudes, as mentioned and discussed above, the incidence of *A. carbonarius* was affected by the different cultivars. That is also supported by the results from the analysis of covariance between the farming systems and the cultivars, where is shown the influence of the two cultivars in the frequency of *A. carbonarius* isolation (Tables 3.4 and 3.5).

The relative percentage of *A. carbonarius* isolation in relation to the remaining species of *Aspergillus* section *Nigri*, was found to be higher in the organic farming system in almost all the experimental vineyards of both cultivars, with the exception of Soultanina vineyards in area C in the year 2011 (Figures 3.25, 3.26 and 3.27). In these

vineyards, the similar relative frequency between the two farming systems must be related to the fact that all the vineyards were treated as “integrated”.

The results of the mycological analysis of the grapes, using the dilution plating method, as presented in Figures 3.28 to 3.40, are in accordance and support the findings of the direct plating method. Indeed, from the statistical analysis of the dilution plating method results, *Aspergillus* section *Nigri* species were also found to be statistically significant lower in integrated farming system, compared to the organic farming system vineyards. Similarly, the effects of the cultivars and areas in *A. carbonarius* CFUs g⁻¹ grapes were in accordance, independent of the method of mycological analysis. However, in 2012, whereas the frequencies of *A. carbonarius* isolation in areas B and C, were at the same levels (Figure 3.17), the *A. carbonarius* populations (CFUs g⁻¹ grapes) were more in area C compared to the area B (Figure 3.34), and especially in organic farming system vineyards (Figure 3.37). It is speculated that this difference may be due to the greater attack from *Lobesia botrana* and *Ceratitis capitata* that was observed in this area during the sampling. The relation of *A. carbonarius* incidence with insect attacks has been established by several studies (Cozzi *et al.*, 2006; Medina *et al.*, 2008; Somma *et al.*, 2012). The skin damage and the exudates from the attacked berries probably favoured the growth of *A. carbonarius* in this area and therefore the *A. carbonarius* populations (CFUs g⁻¹ grapes) were increased.

4.1.3.2. Mycological Analysis of soil

From the mycological analysis of experimental vineyards soil, it results that from among the most dominant species grown on DRBC medium, the *Aspergillus spp.* are the predominant species also in the soil of Sultana vineyards (Figures 3.43 to 3.46).

In the soil of Kotsifali cultivar vineyards, the *Aspergillus spp.* occurs at similar levels with the remaining dominant species (*Penicillium spp.*, *Cladosporium spp.*, *Alternaria spp.*, Yeasts etc.) (Figures 3.41 and 3.42). A widest variety of fungal species were found in soil, in relation to the grapes, while a significant number of *A. flavus* and *A. ochraceus* were also found. Indeed, a large number of fungal species occur in soil during their life cycle, without necessarily having an active role (Bridge and Spooner, 2001).

Similar to the grapes, *A. carbonarius* incidence in Soultanina cultivar vineyards soil is significantly higher compared to the Kotsifali cultivar vineyards (Figure 3.47). This can be explained by the differences in sensitivity between the two cultivars mentioned above, but also by the different temperatures of soil surface, due to the different trellis systems which affect the sun exposure of soil, as already discussed above (Jaime-Garcia and Cotty, 2010). According to Leong *et al.* (2006b), the *A. carbonarius* incidence in soil is also affected by the vine debris and berries falling on the ground. In the Soultanina table grape vineyards, it is a common practice for the removal of any insect attacked or infected berries before harvest, which fall on the soil and constitute an excellent growing medium for the fungus.

Between the two areas of Soultanina cultivar, a significant difference in the occurrence of *A. carbonarius* was found, with the lower incidence in the area C (Figure 3.47). This can be explained by the intensive use of pesticides in this area in the year 2011, as is discussed in the next paragraph.

Regarding the farming system effect, *A. carbonarius* incidence is higher in the organic farming system compared to the integrated farming system vineyards soil, for

both cultivars, all areas and experimental years, with a statistically significant difference in Soultanina cultivar vineyards, apart from the area C in the year 2011 (Figures 3.48, 3.49 and 3.50). As it is also mentioned above, in that area the intensive use of fungicides against *Plasmopara viticola*, affected the incidence of *A. carbonarius*. This is in accordance with studies that found that the use of pesticides and especially fungicides could affect the incidence of fungal species in soil (Klingen *et al.* 2002; Kuthubutheen and Pugh, 1979). However, the incidence of *A. carbonarius* in the soil of the aforementioned area C in the year 2011 was not affected by the intensive use of fungicides to the same extent as in grapes (Figure 3.37).

The relative percentage of *A. carbonarius* populations (CFUs g⁻¹ soil) in relation to the remaining *Aspergillus spp.*, was higher in the organic farming system, compared to the integrated farming system vineyards, for both cultivars, and all the experimental areas and years. A remarkable percentage of *A. flavus* and *A. ochraceus* were also found in soil (Figures 3.51, 3.52 and 3.53).

As results from the Pearson product-moment correlation coefficient calculation, a significant correlation of *A. carbonarius* incidence in grapes and soil, was found in all the years and experimental areas, apart from the area A the year 2012, and the Area C the year 2011, when the intensive use of fungicides differentially affected the occurrence of *A. carbonarius* in grapes and soil (§ 3.15). This supports the aspect that the surface soil beneath the vines is the primary source of grape products contamination by *Aspergillus spp.* (Leong *et al.*, 2006b). The relation of the soil and grapes contamination of *Aspergillus* section *Nigri* species is also documented by Pateraki

(2008) who refers to a higher contamination in grapes touching the soil compared to grapes cited higher on the vine.

4.1.4. ADDITIONAL RESEARCH

4.1.4.1. Ochratoxin A production ability of *A. carbonarius* isolates

The *A. carbonarius* isolates from the aforementioned mycological analysis of grapes and soil, were examined for their ability to produce Ochratoxin A, using the Coconut Cream Agar method (Davis *et al.*, 1987; Dyer and McCammon, 1994). The percentages of ochratoxigenic, in relation to the total, *A. carbonarius* isolates were similar, per area, for both of the years 2011 and 2012. A higher average of the two years percentage (91.8%), of ochratoxigenic *A. carbonarius* isolates was found in area A (Kotsifali cultivar), in relation to the areas of Soultanina cultivar. The average of the two years percentages of ochratoxigenic *A. carbonarius* isolates were similar between the areas B (72%) and C (77.1%) (Figure 3.55). The higher percentage of *A. carbonarius* ochratoxigenic isolates in Kotsifali cultivar is assumed to be due to the lower incidence of the fungus in the vineyards of this cultivar, the unfavourable conditions as mentioned above and hence the greater antagonism with other fungal species. Indeed, the ability of mycotoxigenic fungi to produce toxins is related with their defence against environmental conditions, other fungi and bacteria (Benbrook, 2005).

4.1.4.2. Physicochemical analysis of grape samples

The physicochemical characteristics (sugar content, total acidity, pH) (Tables 3.6 and 3.7) were determined in order to examine any correlation with *A. carbonarius* incidence on grape samples. Despite the fact that the potential of *Aspergillus spp.* is affected by the sugar content and tartaric acid, with an increase from berry set until harvest (Bejaoui *et al.*, 2006; Leong, *et al.*, 2006c; Tjamos *et al.*, 2006; Ponsone, *et al.*, 2007), in this study, all samples were collected at the maturity stage, and the physicochemical characteristics had no effect on the incidence of *A. carbonarius*.

4.1.4.3. Effect of crop protection products on *A. carbonarius* growth

Statistically significant differences in the effect on *A. carbonarius* growth, of the most common crop protection products (chemical fungicides and organic compounds), used by the producers of the experimental vineyards was found, ranged from no growth to growth similar to the control (Table 3.8 and Figure 3.56). The results of the fungicides that had also been studied in the past were indeed similar in the previous studies (Belli *et al.*, 2006b; Tjamos *et al.*, 2004).

As results from the statistical analysis of the mean daily growth of *A. carbonarius* in treatments between the products that are approved and not approved in the organic farming system, the chemical fungicides that are used in the integrated farming system, independently of the fungus target, were statistically significant more effective against *A. carbonarius*, in relation with the crop protection products, approved for use in the organic farming system (Figure 3.57).

Statistically significant differences among the three fungicide categories (independent of the farming system used), in relation with their fungus target (*Uncinula necator*, *Plasmopara viticola*, *Botrytis cinerea*), were found in their effectiveness against *A. carbonarius*. The fungicides used against *Botrytis cinerea* were the most effective against *A. carbonarius*, followed by *Plasmopara viticola* fungicides, while the fungicides that used against *Uncinula necator* were the least effective (Figure 3.58).

This difference in the effectiveness of the three fungicide categories, could explain the decrease of *A. carbonarius* incidence in the years 2011 and 2012, where an intensive use of fungicides against *Plasmopara viticola*, chemical and organic (copper compounds), occurred. This is in accordance with Battilani and Logrieco (2006c), who refer to the fact that the application of fungicides active against *A. carbonarius* minimizes the development of the fungus on grapes.

On the contrary, in the experimental year 2010, the minimal use of the aforementioned fungicides, and the only use of fungicides used against *Uncinula necator*, and especially sulphur as also found by Lo Curto *et al.* (2004) as low-effective, commonly used either in organic or integrated Kotsifali vineyards, can explain the similar incidence of *A. carbonarius* between the two farming systems in Kotsifali vineyards.

4.1.4.4. Effect of essential oils on *A. carbonarius* growth

Different essential oils have been tested against fungal species (Thanaboripat *et al.* 2007; Combrinck *et al.*, 2011; Tzortzakis *et al.*, 2011; Camele *et al.*, 2012) and bacteria (Tajkarimi *et al.*, 2010; Alexopoulos *et al.*, 2011) infecting fruits post-harvest. In the

framework of organic post-harvest control of *A. carbonarius* on grapes, considering the difficulty of application in the field, the effectiveness of five essential oils, in reducing *A. carbonarius* growth were examined *in vitro*.

Only the essential oils of *Origanum dictamnus* and *Origanum vulgare* were effective, in the concentrations that were tested. The essential oils of both herbal species, belonging to the same genera, mainly contain the compound carvacrol (Liolios *et al.*, 2010; Tibaldi *et al.*, 2011), found by Martinez-Romero *et al.* (2007) to also be active against *Botrytis cinerea*. The essential oils of *Laurus nobilis* and *Salvia officinalis* were found to slightly enhance the growth of *A. carbonarius*, while the essential oil of *Eucalyptus globulus* had no effect.

4.1.4.5. Ochratoxin A in wine

In an effort to correlate the incidence of *A. carbonarius* between the two farming systems being examined, and the Ochratoxin A (OTA) contamination of wines, 40 wine samples produced in wineries located in area A in the experimental years 2011 and 2012, from Kotsifali cultivar grapes cultivated under the organic (20 samples) and integrated farming systems (20 samples) were analysed.

From this study it resulted that, in all wines tested, independent of the farming system, the OTA concentration was at very low levels ($<0.10 \mu\text{g l}^{-1}$), respective to the low occurrence of *A. carbonarius* on Kotsifali grapes in that area, in the years of production.

More analytically, OTA concentration of organic and integrated farming systems wines ranged from <0.05 to $0.10 \mu\text{g l}^{-1}$ wine, with a median = $0.07 \mu\text{g l}^{-1}$ wine and from <0.05 to $0.07 \mu\text{g l}^{-1}$ wine, with a median = $0.06 \mu\text{g l}^{-1}$ wine, respectively (Table 3.10). This is in accordance with the results of similar studies conducted in Greek red dry wines by Soufleros *et al.* (2003), Stefanaki *et al.* (2003) and Labrinea *et al.* (2011) and found a median of 0.09, 0.09 and $0.07 \mu\text{g l}^{-1}$ wine respectively. Similar levels of OTA were also found in Italy (Pietri *et al.*, 2001; Brera *et al.*, 2008) and Turkey (Anli *et al.*, 2005).

Regarding the influence of the farming systems to the wines OTA contamination, in contrast to Chiodini *et al.* (2006) study of a similar number of wines produced under organic and conventional systems and Labrinea *et al.* 2011, reporting that there was no difference in the concentration of OTA between the organic and conventional farming systems, in this study, that all samples originated from the same area, a statistically significant difference between organic and integrated farming systems grapes was found.

The wine samples produced from organic farming grapes, contained statistically significant higher OTA levels, compared to the sample wines from integrated farming system. This is in accordance with Visconti *et al.* (2008) who refer that among other factors, cropping systems affect OTA occurrence on grapes. Winter (2008) also states that mycotoxin levels in organic foods may be higher, as the usage of synthetic fungicides and insecticides in non-organic production can reduce the fungal populations.

Zimmerli and Dick (1996) also correlate the higher incidence and concentrations of OTA in wines with the different practices (e.g. the use of pesticides) that are used in viticulture. Indeed, in the present study, the results of the wine OTA analysis are in accordance with the results of grape mycological analysis, which revealed a higher incidence of *A. carbonarius* in organic farming system vineyards, compared to the integrated farming system vineyards of Kotsifali cultivar, in the respective experimental years 2011 and 2012, as presented and discussed above. This correlation is supported by Pitt and Hocking (2009) who refer to a similar relation between the fungal incidence percentage and the potential for infection and the risk of mycotoxin occurrence, but also by Cabanes *et al.* (2002) referring the contribution of *A. carbonarius* in the OTA contamination in wine.

4.2. CONCLUSIONS

The main target of the present study was to investigate the effects of the farming systems that are applied in the Cretan vineyards, either for wine or table grapes production, on the incidence of *A. carbonarius*, predominant producer of ochratoxin A in viticultural products.

Two cultivars grown in the Prefecture of Heraklion (Crete, Greece) were studied; Kotsifali (wine grape local cultivar for quality wine production) in area A (Peza) and Soultanina (the main table grape cultivar) in areas B (Malevizi) and C (Messara). The interference of the cultivars and areas on the farming system effects in the incidence of *A. carbonarius* were also investigated.

The main outcomes of the present study are listed below:

1. The *Aspergillus* spp. were the most frequently isolated among the dominant fungal species/genera (*Penicillium* spp., *Cladosporium* spp., *Alternaria* spp., *Botrytis* spp., *Rhizopus* spp. and Yeasts).
2. Among the *Aspergillus* section *Nigri* species the percentage of *A. carbonarius* was relatively low. The relative percentage of *A. carbonarius* isolation in relation to the remaining species of *Aspergillus* section *Nigri*, was found to be higher in the organic farming system vineyards.
3. *A. carbonarius* incidence was statistically significant higher in the Soultanina cultivar vineyards compared to the Kotsifali cultivar vineyards, independent of the farming system.

4. The differences in the incidence of *A. carbonarius* between the cultivars are correlated with the different characteristics of the grapes, mainly thickness of the berry skin and susceptibility to berry splitting.
5. The differences in the cultivation techniques between the cultivars resulted in different frequency of *A. carbonarius* isolation.
6. The trellis system affected *A. carbonarius* incidence, with lower levels in the trellis system that allows better exposure of the grapes to the sun light and reduce the relative humidity in the vine foliage.
7. The absence of a specific growing protocol in conventional farming system, in contrast to the organic and integrated farming systems, resulted to heterogeneous and variable outcomes.
8. The different areas of Soultanina cultivar vineyards had not effect in the incidence of *A. carbonarius*. In a restricted geographical area, the factor that differentiates the *A. carbonarius* incidence is the altitude of the vineyards under examination, with the lower incidence at the higher altitudes.
9. The different farming systems affected the incidence of *Aspergillus* section *Nigri* species in soil. The incidence of *A. carbonarius*, as well as the remaining *Aspergillus* section *Nigri* species, was higher in organic farming system vineyards.
10. A correlation between the incidence of *A. carbonarius* on grapes and in the soil of the vineyards was found.

11. The incidence of *A. carbonarius* in soil was affected by the different cultivation techniques. The different sun exposure and hence the higher temperature of surface soil (different trellis systems), the fungicides applications etc., had a significant impact.
12. The intensive use of fungicides against *Plasmopara viticola*, either in organic or integrated vineyards, significantly reduced the incidence of *A. carbonarius* in grapes and soil.
13. The fungicides used against *Botrytis cinerea* and *Plasmopara viticola* are more effective against *A. carbonarius*, compared to the fungicides used against *Uncinula necator*.
14. The crop protection products, approved for use only in organic farming system, are less effective.
15. The essential oils of *Origanum dictamnus* and *Origanum vulgare* are effective against *A. carbonarius*.
16. The percentage of the ochratoxigenic isolates of *A. carbonarius* was higher in the area with the lower incidence.
17. The higher incidence of *A. carbonarius* result to a higher potential risk of ochratoxin A occurrence.
18. The OTA concentration in wines produced from Kotsifali cultivar grapes grown in the experimental area A, was at low levels, respective to the low incidence of *A. carbonarius* in that area.

19. Despite the low levels, the OTA concentration was found significantly higher in wines from organic farming system vineyards.

4.3. GENERAL CONCLUSIONS AND FURTHER CONSIDERATIONS

It is proved in the present study that the different farming systems have a significant influence on the incidence of *A. carbonarius*, with the integrated farming system to be the most effective regarding the control of *A. carbonarius* and prevention/reduction of ochratoxin A occurrence in viticultural products.

Nevertheless, the grape cultivar has a significant impact on *A. carbonarius* incidence on grapes. Differences among the cultivars grown under the same farming system are related to the different sensitivity of the cultivars, the intensiveness of the applied cultivation practices (e.g. crop protection etc.) and cultivar-related techniques (trellis system, infected berry trimming etc.).

Finally, the research area differentiates the incidence of *A. carbonarius* independent of the farming system with the altitude being the critical factor.

Further research is necessary for better understanding of the interrelations between the factors found to affect *A. carbonarius* incidence on grapes in the present study.

The integration of fungicides with good agricultural practices must be tested, in order to provide useful data for an effective control strategy of OTA producing fungi during the production process, eliminating the risk of pesticide residues. The effectiveness of

different fungicides against *A. carbonarius* in relation with the respective pre-harvest interval must be taken under consideration. This is more critical in table grape cultivars.

The factor of the different grape cultivars, in the incidence of *Aspergillus spp.* must be studied under the same farming system and the same area, while the effect of the different areas must be studied in the same cultivars, cultivation techniques and farming system.

The co-vinification of different grape cultivars is an offend practice used in table wine production. The comparative studies of the used cultivars regarding the *A. carbonarius* incidence could be a useful tool for the oenologists, in order to organise properly the vinification process.

In cultivars used for dessert wine production, e.g. the cultivar Liatiko in the Prefecture of Heraklion, the grapes are harvested full mature or over mature. In some areas the grapes remain after harvest exposed to the sun, in order to arise the sugar content in the must. The impact of these practices in *A. carbonarius* incidence and OTA concentration must be investigated and considered.

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APPENDIX A



Plate A.1. Suberification of hole made on berry of Kotsifali cultivar by bird attack.



Plate A.2. Temperature and Relative Humidity Data Logger (HOBO – Onset, USA), placed inside the canopy of Soultanina vines, in an experimental vineyard.



Plate A.3. Vines V-type trellis system.



Plate A.4. Vines vertical-type trellis system.



Plate A.5. Insect attacked and infected berries trimming before harvest. Berries fall on the soil and constitute an excellent growing medium for the fungi.

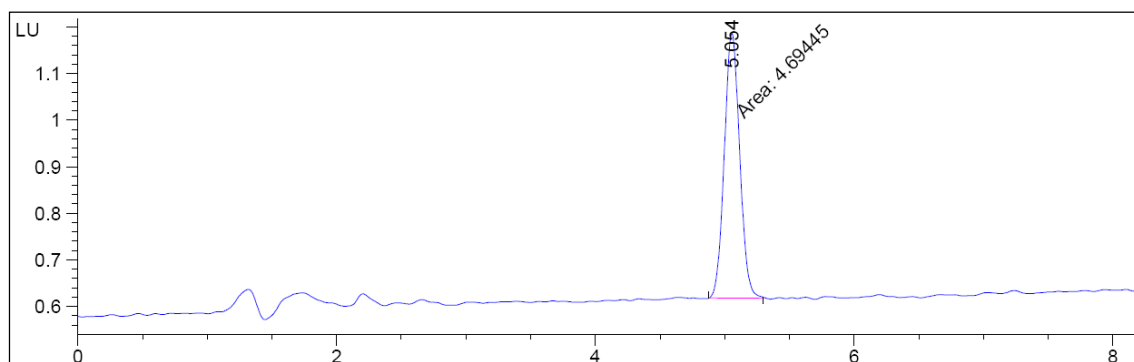


Figure A.6. HPLC chromatogram, with ochratoxin A being detected at about 5 min.

APPENDIX B

METEOROLOGICAL DATA OF RESEARCH AREAS FOR THE PERIOD 2010-2012

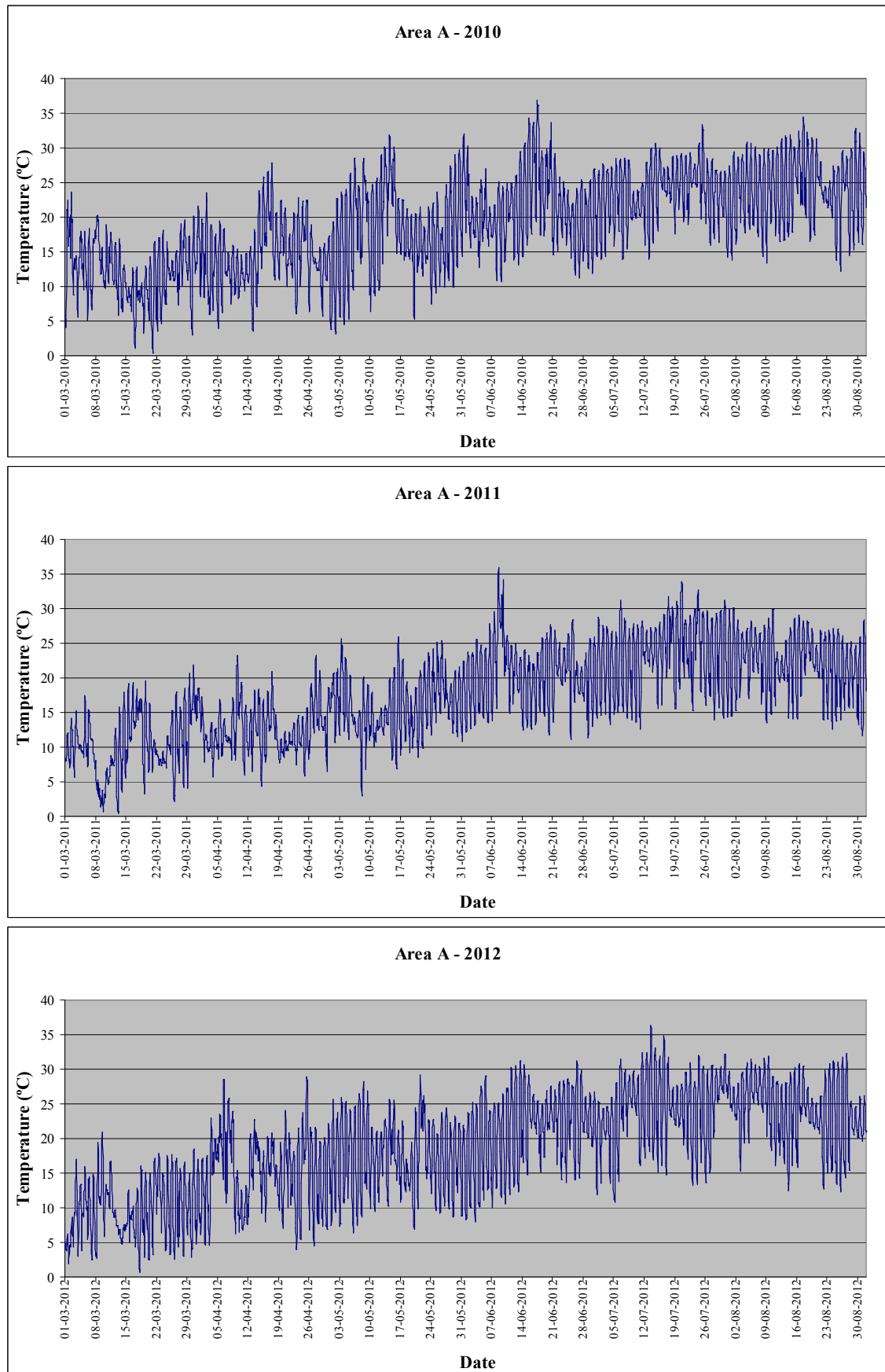


Figure B.1. Temperature (°C) during growing seasons of the years 2010, 2011 and 2012 in the Area A (N.AG.RE.F. – PPI of Heraklion).

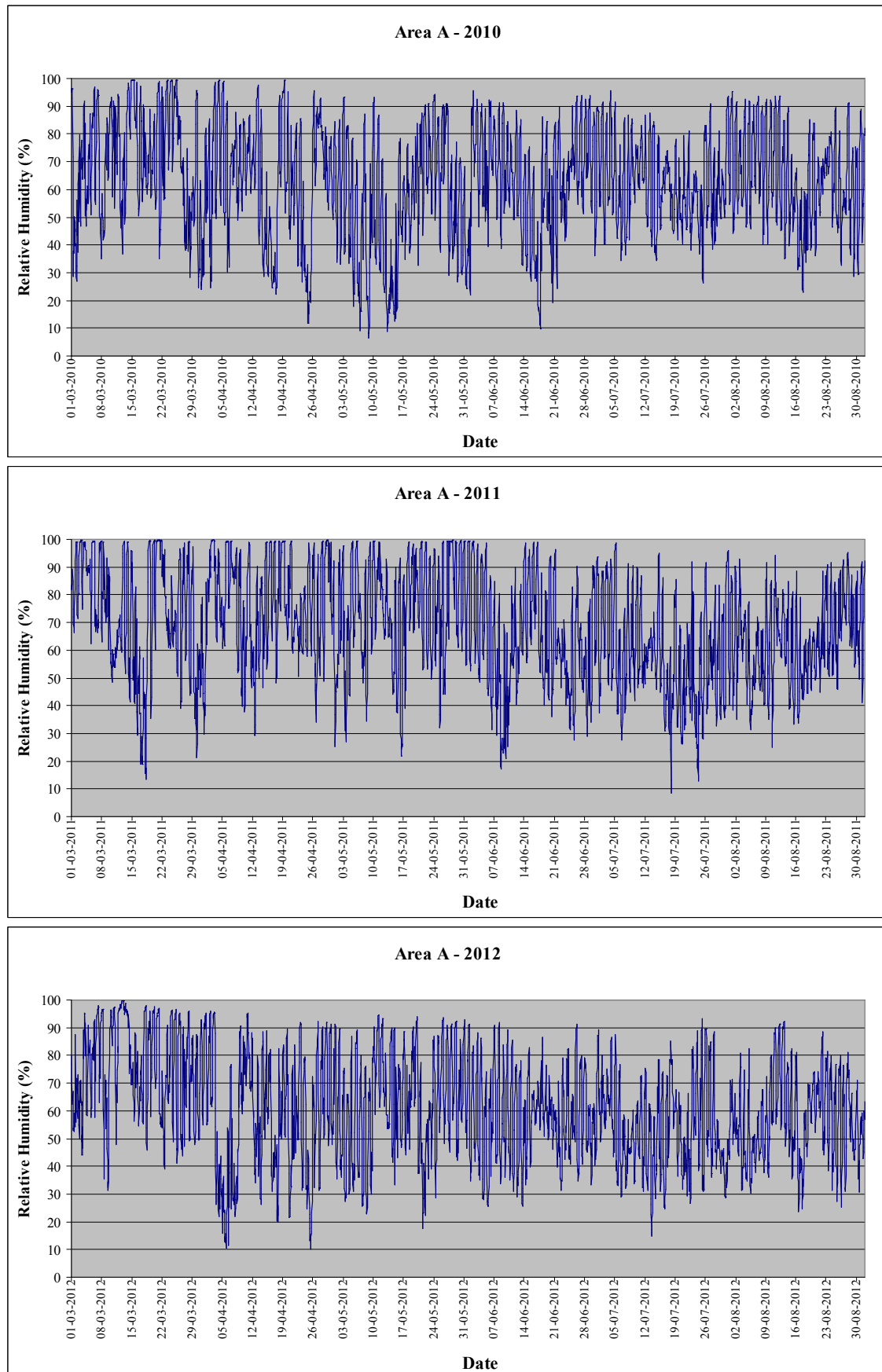


Figure B.2. Relative humidity (%) during growing seasons of the years 2010, 2011 and 2012 in the Area A (N.AG.RE.F. – PPI of Heraklion).

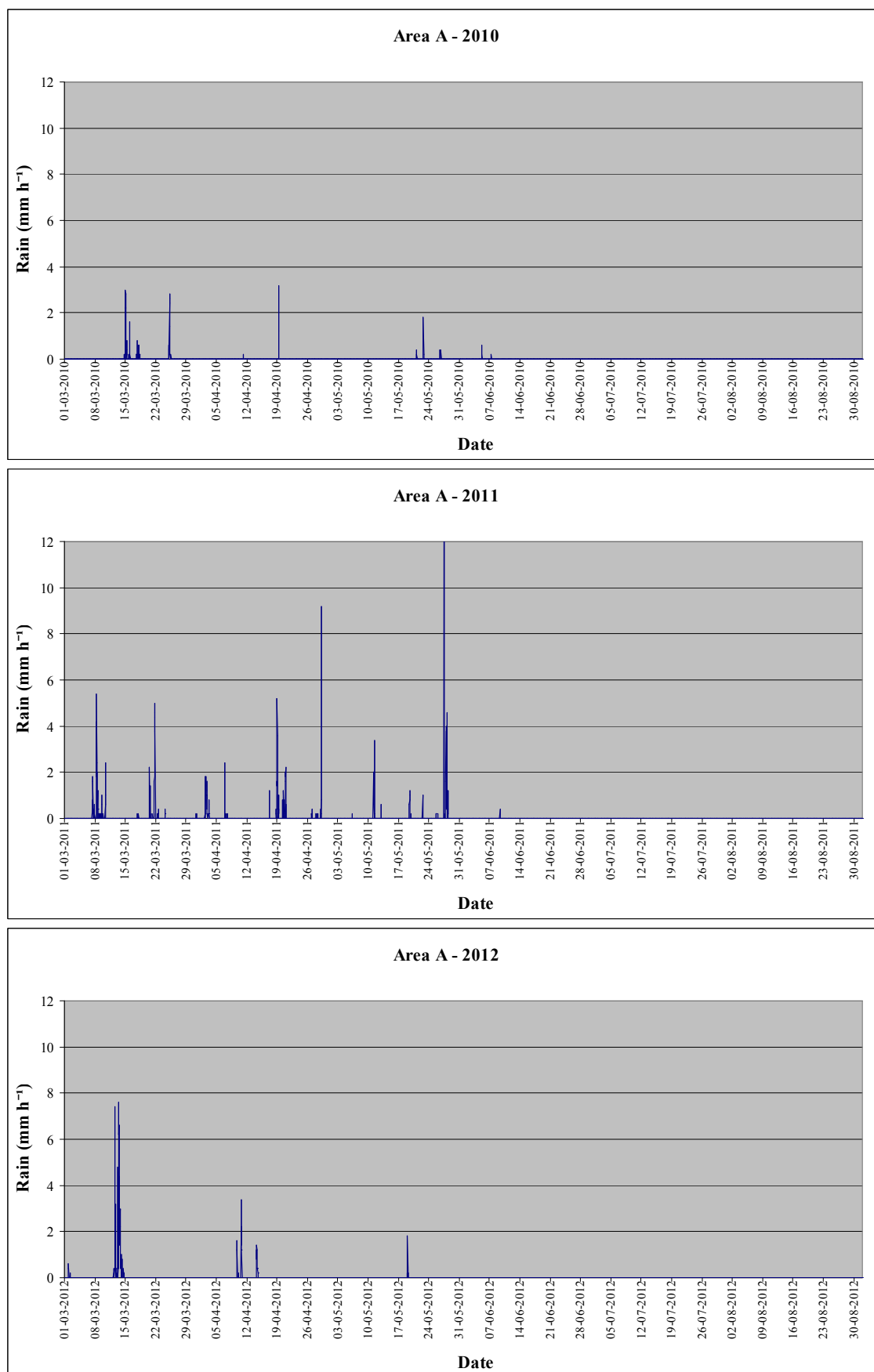


Figure B.3. Rainfall (mm h⁻¹) during growing seasons of the years 2010, 2011 and 2012 in the Area A (N.AG.RE.F. – PPI of Heraklion).

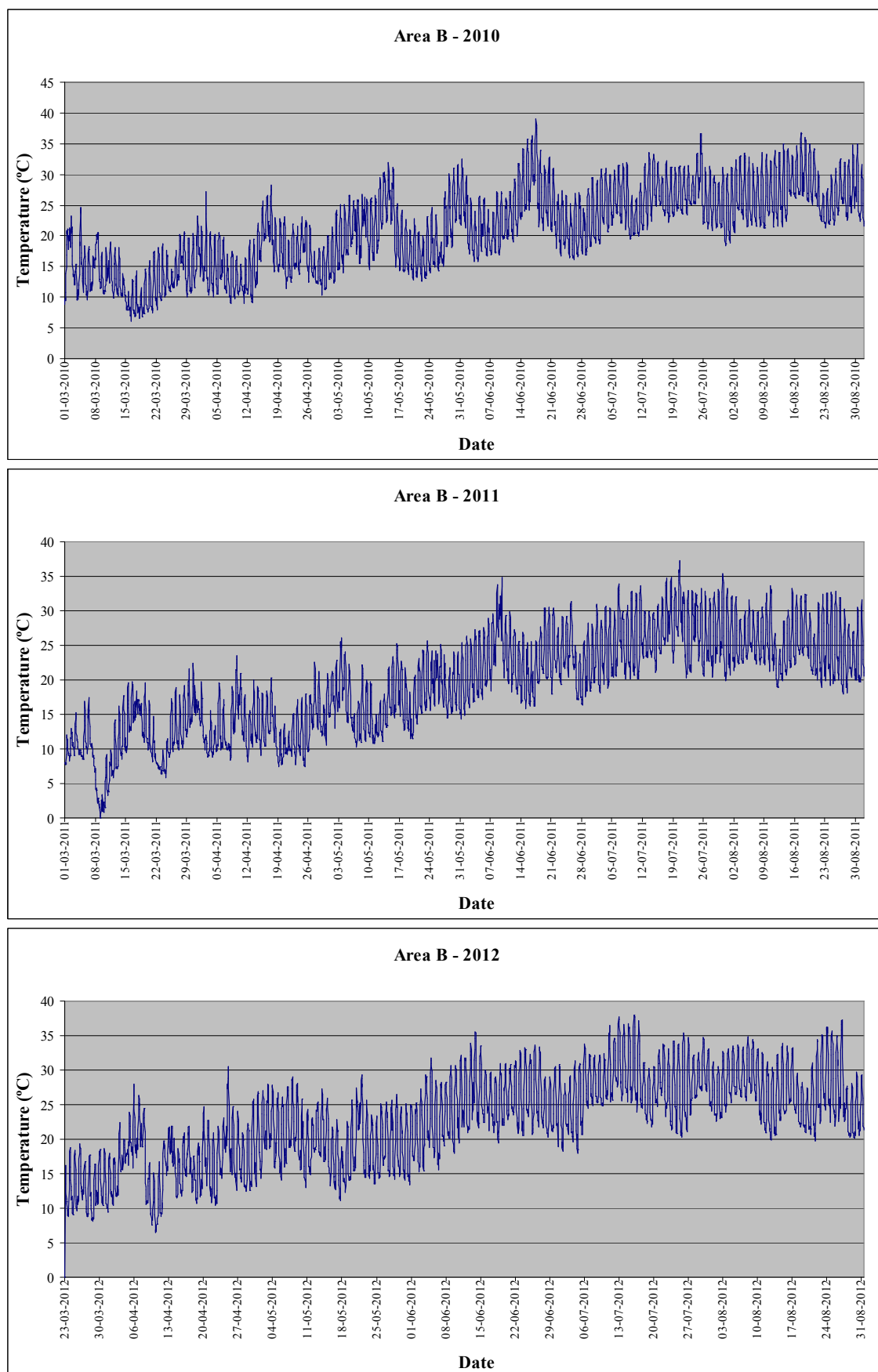


Figure B.4. Temperature (°C) during growing seasons of the years 2010, 2011 and 2012 in the Area B (N.AG.RE.F. – PPI of Heraklion).

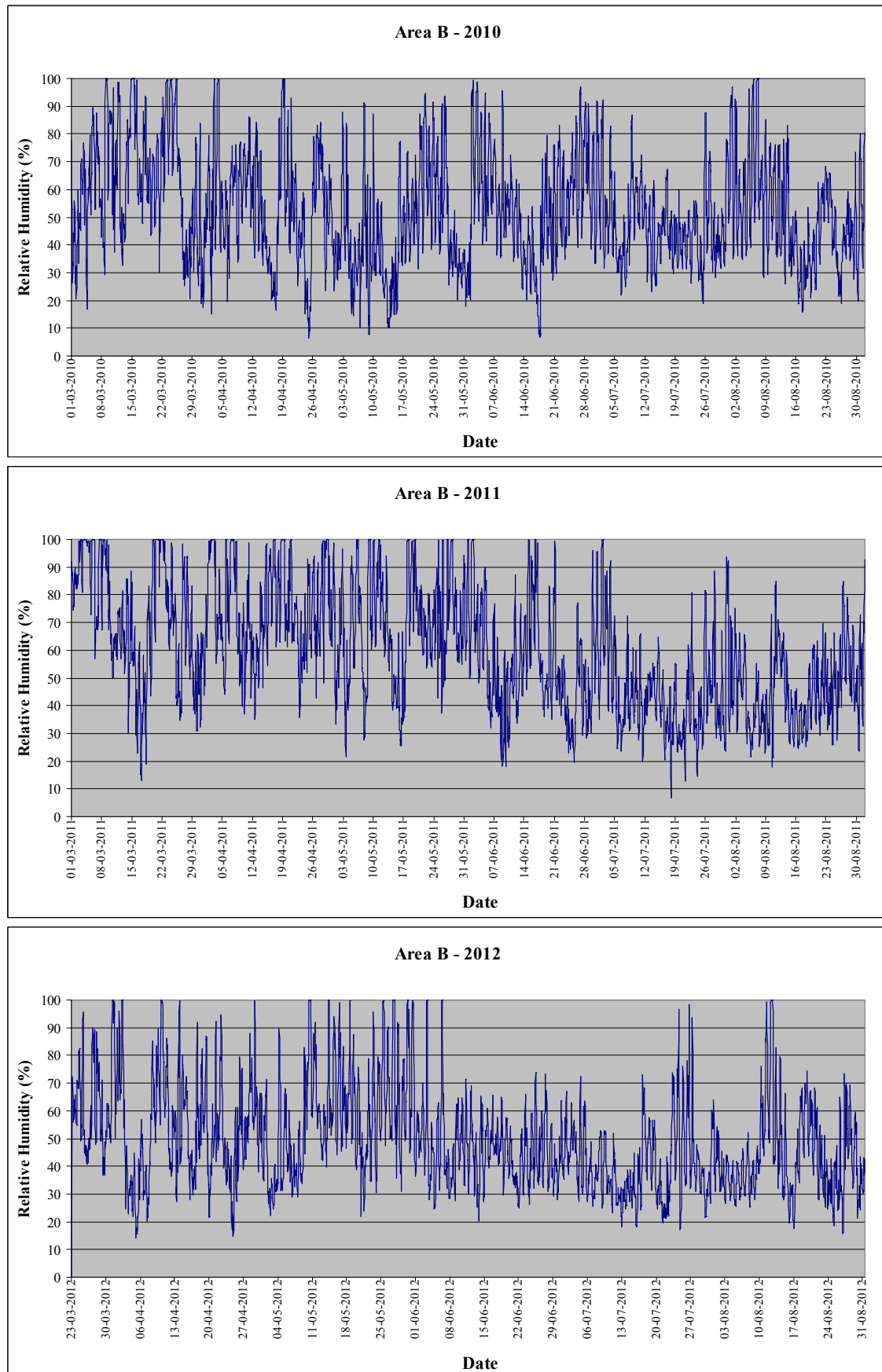


Figure B.5. Relative humidity (%) during growing seasons of the years 2010, 2011 and 2012 in the Area B (N.AG.RE.F. – PPI of Heraklion).

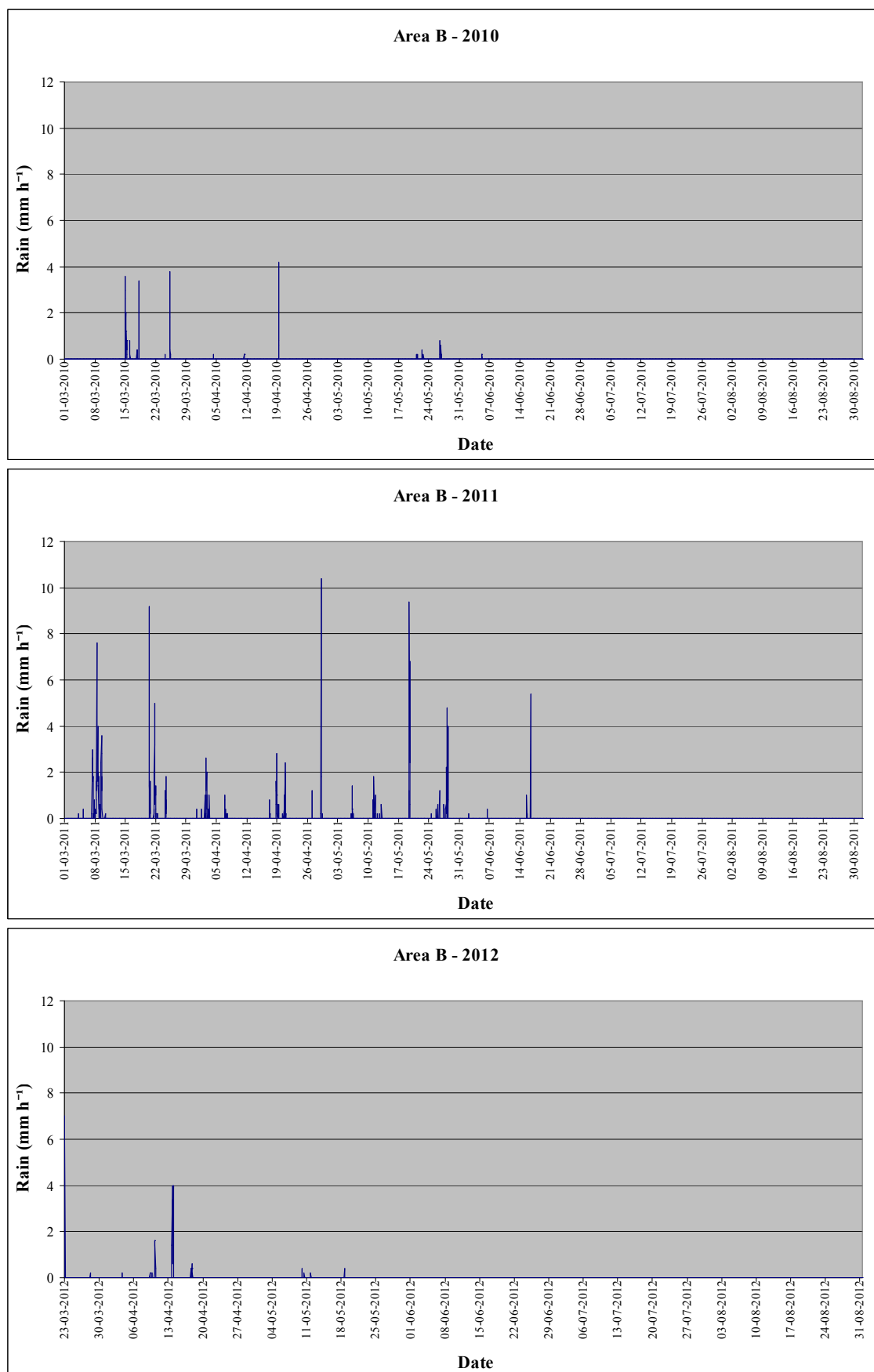


Figure B.6. Rainfall (mm h⁻¹) during growing seasons of the years 2010, 2011 and 2012 in the Area B (N.AG.RE.F. – PPI of Heraklion).

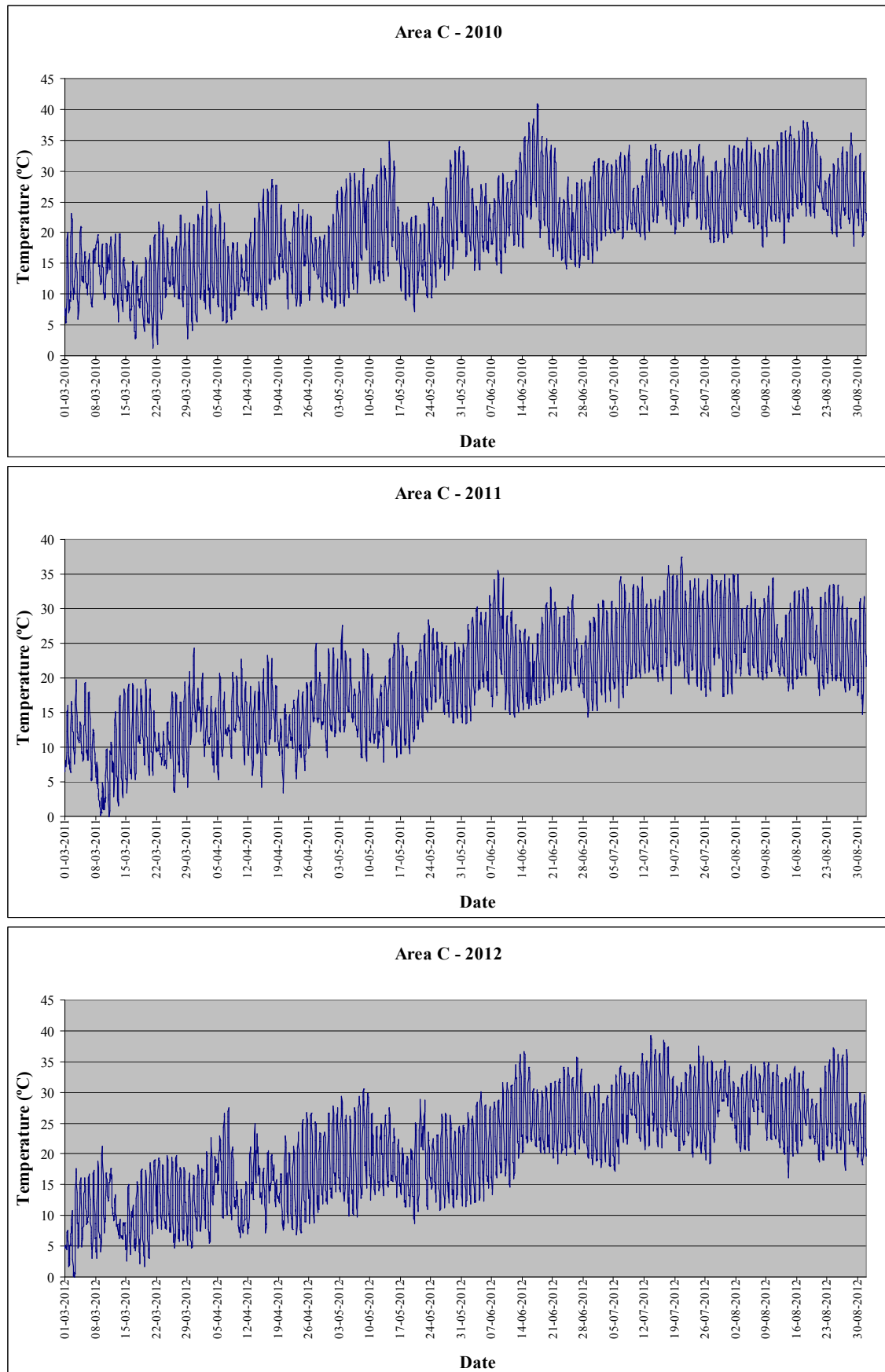


Figure B.7. Temperature (°C) during growing seasons of the years 2010, 2011 and 2012 in the Area C (N.AG.RE.F. – PPI of Heraklion).

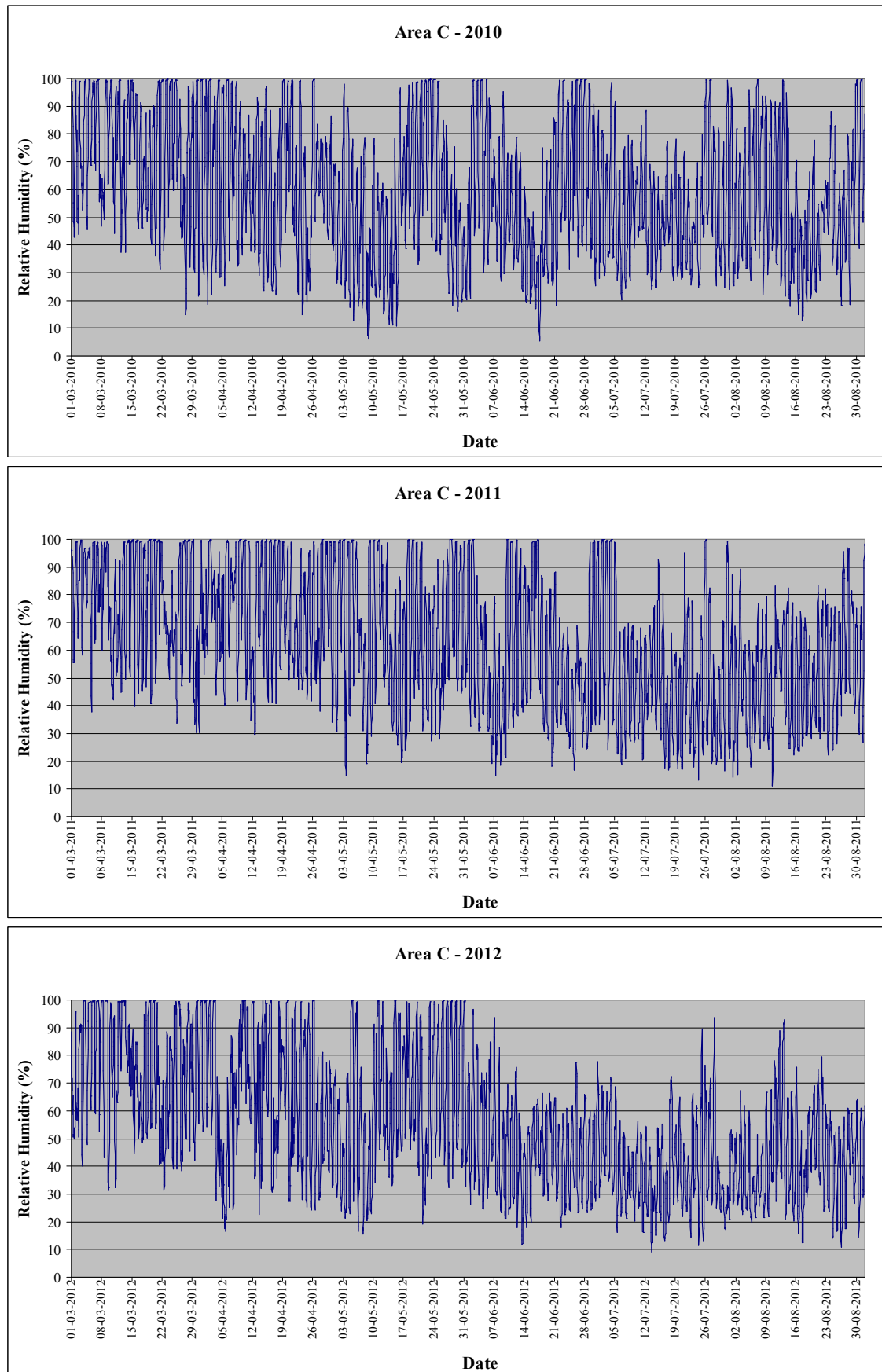


Figure B.8. Relative humidity (%) during growing seasons of the years 2010, 2011 and 2012 in the Area C (N.AG.RE.F. – PPI of Heraklion).

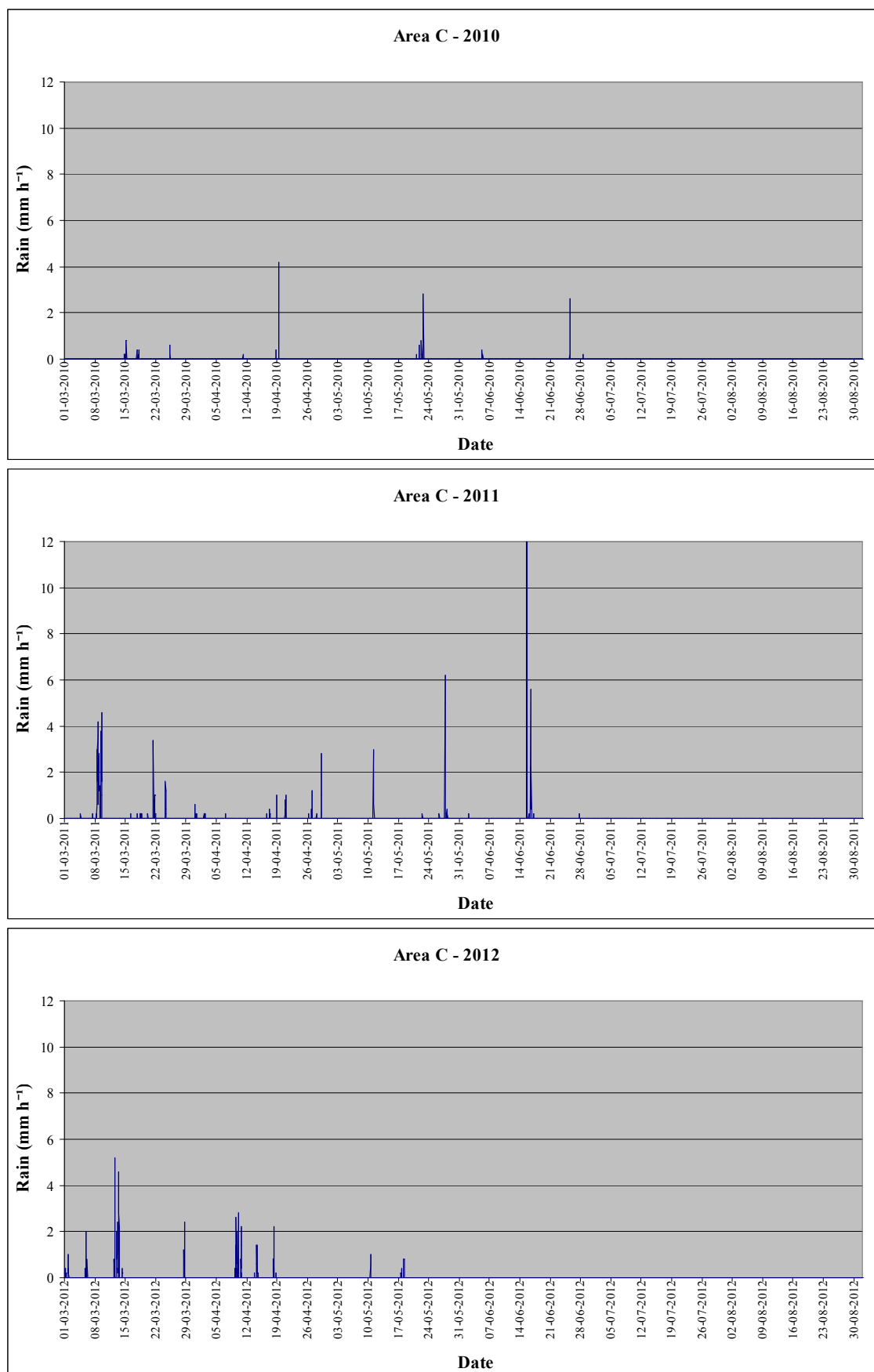


Figure B.9. Rainfall (mm h⁻¹) during growing seasons of the years 2010, 2011 and 2012 in the Area C (N.AG.RE.F. – PPI of Heraklion).

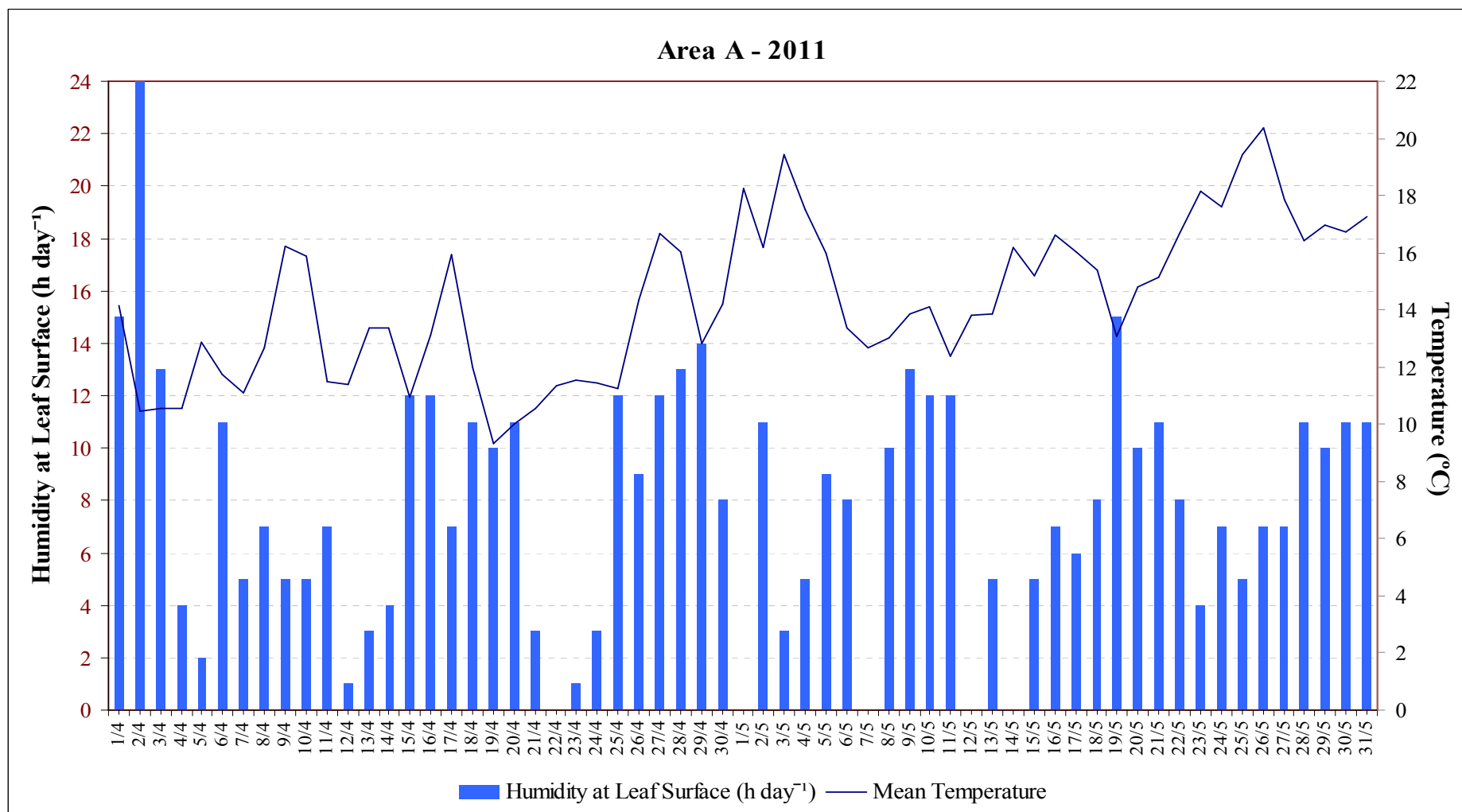


Figure B.10. Humidity at leaf surface (h day⁻¹) and mean temperature (°C) during April and May of 2011, in the Area A (N.AG.RE.F. – PPI of Heraklion).

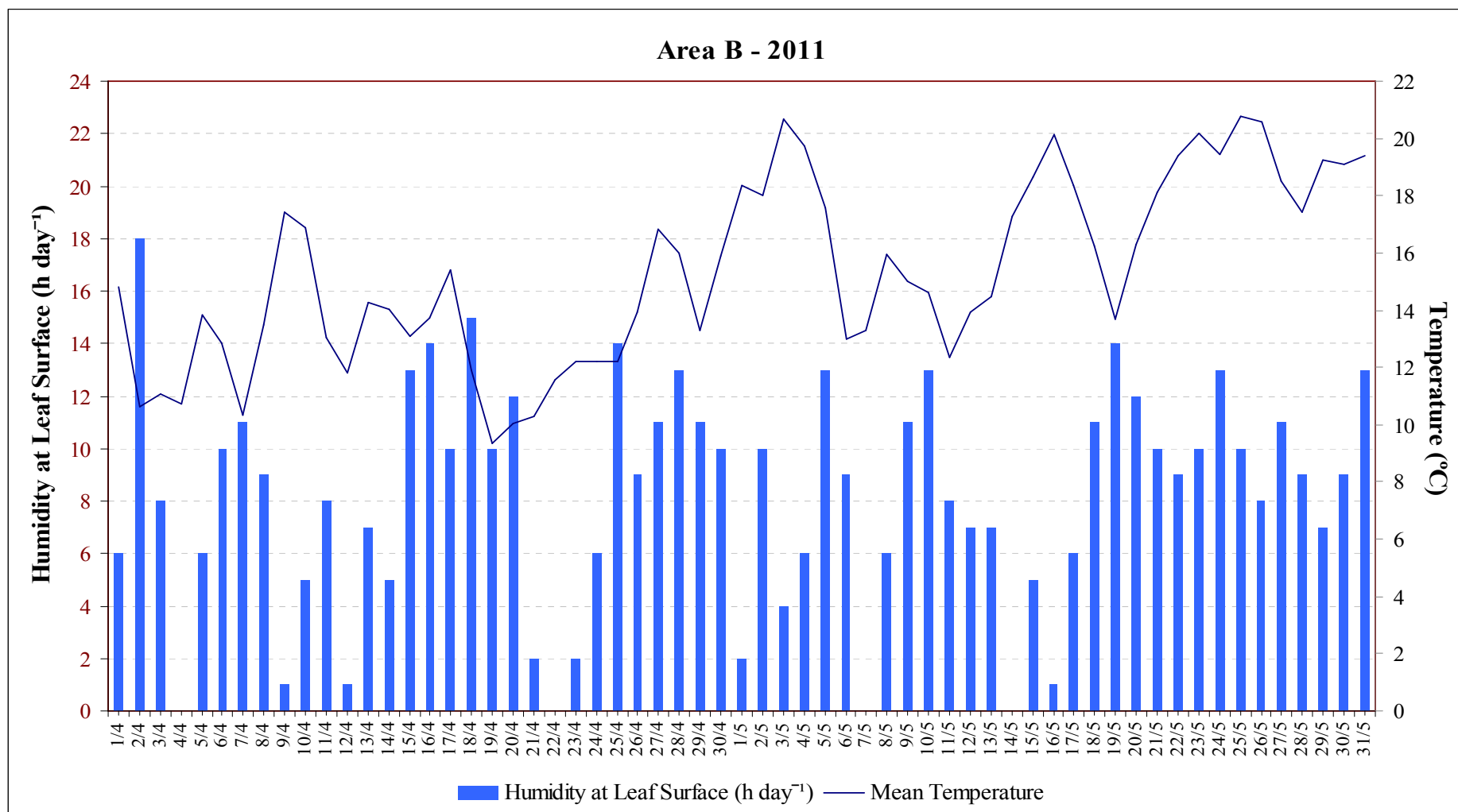


Figure B.11. Humidity at leaf surface (h day^{-1}) and mean temperature ($^{\circ}\text{C}$) during April and May of 2011, in the Area B (N.AG.RE.F. – PPI of Heraklion).

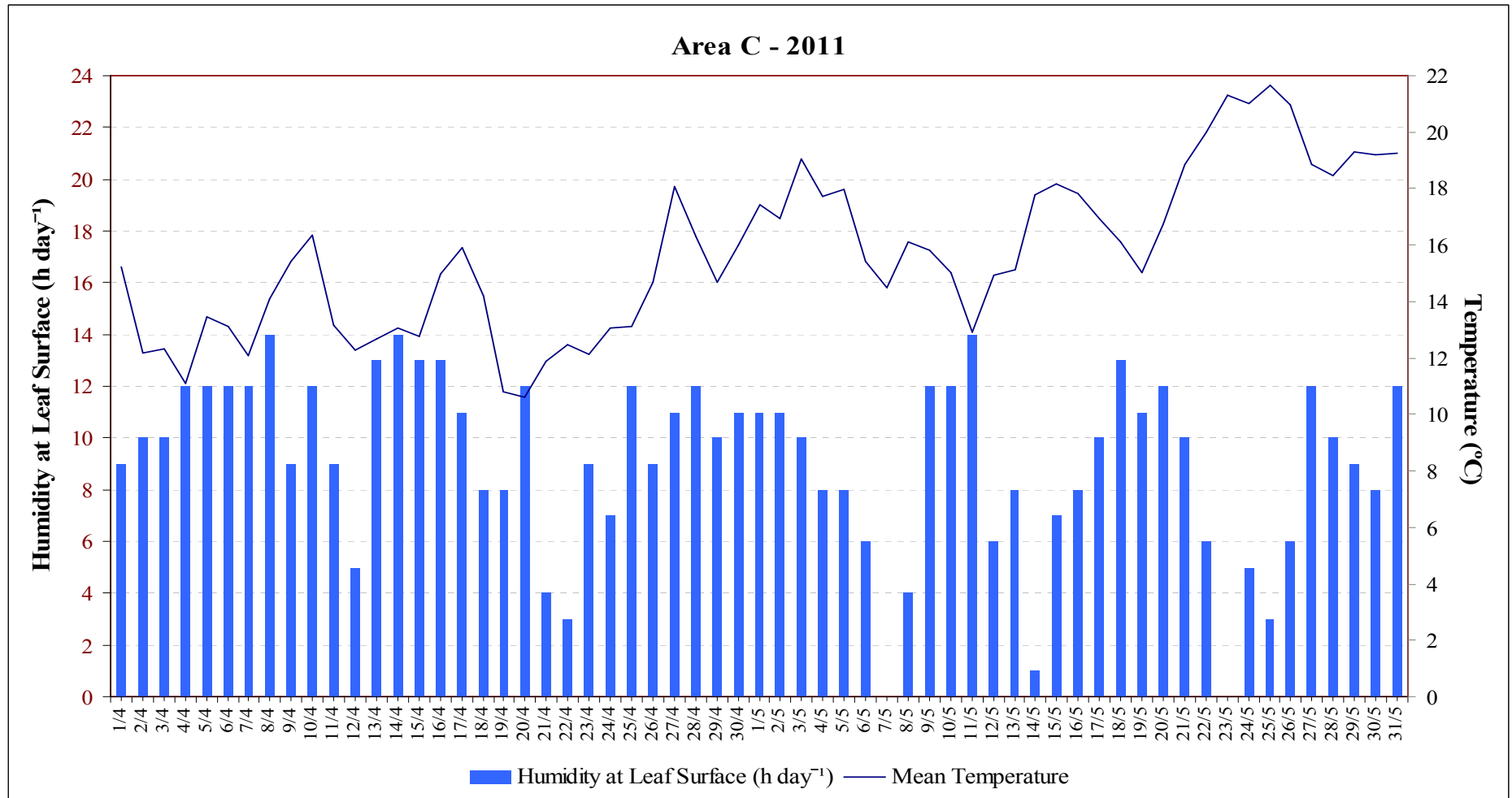


Figure B.12. Humidity at leaf surface (h day^{-1}) and mean temperature ($^{\circ}\text{C}$) during April and May of 2011, in the Area C (N.AG.RE.F. – PPI of Heraklion).

APPENDIX C

**ABSTRACT OF POSTER SUBMITTED IN THE 25TH MEETING OF
GREEK SOCIETY OF HORTICULTURAL SCIENCES, THE YEAR
2011, IN CYPRUS (IN GREEK).**

**THE INFLUENCE OF FARMING SYSTEMS IN THE INCIDENCE OF
ASPERGILLUS CARBONARIUS IN VITICULTURAL PRODUCTS**

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Abstract

In recent years there has been a continuous increasing concern regarding the health of consumers and their exposure to foods containing mycotoxins, which are secondary metabolites of various fungal species. One of the major mycotoxins, with unfavourable consequences to humans and animals health, and can be found in a wide range of foods as cereals, nuts, spices, meat, viticultural products etc., is ochratoxin A, produced mainly by the species of *Aspergillus* and *Penicillium* genera, and especially in viticultural products by *A. carbonarius*. The aim of the present study, conducted the growing season of the year 2010, in two of the main viticultural areas of Heraklion Prefecture, was the determination of farming systems (Organic and Integrated) influence in the incidence of *A. carbonarius* on table grapes of Soultanina cultivar. A mycological analysis of grape berries, from grape samples taken at harvest time, from 12 vineyards, of organic and integrated farming systems carried out, using the direct plating method, on Malt Extract Agar (MEA) medium. *A. carbonarius* isolated and identified by microscopic and stereoscopic observation based on morphologic characteristics, after seven days of incubation at 25 °C. In viticultural area of Malevizi, *A. carbonarius* isolated, from the 58.67% of grape samples from organic farming vineyards and from the 32% of grape samples of integrated farming system vineyards. Respectively, in viticultural area of Messara, *A. carbonarius* isolated from the 47.33% of grape samples from organic farming system vineyards and from 32.67% of grape samples from integrated farming system vineyards. According to the results, the incidence of *A. carbonarius* seems to be affected of the farming system, as in both of research areas, the percentage of isolation was significant more frequent in organic farming system vineyards compared to the integrated farming system vineyards.