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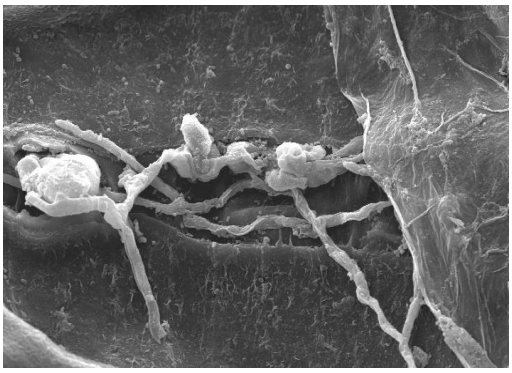
AGRICULTURAL UNIVERSITY OF ATHENS

Monitoring of ochratoxin A production of *Aspergillus carbonarius* isolates from Greek grapes through gene expression studies under different ecophysiological factors



PhD Thesis

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SCHOOL OF FOOD, BIOTECHNOLOGY AND DEVELOPMENT

DEPARTMENT OF FOOD SCIENCE AND HUMAN NUTRITION

LABORATORY OF FOOD MICROBIOLOGY AND BIOTECHNOLOGY

Athens 2018

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ΤΡΟΦΙΜΩΝ

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Διδακτορική Διατριβή

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Αθήνα, 2018

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Στους γονείς μου

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Summary

Ochratotoxin A (OTA) is a secondary metabolite which is one of the most widely distributed mycotoxins since dietary exposure causes major health risks. Contamination of grapes with OTA is mainly caused by black Aspergilli, among which *Aspergillus carbonarius* is recognized as the main OTA producer in grapes and their derivatives (grape juice, raisins and wine). This potential pathogenic fungus has wide distribution in the environment and is characterized by a remarkable ability to proliferate under a variety of stress conditions, enabling thus its growth in a wide range of environmental conditions and substrates. *A. carbonarius* frequent occurrence could be attributed to its rapid growth resulting in grape contamination especially in the late veraison stage. Nowdays, OTA control still remains a challenge of continuous efforts in order to meet food safety standards in the production of wine and table grapes.

Current knowledge suggests that OTA production is under the control of various ecophysiological interactions at molecular level. Therefore, in the last years attention has been given on fungal genetic parameters in conjunction with environmental and biological factors that influence OTA production. The aim of this thesis was to employ a gene expression approach on *A. carbonarius* isolates of different phenotypic responses concerning OTA production in order to elucidate OTA related gene expression under different ecophysiological factors.

Initially, the correlation between OTA production and different parameters of fungal growth determination (i.e., radial growth extension, biomass, dry weight, mycelium colony area) was measured in relation to time in a synthetic grape juice medium (SGM) (Chapter 3). Results showed that different growth responses were correlated and intra-species differences were highlighted. It was also found that higher OTA producers seemed to be better correlated with the measured growth parameters. Finally, a preliminary modelling approach was used to evaluate toxin formation taking inter-specificity into consideration, presenting high correlation between OTA kinetics and fungal growth.

Further on, the transcription of the OTA key biosynthetic genes AcOTA*pks* and AcOTA*nrps*, and global regulator *laeA* OTA related genes was investigated using Reverse Transcription quantitative PCR (RT-qPCR) (Chapter 4). The transcription levels of OTA key biosynthetic genes were assessed during growth of *A. carbonarius* in liquid SGM medium at different temperatures, water activity levels and incubation time. Gene expression was monitored at the same time points along with fungal biomass and OTA accumulation at three, six and nine days of incubation. Nevertheless, no general trend could be established regarding the effect of incubation temperature, water activity (a_w) or time on gene expression. Moreover, the isolate specificity impact which was observed, underlined the complexity of gene expression responses. Early activation of both OTA biosynthetic key genes underlined the predictive nature of RT-qPCR analysis, since genes as molecular indicators were indeed expressed a few days before any OTA could be detected.

In addition, the effect of clove, cinnamon, lemongrass and mandarin essential oils (EOs) on *A. carbonarius* growth and OTA regulation was also investigated (Chapter 5). The expression of the specific OTA related genes (AcOTA*pks*, AcOTA*nrps*, and *laeA*) was

characterized in SGM solid growth medium in response to EOs application. The antifungal activity of the selected EOs was affected by the different fungal isolates and media substrates (MEA, CYA, SGM). The influence of different EOs was dose dependent concerning their inhibitory effect. In most cases, relative expression profiles presented similar trends with OTA production among treatments and control samples, indicating a possible connection between transcript levels of the structural genes and ochratoxigenic activity. These results suggest that the specific genes may play a key role in the mechanism by which essential oils inhibit OTA production by *A. carbonarius.*

Finally, co-cultivation of *A. carbonarius* with Lactic Acid Bacteria (LAB) effectively reduced OTA production *in vitro* in a strain-dependent manner (Chapter 6). The results indicated a significant diversity of growth profiles and toxin production in mixed fungal/LAB co-cultures. The *in situ* effect of fungal/LAB co-cultivation on grape berries was also studied in parallel to bacterial ability for attachment on grape skin surface and toxin inhibition with promising results. Finally, gene expression studies indicated a mixed response for all genes highlighting biological variability for both fungal and LAB microorganisms.

Scientific field: Food Mycology and Safety

Key words: *Aspergillus carbonarius*, Ochratoxin A, grapes, gene expression, transcription, toxin control, essential oils, fungal growth inhibition

Έλεγχος της παραγωγής ωχρατοξίνης Α σε στελέχη *Aspergillus carbonarius* από ελληνικές ποικιλίες σταφυλιών μέσω μελέτης της γονιδιακής έκφρασης υπό την επίδραση διαφορετικών οικοφυσιολογικών παραγόντων

Περίληψη

Η ωχρατοτοξίνη Α (ΩΤΑ) είναι ένας δευτερογενής μεταβολίτης που αποτελεί μια από τις πλέον διαδεδομένες μυκοτοξίνες, καθώς η παρουσία της στη διατροφική αλυσίδα προκαλεί σοβαρούς κινδύνους στην ανθρώπινη υγεία. Η επιμόλυνση των σταφυλιών με ΩΤΑ οφείλεται κυρίως στην παρουσία των μαύρων Ασπέργιλλων (*Aspergillus* section *Nigri*) μεταξύ των οποίων ο μύκητας *Aspergillus carbonarius* έχει αναγνωριστεί ως ο κύριος παραγωγός ΩΤΑ στα σταφύλια και στα παράγωγα προϊόντα (χυμός σταφυλιών, σταφίδες και οίνος). Ο μύκητας είναι δυνητικά παθογόνος, παρουσιάζει ευρεία διασπορά στο περιβάλλον και χαρακτηρίζεται από αξιοσημείωτη ικανότητα να ανθίσταται σε διάφορους παράγοντες καταπόνησης, γεγονός που του επιτρέπει να επιβιώνει σε μεγάλο εύρος κλιματολογικών συνθηκών και θρεπτικών υποστρωμάτων. Η συχνή παρουσία του μύκητα οφείλεται στην ταχεία ανάπτυξή του, η οποία συντελεί στην επιμόλυνση των σταφυλιών, ειδικότερα προς το τέλος του σταδίου του περκασμού (veraison). Σήμερα, η ΩΤΑ εξακολουθεί να αποτελεί σημαντική πρόκληση στο πλαίσιο των συνεχών προσπαθειών τήρησης των προδιαγραφών ασφάλειας των τροφίμων για την παραγωγή ποιοτικών οίνων και επιτραπέζιων σταφυλιών.

Οι πρόσφατες μελέτες δείχνουν ότι η παραγωγή της ΩΤΑ είναι συνέπεια διαφόρων οικοφυσιολογικών αλληλεπιδράσεων σε μοριακό επίπεδο. Επομένως, τα τελευταία χρόνια το ενδιαφέρον της επιστημονικής κοινότητας έχει εστιαστεί στη μελέτη των γενετικών παραμέτρων που σε συνδυασμό με περιβαλλοντικούς και βιολογικούς

παράγοντες επηρεάζουν την παραγωγή ωχρατοξίνης. Στο πλαίσιο της εν λόγω διδακτορικής διατριβής μελετήθηκαν διαφορετικοί φαινότυποι του μύκητα *A. carbonarius* ως προς το δυναμικό παραγωγής τοξίνης, εστιάζοντας στην έκφραση των γονιδίων εκείνων που σχετίζονται με την παραγωγή ΩΤΑ υπό την επίδραση διαφορετικών οικοφυσιολογικών παραγόντων σε επίπεδο μεταγραφής.

Αρχικά μελετήθηκε η συσχέτιση μεταξύ παραγωγής ΩΤΑ και διαφορετικών μεθόδων προσδιορισμού της ανάπτυξης (μεταβολή της διαμέτρου, της βιομάζας, του ξηρού βάρους και της επιφάνειας της μυκηλιακής αποικίας) του μύκητα *A. carbonarius* σε σχέση με το χρόνο, χρησιμοποιώντας συνθετικό υπόστρωμα χυμού σταφυλιών (SGM) (Κεφάλαιο 3). Τα αποτελέσματα έδειξαν ότι όλες οι μέθοδοι προσδιορισμού της ανάπτυξης που μελετήθηκαν συσχετίστηκαν τόσο με την παραγωγή τοξίνης όσο και μεταξύ τους, τονίζοντας επίσης την ενδο-στελεχιακή παραλλακτικότητα που παρατηρήθηκε. Διαπιστώθηκε επίσης ότι οι απομονώσεις του μύκητα *A. carbonarius* που παρουσίασαν υψηλότερη παραγωγή τοξίνης συσχετίστηκαν καλύτερα με τις εκάστοτε παραμέτρους ανάπτυξης. Τέλος, έγινε προσπάθεια ανάπτυξης πρωτογενών μοντέλων συσχέτισης της ΩΤΑ με το χρόνο, λαμβάνοντας υπόψη τη διαστελεχική παραλλακτικότητα, τα οποία έδειξαν υψηλή συσχέτιση της κινητικής της ΩΤΑ με την ανάπτυξη του μήκυτα.

Στη συνέχεια διερευνήθηκε η έκφραση σε μεταγραφικό επίπεδο των βιοσυνθετικών γονιδίων της ΩΤΑ, AcOTA*pks*, AcOTA*nrps* και του μεταγραφικού παράγοντα *laeA*, που σχετίζεται με την παραγωγή της ΩΤΑ, χρησιμοποιώντας τη μέθοδο της αλυσιδωτής αντίδρασης της πολυμεράσης πραγματικού χρόνου (RT-qPCR) (Κεφάλαιο 4). Τα επίπεδα μεταγραφής των παραπάνω γονιδίων εκτιμήθηκαν κατά την ανάπτυξη του μύκητα *A. carbonarius* σε υγρό θρεπτικό μέσο SGM σε διαφορετικές τιμές θερμοκρασίας, ενεργότητας ύδατος και χρόνου ανάπτυξης. Η γονιδιακή έκφραση

προσδιορίστηκε στα ίδια χρονικά σημεία μαζί με τη βιομάζα του μύκητα και την παραγωγή τοξίνης. Τα αποτελέσματα έδειξαν μεμονωμένες συσχετίσεις και όχι κάποια ευκρινή τάση σε σχέση με την επίδραση της θερμοκρασίας, της ενεργότητας ύδατος ή του χρόνου ανάπτυξης επί της γονιδιακής έκφρασης. Επίσης, τα αποτελέσματα εξαρτήθηκαν από το στέλεχος του μήκητα, γεγονός που υποδεικνύει την πολυπλοκότητα των αποκρίσεων σε επίπεδο γονιδιακής έκφρασης Η πρώιμη ενεργοποίηση των δύο βασικών βιοσυνθετικών γονιδίων της ωχρατοξίνης υπογράμμισε την προρρητική φύση της ανάλυσης RT-qPCR, εφόσον τα εν λόγω γονίδια, ως μοριακοί δείκτες εκφράζονται πριν την φαινοτυπική ανίχνευση της τοξίνης.

Επιπλέον, μελετήθηκε η επίδραση των αιθερίων ελαίων γαρίφαλλου, κανέλλας, λεμονόχορτου και μανταρινιού στην ανάπτυξη του μύκητα *A. carbonarius* και στη βιοσύνθεση της ΩΤΑ (Κεφάλαιο 5). Η έκφραση των σχετιζόμενων με την παραγωγή τοξίνης γονιδίων προσδιορίστηκε σε υγρό υπόστρωμα SGM στο οποίο είχαν προστεθεί διαφορετικές συγκεντρώσεις των ανωτέρω αιθερίων ελαίων. Η αντιμυκητιακή τους δράση επηρεάστηκε από τις διαφορετικές απομονώσεις του μύκητα *A. carbonarius* καθώς και τα διαφορετικά υποστρώματα (MEA, CYA, SGM). Η ανασταλτική δράση των διαφορετικών αιθερίων ελαίων ήταν ανάλογη της συγκέντωσης που χρησιμοποιήθηκε σε κάθε περίπτωση. Στις περισσότερες περιπτώσεις, η γονιδιακή έκφραση παρουσίασε παρόμοια τάση με την παραγωγή τοξίνης μεταξύ των μεταχειρήσεων (παρουσία αιθερίου ελαίου) και του μάρτυρα, γεγονός που μπορεί να υποδεικνύει πιθανή συσχέτιση μεταξύ των επιπέδων μεταγραφής των γονιδίων και της αντι-ωχρατοξινογόνου δράσης. Τα αποτελέσματα υποδηλώνουν την πιθανή επίδραση των συγκεκριμένων αιθερίων ελαίων στην έκφραση των υπό μελέτη γονιδίων και επομένως στην παραγωγή ΩΤΑ.

Τέλος, μελετήθηκαν οι επιπτώσεις στην ανάπτυξη του μύκητα *A. carbonarius* και στην παραγωγή ΩΤΑ κατά την *in vitro* συγκαλλιέργεια του μύκητα με διαφορετικά στελέχη του οξυγαλακτικού βακτηρίου *Lactobacillus plantarum* (Κεφάλαιο 6). Τα αποτελέσματα έδειξαν σημαντική μείωση της ανάπτυξης του μύκητα και της παραγωγής ΩΤΑ σε όλες τις περιπτώσεις συγκαλλιέργειας με τα οξυγαλακτικά βακτήρια. Διερευνήθηκε επίσης *in situ* η ικανότητα προσκόλλησης των βακτηριακών κυττάρων στην επιφάνεια των σταφυλιών και η επίδρασή τους στην αναστολή της παραγωγής ΩΤΑ με θετικά αποτελέσματα. Τέλος, η μελέτη της γονιδιακής έκφρασης έδειξε μικτές αποκρίσεις για όλα τα εξεταζόμενα γονίδια γεγονός που τονίζει την επίδραση της παραλλακτικότητας του στελέχους, όσον αφορά στις διαφορετικές απομομώσεις του μύκητα *A. carbonarius* αλλά και τα διαφορετικά στελέχη του βακτηρίου *L. plantarum* που χρησιμοποιήθηκαν.

Επιστημονική περιοχή: Μικροβιολογία και ασφάλεια τροφίμων

Λέξεις κλειδιά: *Aspergillus carbonarius*, Ωχρατοξίνη Α, σταφύλια, γονιδιακή έκφραση, μεταγραφή, έλεγχος τοξίνης, αιθέρια έλαια, αναστολή ανάπτυξης

Contents

CHAPTER 1

General introduction, scope and outline of the thesis

1.1 *Fungal secondary metabolites production regulation*

In nature, filametous fungi are challenged by multiple biotic and abiotic stressors, such as environmental conditions, nutrient deprivation, or even the interaction with other organisms. As a physiological response to all the above, fungi produce a diverse number of secondary metabolites (SMs). Fungal genes encode enzymes which are responsible for the main synthesis steps of SMs (Keller et al., 2005) that can be divided into four main chemical classes: polyketides, terpenoids, shikimic acid derived compounds, and non-ribosomal peptides (Pusztahelyi et al., 2015). Polyketides are the most abundant fungal secondary metabolites. Analysis of available fungal genomes revealed that ascomycetes have more genes for secondary metabolism than basidiomycetes, whereas zygomycetes have none (Collemare et al., 2008). Ascomycetes genomes code on average 16 polyketide synthases (PKS), 10 non-ribosomal protein synthases (NRPS), 2 tryptophan synthetases (TS), and 2 dimethylallyl tryptophan synthetases (DMATS) with crucial importance in SM synthesis.

Fungal secondary metabolites production regulation does not follow a strict hierarchical regime since inter-connection and even overlapping of metabolic pathways exist. Their physiological function in nature exists in multiple levels of regulation, including epigenetic control (Li and Reinberg, 2011; Venkatesh et al., 2015), signal transduction pathways and pathway-specific transcription factors (TFs), whereas global regulators translate environmental cues into SMs (Alkhayyat and Yu, 2013). Moreover, pathway-specific transcription factors typically control transcriptional activation for most of structural genes (Yin and Keller, 2011). The modifications in several regulatory levels at the same time could possibly lead to the induction of silent SM gene clusters (Bvadhage and Schoeckh, 2010).

Fungi are organisms that are prone to changes in environmental conditions which influence SM formation (Fountain et al., 2014). Fungal genes involved in stress factors (such as temperature, a_w, etc.) are highly represented in phytopathogenic fungi (Karányi et al., 2013) and fungal toxins often play a key role in triggering these responses. The transaction from stimulus to response is based on signaling pathways, which regulate gene expression and activation of secondary metabolism (Fox and Howlett, 2008).

Mycotoxins stand among the most important SMs of filametous fungi. These metabolites are mainly low molecular weight compounds, not directly essential for fungal growth and/or survival. Secondary metabolism has proven to be advantageous for the fitness and proliferation of the producing fungus (Howard and Valent, 1996; Fox and Howlett, 2008). Studies have suggested that mycotoxins serve as a molecular arsenal to aid the fungus in successful competition against other microorganisms in its natural environment (Keller et al., 2005). On the other hand, pigments, a kind of secondary metabolic products, have a broad range of functionality, ranging from protecting the fungus from UV damage to serve as fungal virulence factor. As already mentioned, mycotoxins biosynthesis as SM are under the control of common global transcriptional factors implicated in responding to environmental cues (pH, nutrition, light, and temperature) and stresses (Hoffmeister and Keller, 2007). Several developmental pathways have been linked to mycotoxin regulation as reported for the velvet complex (VelB/VeA/LaeA) (Bayram et al., 2008) which coordinates light signal with fungal development and secondary metabolism. This complex consists of proteins fully finctional in the nucleus under dark conditions (Knox et al., 2015). The nuclear protein LaeA is a master regulator of secondary metabolism in Aspergilli (Bow and Keller, 2004; Keller et al., 2006). Deletion of *laeA* gene led to conidial reduction and OTA decrease in *A. carbonarius* (Crespo-Sempere et al., 2013). Interestingly, no global

regulator has been identified for other environmental factors such as temperature, as it has for light regulation. These regulators act as an upper level governor of mycotoxin biosynthesis in comparison to the pathway-specific regulators (Ehrlich et al., 2003; Yu and Keller, 2005). This system of regulation usually mediates its function through genes that are associated with physiological responses, but as a consequence, it influences the fate and amount of the mycotoxin produced (Martin, 2000; Yu et al., 2011).

Functional analysis of biosynthetic gene clusters usually relies on disrupting key genes in the cluster and examining the resulting secondary metabolic profile. However, genes in many clusters are expressed at extremely low levels and often particular metabolites cannot be detected in fungi cultured under standard conditions (Brakhage, 2013). Another challenge is that many SMs are not readily produced under laboratory conditions although several approaches have been successfully employed (Yaegashi et al., 2014; Wiemann and Keller, 2014).

Knowledge about the influence of upstream regulatory mechanisms, like global regulatory factors or signal transcription pathways, is also important for assessing biosynthesis data. It has to be kept in mind however that the correlation between transcription and biosynthesis is not given. Transcription data about the regulation of mycotoxins in relation to the food environment have provided important hints to improve the safety of a certain food with respect to mycotoxin biosynthesis. From the food safety perspective, the evaluation of the induction of the OTA biosynthesis genes can be a good indicator for determining the risk from specific toxigenic species, since the gene transcription always precedes phenotypic production (Zhang et al., 2016).

Molecular monitoring approaches would help to understand the complex mechanisms of mycotoxin production in a natural habitat. Experiments already conducted have used a

series of technical methods like Real Time PCR, droplet digital PCR (ddPCR), Microarray or RNA-seq. Nucleic acid amplification and detection techniques are among the most valuable tools in biological research today. Scientists in all areas of life science basic research, biotechnology, medicine, diagnostics and more, utilize these methods in a wide range of applications. For some applications, qualitative nucleic acid detection is sufficient. Other applications, however, demand a quantitative approach. Real-time PCR can be used for both qualitative and quantitative analysis (Filion, 2012), but choosing the best method for a specific application requires a broad knowledge of this technology. Quantitative PCR (qPCR) assays are widely used for the quantitative measurement of gene copy. In combination with Reverse-transcription PCR (RT-PCR), qPCR assays can be used to precisely quantify changes in gene expression. Thus, an increase or decrease in gene expression in response to different environmental conditions could be quantified by measuring changes in cellular mRNA levels (Bustin and Nolan, 2013).

1.2 *Aim of the PhD thesis*

In general, synthesis of mycotoxins is closely related to primary metabolic pathways, since the actual toxin production is modulated by both ecophysiological and genetic factors. Even though several molecular studies have been carried out recently for other mycotoxins, the genes and the enzymatic stages involved in the biosynthesis pathway of OTA have remained long unknown and still the length and composition of OTA clusters are not completely defined. Despite the fact that *A. carbonarius* stands among the most prevalent mycotoxigenic fungi and OTA as one of the most toxic metabolites relevant to food safety, there is limited information concerning the way this secondary metabolite incorporates the ecophysiological factors through regulation of related genes.

In food safety assessment, knowledge about the variables that influence the regulation of mycotoxin biosynthetic genes is of utmost importance. Therefore, new parameters have to be investigated to fully elucidate the mechanism of the biosynthetic pathway and clarify the molecular regulation on the basis of OTA production. Hence, knowledge of the genetic pathway and regulators that activate or inhibit (directly or indirectly) the biosynthetic genes could provide the means for the development of prevention strategies, diagnostic methods and remediation measures concering toxin production.

Taking this into consideration, this thesis focused on the study of OTA production by *A. carbonarius* intergating gene expression in relation to interacting abiotic and biotic factors, focucing on two key genes localized in the ochratoxigenic biosynthetic cluster, and one global regulator.

The main research objectives of this thesis are the following:

1. To perform an initial screening study on *A. carbonarius* grape isolates from an intra-strain perspective, employing different growth assessment methods (diameter, biomass, dry weight, colony density, colony area) and investigate their correlation with OTA production potential under optimal growth conditions on a synthetic grape medium (SGM).

2. To employ Reverse Transcription Real Time PCR (RT-qPCR) in order to study the influence of temperature, water activity (a_w) and time on OTA related gene expression on fungal isolate level.

3. To evaluate the impact of essential oils (EOs) antifungal activity on growth behavior and the effect of their anti-ochratoxigenic activity on gene transcription level.

4. To investigate the efficacy of LAB strains as biocontrol agents against *A. carbonarius* growth and OTA accumulation on molecular level.

CHAPTER 2

Literature Review

2.1 MYCOTOXINS PRODUCTION

2.1.1 Relation between growth, environmental factors and regulation

Production of fungal secondary metabolites possibly follows a pathway parallel to growth with a slight delay. However, regulation of secondary metabolism and the relationship between the rates of primary and secondary metabolism are still ambiguous (Calvo et al., 2002; Garcia et al., 2013). Moreover, as secondary metabolites, mycotoxins could be affected by factors that are not correlated with fungal growth (Cendoya et al., 2014, 2017; Vipotnik et al., 2017) and are directly regulated by other metabolic pathways. Even though in cases where environmental factors (temperature, water activity, CO_2, nitrogen sources, light, etc.) or co-existed microorganisms have been showed to exert little effect on growth, they have been proved to pose significant impact on the regulation of toxin production through biosynthetic gene expression (Fig. 1A). In a recent study, it was reported that temperature and a_w modifications affect aflatoxin biosynthetic pathway in *A. flavus* and *A. parasiticus* (Medina et al., 2014). In another work (Zong et al., 2015), it was indicated that patulin biosynthesis was mainly regulated at transcriptional level by ecophysiological factors such as carbon, nitrogen and pH, since gene up-regulation was observed under conditions that favor toxin production and down regulation under conditions that did not support patulin accumulation. Later, De Clercq et al. (2016) studied the impact of environmental conditions on patulin production and corroborated that conditions affecting toxin acted at transcriptional level on *idh* gene. Temperature has also been reported to act as the key factor affecting aflatoxin B1 (AFB1) production, strictly related to the induction of biosynthetic genes, but not aflatoxin regulatory ones, suggesting that *aflR* gene is not a good indicator for toxin prediction (Gallio et al., 2016). Furthermore, Liu et al. (2017) observed complex influence of temperature and a_w on gene regulation since significant

differences were revealed between AFB_1 production and *laeA* expression. Several reports have been published on the impact of individual environmental factors on mycotoxin production (Tannous et al., 2016; Peromingo et al., 2016). Nevertheless, it is also the combined effect of these abiotic factors that has a significant contribution in toxin production (Akbar et al., 2016; Medina et al., 2017).

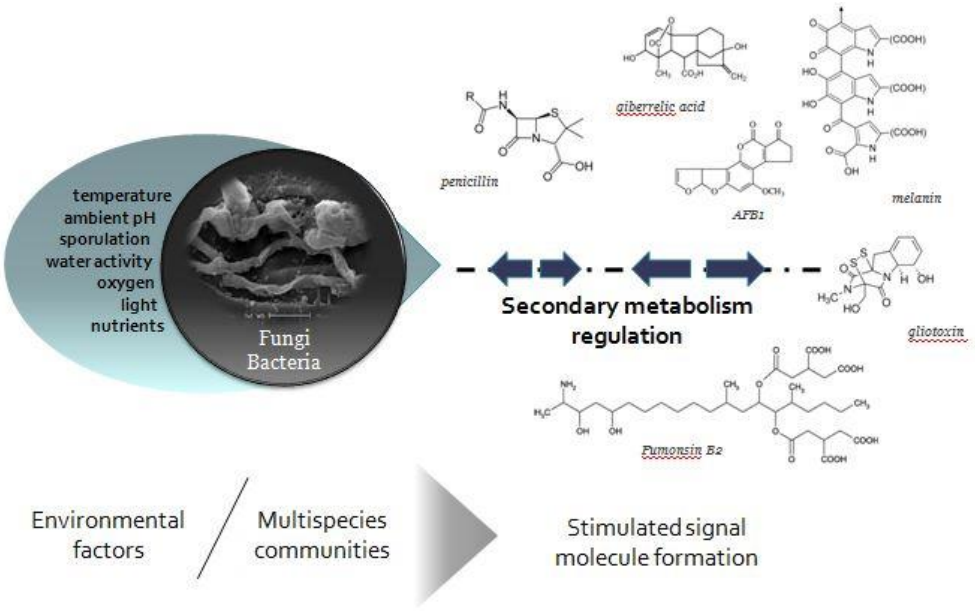

Fig. 1A. Ecophysiological factors along with multispecies interactions form the stimulation of secondary metabolism regulation (modified from Netzker at al., 2017)

Temperature and a_w represent two environmental key factors affecting both the rate of fungal spoilage of food commodities and mycotoxin accumulation. In this regard, plenty of studies have reported optimal environmental conditions of fungal growth and toxin production (Esteban et al., 2006; Spadaro et al., 2010; Lahouar et al., 2016). However, the knowledge of interaction among marginal and suboptimal environmental conditions could provide useful information for the prediction of possible risk and elucidate possible modes of action (Abdel-Hadi et al., 2011; Rodriguez et al., 2014). Despite the existing studies to explain toxin induction on molecular level, several attempts have

been undertaken in the last years to relate key environmental factors to the transcriprional regulation of mycotoxin biosynthetic genes. For instance, Fanelli et al. (2012) studied *Fusarium proliferatum* and revealed correlation between FUM1 fumonsin biosynthetic gene and toxin production. On the other hand, Bernaldez et al. (2017) investigated the interaction of temperature and a_w on AFB_1 studying *aflR* gene expression and reported that both factors presented a strong impact on toxin production, but no specific trend could be established on gene expression level.

The investigation of these environmental parameters which induce toxin production would allow the development of control strategies to minimize mycotoxin contamination. In many cases, inter and intra specific variability of mycotoxigenic fungi restricted the development of models for mycotoxin prediction, since it was not possible to obtain general conclusions and define optimal conditions for mycotoxin production since inter and intra strain variability was observed for different ecophysiological conditions. In a recent work (Marino et al., 2014), it was suggested that OTA synthesis by *A. niger* appeared to be an intrinsic strain dependent mechanism. Due to this complexity, contradictory results on mycotoxin production have been reported in the literature (Moussa et al., 2013; Yogendrarajah et al., 2016).

2.1.2 Current trends in mycotoxin control

2.1.2.1 Essential Oils (EOs)

In the last decade, over 25% of the world's agricultural commodities were estimated to be contaminated with mycotoxins (Stepien et al., 2007; Koopen et al., 2010). Therefore, the scientific community has focused on strategies about mitigating the concentration of mycotoxins in food. As a common practice, several synthetic preservatives have been effectively used in the management of food contamination by fungi, which despite their

continuous application has led to the development of fungal resistance (Brul et al., 1999) and numerous potential adverse environmental and health effects (Damalas et al., 2011). For this reason, the food industry has been put under pressure to find ways to inhibit the growth of toxigenic fungi and the presence of mycotoxins in raw materials and end products, while at the same time consumers demand high quality, preservative free, safe and mild processed foods with extended shelf life. Hence, there is a continuous need for alternative ways to control fungi such as biological agents and/or natural substances.

Among natural products, essential oils (EOs) from aromatic plants present promising perspectives as food additives and are widely accepted by consumers because of their relatively low toxicity, high volatility, transient nature and biodegradability. In the last years, several EOs from different plants have been proved efficient against fungal growth as an eco-friendly alternative of synthetic fungicides. The antifungal potential of EOs and their possible role as natural control agents in agriculture has been described in detail against *Aspergillus* spp., *Fusarium* spp., *Mucor* spp., *Alternaria alternate* (Sharma and Tripathi, 2006; Tzortzakis and Economakis, 2007; Viuda-Martos et al., 2008; Singh et al., 2010; Tian et al., 2012). However recent studies focus on the investigation of their effects not only against food-born fungi but also against their associated mycotoxins. Significant anti-toxigenic activity of different EOs such as *Rosmarinus officinalis, Salvia officinalis, Eucalyptus glovus, Origanum bacilicum, Mentha spicata, Zingibe officinale, Thymus vularis, Curcum longa*, has been reported (Yamamoto-Ribeiro et al., 2013; Prakash et al., 2015; Cisarova et al., 2016; Dwivedy et al., 2016; Kendia et al., 2016; Nazzaro et al., 2017; Pandey et al., 2016;).

In addition, many studies have been performed using essential oils from aromatic plants as potential aflatoxin inhibitors of *A. flavus* and *A. paraciticus* (Passone et al., 2013;

Esper et al., 2014; Tian et al., 2014; Soares et al., 2016). Moreover, Dambolena et al. (2010) reported that control of fumonisin production by EOs was a result of fungal growth inhibition. Few reports are also available on the effects of EOs on anti-ochratoxigenic potential. Hua et al. (2014) reported EOs antifungal and anti-toxigenic effect on *A. ochraceus*, suggesting that toxin inhibition by EOs was mainly due to fungal biomass reduction. Rao et al. (2015) explored the impact of EOs on the reduction of *Penicillium verrucosum* and *P. nordicum* biomass and OTA production, observing a positive correlation between the inhibitory effect of essential oils on fungal growth and toxin production.

In contrast, Mossini et al. (2009) conducted *in vitro* trials to investigate OTA production by *P. verrucosum* and *P. brevicompactum* under the influence of oil extracts and reported significant reduction in fungal growth and sporulation, although no OTA inhibition was observed. Meanwhile, Passone et al. (2012) observed complete OTA inhibition of *Aspergillus* section *Nigri* even though total fungal growth inhibition did not occur. These data underline a different mode of action compared to antifungal mechanisms on cell membrane disruption, alteration, inhibition of cell wall formation, disfunction of fungal mitochondria (Nazzaro et al., 2017). EOs and their components can also inhibit the expression of some enzymes considered as key elements in the catabolism of carbohydrates. Concerning mycotoxin synthesis, EOs could possibly act in the regulation mechanism of toxin biosynthetic pathway. Jahanshiri et al. (2015) reported the effect of eugenol on down-regulating the expression of AFB1 key genes of *A. parasiticus*. These findings are also in accordance with Caceres et al. (2016) who showed that eugenol treatment resulted in a complete inhibition of the expression of all but one genes of the AFB1 biosynthesis cluster in *A. parasiticus*. Moreover, Hu et al. (2017) found that turmeric EO inhibited the aflatoxin

biosynthetic gene expression in *A. flavus* and referred to toxin reduction as consequence of down-regulation in the transcription level of structural genes. Recently, it was shown that *Eugenia cariophyllus* EO can not only decrease *A. carbonarius* growth but also affect OTA biosynthetic pathway by suppressing key biosynthetic genes resulting in a significant toxin reduction (Lappa et al., 2015, 2017). It must be emphasised that literature data are still limited in this research field and further studies must be undertaken to elucidate the mode of action of EOs and their use in a more useful and safer way for mycotoxin control, since stimulation of fungal growth and/or toxin production has been occasionally observed (Murthy et al., 2009).

2.1.2.2 Biological control

Biological control studies using microbial antagonists against mycotoxigenic fungi have also been undertaken (Tsitsigiannis et al., 2012; Fapohunda et al., 2017; Medina et al., 2017) and proved to be an effective way to reduce the use of chemical compounds. Yeasts and bacteria such as *Bacillus subtilis, Rhizobacteria* spp., *Rhodotorula* spp., *Debaryomyces hansenii, Aureobasidium pullulans*, are well known for their antifungal potential (Salas et al., 2017) and inhibition of mycotoxin production (Samsudin and Magan, 2015; Alberts et al., 2016; Souza et al., 2017; Zheng at al., 2017; Peromingo et al., 2018).

While early reports indicated that competition of space or nutrients on food commodities play a major role in biocontrol activity, recent studies have suggested much more complex interactions among antagonists including mycotoxin molecular regulations. Several microorganisms proposed as effective agents inhibiting the growth of filamentous fungi may also have a direct inhibitory effect on mycotoxin production which is independent of their growth suppressing effect (Verheecke et al., 2015).

Recently, Li et al. (2017) reported growth and patulin biosynthesis inhibition of *P. expansum* through reduction of gene expression of isoepoxydon dehydrogenase by *Saccharomyces cerevisae*. In addition, Al-Saad et al. (2016) investigated the impact of bacterial biocontrol agents on aflatoxin structural and regulatory genes of *A. flavus* under different environmental and nutritional regimes. Results showed that some bacteria stimulated AFB1 production whereas some other could not inhibit toxin production even though relative gene expression was suppressed. More recently, Ponsone et al. (2016) evaluated the effectiveness of potential biocontrol yeasts against *Aspergillus* section *Nigri* and ochratoxin A occurring under commercial greenhouse grape production conditions, indicating toxin reduction and highlighting harvesting time or year of the study as affecting parameters.

Lactic acid bacteria (LAB) have been proved a promising biological agent, since many published reports refer to their potential to inhibit fungal growth (Olivera et al., 2014; Russo et al., 2017) and mycotoxin production (Vinderola and Ritieni, 2015). The biocontrol potential of LAB, isolated from various sources, has been demonstrated in the prevention of fungal infections (Crowley et al., 2013; Gajbhiye and Kapadnis, 2016) and interaction with mycotoxin production (Lipinska et al., 2016). Biocontrol of aflatoxin using LAB has been investigated by Ghazvini et al. (2016) who reported *Bifidobacterium bifidum* and *Lactobacillus fermentum* as suitable biocontrol agents against the growth and aflatoxin production by *Aspergillus* parasiticus. In another work, Khachuri et al. (2014) reported the potential of *Lactobacillus plantarum* to adhere on the surface of olives forming biofilms, which affected the attachement of other undesirable microorganisms, increasing thus the antioxidant activity and consequently weakening the production of AFB1. Moreover, Ngang et al. (2015) studied the antifungal potential of LAB on three ochratoxin-A producing fungi namely, *A.*

carbonarius, *A. niger*, and *A.ochraceus* reporting growth and OTA reduction both *in vitr*o and also during cocoa fermentation. In a recent study, the transcriptomic and morphological profile of *A. fumigatus* was investigated under the effect of cell free supernatant (CFS) by *L. plantarum* suggesting a global metabolic shutdown (Crowley et al., 2013).

Despite the great progress on antifungal biocontrol research during the last decades, it was proved difficult to select one microbial species capable of controlling major post-harvest pathogens and suppressing at the same time the production of mycotoxins. This could be attributed to the fact that some bacterial may exert fungal antagonistic activities, whereas some other may interfere with mycotoxin synthesis in a different way resulting in toxin stimulation (Zhu et al., 2015; Zhang et al., 2017). Moreover, strain variabity along with nutritional and temporal studies concerning environmental factors are some parameters that must be involved in future research (Samsudin et al., 2017).

2.2 Ochratoxin A

2.2.1 Physico-chemical properties

Ochratoxin A (OTA) is a secondary metabolite predominantly produced by a group of different filamentous fungi belonging to the genera *Aspergillus* and *Penicillium.* OTA ($C_{20}H_{18}ClNO_6$; IUPAC name: N-{[(3R)-5-chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydro-1H-isochromen-7-yl]carbonyl}-L-phenylalanine) is an odorless, crystalline solid agent (melting point: 168–173^{o}C) with poor aqueous solubility. It is a weak organic acid with a pKa value of 7.1 and a molar mass of 403.8 g mol^{-1}. With crystalline structure varying from colorless to white, this molecule presents an intense green

fluorescence under UV light in acid medium and blue fluorescence in alkaline conditions (El Khoury et al., 2010).

It is resistant to acidic conditions and high temperatures and it is only partially degraded at normal cooking conditions (Müller, 1982). Once foodstuffs are contaminated, it is very difficult to totally remove the toxin. Hence, common food processing treatments fail to substantially reduce its presence in foods and beverages since it can resist three hours of high pressure steam sterilization at 121.1 °C and even at 250 °C its destruction is not complete (Boudra et al., 1995).

2.2.2 Occurance and toxicological profile

OTA is a highly abundant food and animal feed contaminant as naturally occurring foodborne mycotoxin and is frequently detected in a wide variety of agricultural commodities worldwide. Many drinks (e.g., wine, beer, coffee, tea, milk, etc.), common meals (meat and dairy products) wheat and maize contain high or low amounts of OTA (Hashimoto et al., 2016; Alshannaq and Yu, 2017; Limay-Rios et al., 2017; Gil-Serna et al., 2018; Peromingo et al., 2018). Recent studies also revealed its presence in herbal medicines (Shim et al., 2014), food coloring agents (Solfrizzo et al., 2015), spices (Do et al., 2015; Jeswal and Kumar 2015), bottled water (Mata et al., 2015) and even eggs (Lee et al., 2016). The wide occurrence of OTA and its high thermal stability makes the eradication of this toxin from the food chain very difficult.

The presence of OTA in blood from healthy humans confirms a continuous worldwide exposure. The human dietary exposure has been estimated to 15–60 ng OTA kg^{-1} body weight (b.w.) per week for adult consumers in the EU (EFSA, 2006). Recently, a human biomonitoring study of 33 mycotoxins in the Belgian population revealed that about 1%

of the investigated population exceeded the tolerable daily intake level (Heyndrickx et al., 2015).

Various public health agencies and national/international authorities including the International Agency for Research on Cancer (IARC), the European Food Safety Authority (EFSA), the European Commission, the U.S. Department of Health and Human Services (National Toxicology Program, NTP) have set OTA limits described in the *Codex Alimentarius* of the FAO/WHO (JCFA, 2002, 2007).

The kidney is the main target organ for OTA, as it has been connected to the disease of Balkan endemic nephropathy (BEN) and chronic interstitial nephropathy (CIN) (Pavlovi et al., 1979; Vrabcheva et al., 2004; Bui-Klime and Wu., 2015). Nevertheless, more toxic effects of OTA have been investigated in numerous studies and also extensively reviewed (Koszegi et al., 2015; Heussner and Bingle, 2015) showing neurotoxic, hepatotoxic, teratogenic and immunotoxic effects in various animals and *in vitro*, with renal toxicity and carcinogenesis being the key adverse effects. Meanwhile, a direct genotoxic mechanism involving OTA bioactivation and DNA adduct formation in particular for renal carcinogenesis is still under debate (Zepnik et al., 2003; Petkova-Bocharova et al., 2003).

2.2.3 OTA biosynthetic genes

OTA is biosynthetically a pentaketide derived from the dihydrocoumarins family coupled to β-phenylalanine (El Khoury et al., 2010) (Fig. 2A) Unlike other mycotoxins, the genes involved in OTA biosynthesis have been long unknown. Nevertheless, the considerable increase of genomic data at species level along with the successive studies on molecular aspects in the last decade, has led to the identification of several genes and

their biosynthetic role in OTA production. However, the gene cluster for OTA biosynthesis remains uncomplete until today.

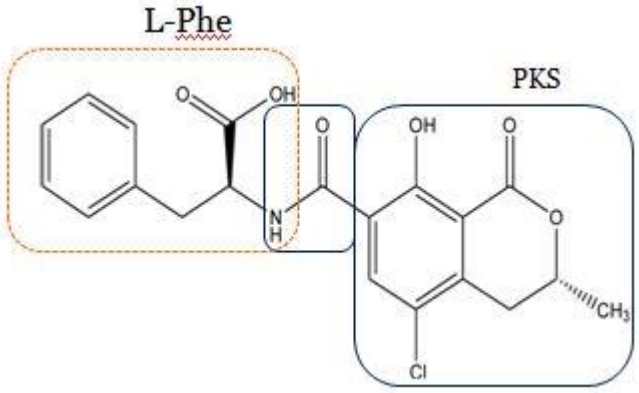

Fig. 2A. OTA molecular srtucture

A putative gene cluster encoding for polyketide synthase (PKS) and a non-ribosomal peptise synthetase (NRPS) was identified in *P. nordicum* revealing *otapksPN* and *otanrpsPN* genes to be involved in OTA biosynthetic pathway (Karolewiez et al., 2005; Karolewiez and Geisen, 2005; Geisen et al., 2006). Lately, the identification of *otapksPV* gene to be responsible for OTA biosynthesis by *P. verrucosum* was confirmed through gene disruption (Abbas et al., 2013; O'Callaghan et al., 2013). Recently, the genome of a *P. thymiola* strain was sequenced and analyzed for the presence of OTA biosynthetic cluster. Interestingly, no proteins homologous to OTA genes from *P. verrucosum* and *P. nordicum* were found. Instead, OTA clusters were more related to *Aspergillus* species that to *P. verrucosum* and *P. nordicum* (Nguyen et al., 2016).

In 2009, a Ac*pks* gene related to OTA production was identified by Gallo and co-workers (Gallo et al., 2009). Later on, and after the complete genome sequence of *A. carbonariu*s ITEM 5010, the investigation of OTA cluster was feasible *in silico*. Recently, the same group confirmed the role of AcOTA*pks* gene in OTA biosynthetic pathway by gene inactivation (Gallo et al., 2014). Meanwhile, inactivation of

AcOTA*nrps* confirmed this specific gene as responsible of the linking of phenylalanine to the polyketide dihydroisocoumarin (Gallo et al., 2012). Even more recently, a flavin-halogenase was found to be implicated in OTA biosynthesis of *A. carbonarius*. Inactivation of the encoding gene AcOTA*hal* confirmed its role in toxin biosynthetic pathway, indicating a strong correlation of the gene with the kinetics of OTA accumulation in *A. carbonarius* (Ferrara et al., 2016). So far the most recent information on OTA biosynthetic pathway steps is presented in Fig. 2C.

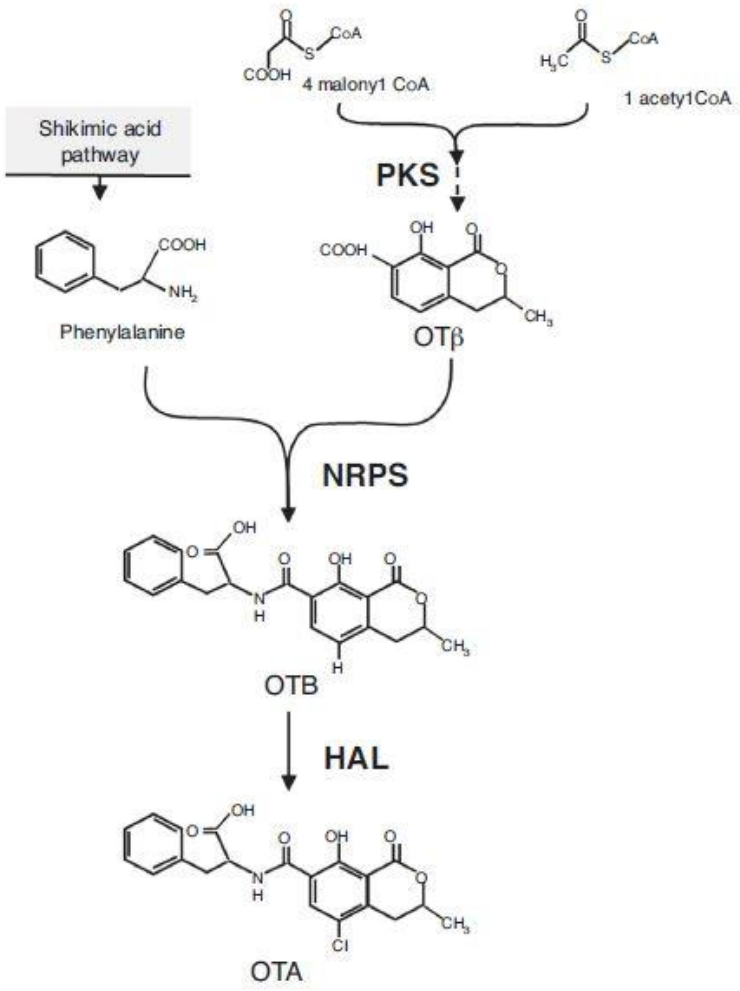

Fig. 2C. Schematic presentation of the order of reactions of OTA biosynthesis in *A. carbonarius* (adapted by Gallo et al., 2017)

A. niger is the OTA producing fungus phylogenetically most related to *A. carbonarius*. An15g07920*pks* gene of the putative OTA cluster was recently described and its crucial role in the biosynthesis of OTA was proved (Ferracin et al., 2012; Zhang et al., 2016). The whole genome of *A. westerdijkiae* was released by Chakrabortti and Liang (2016) leading to the identification of two putative OTA clusters including a *pks* gene among others. Likewise, *A. ochraceus* genome sequence analysis further revealed two more *pks* genes (Wang et al., 2015), namely AoOTA*pks*-1 that was proved to be directly involved in toxin biosynthesis and AoOTA*pks*-2 that might complement the expression of the former and be involved indirectly in toxin biosynthesis.

Numerous studies have been conducted to elucidate molecular aspects of OTA biosynthesis, but still the length and composition of OTA cluster remains partially clarified (Fig. 2D) Secondary metabolism is a mechanism that may utilize different pathway-specific regulator elements by controlling expression of the corresponding biosynthetic genes. Environmental signals are transduced to this toxin biosynthesis mechanism by global regulators and multiprotein complexes even though these regulatory processes are often interconnected and overlapping (Gallo et al., 2016).

Among the global regulators, deletion of laeA and veA in *A. carbonarius* resulted in conidiation reduction and decreased OTA production (Crespo-Sempere et al., 2013), confirming the role of the two proteins in the regulation of conidiation and OTA biosynthesis in response to light. Moreover, laeA has been reported to have also a regulatory effect on production of citric acid and cellulolytic enzymes in *A. carbonarius* (Linde et al., 2013).

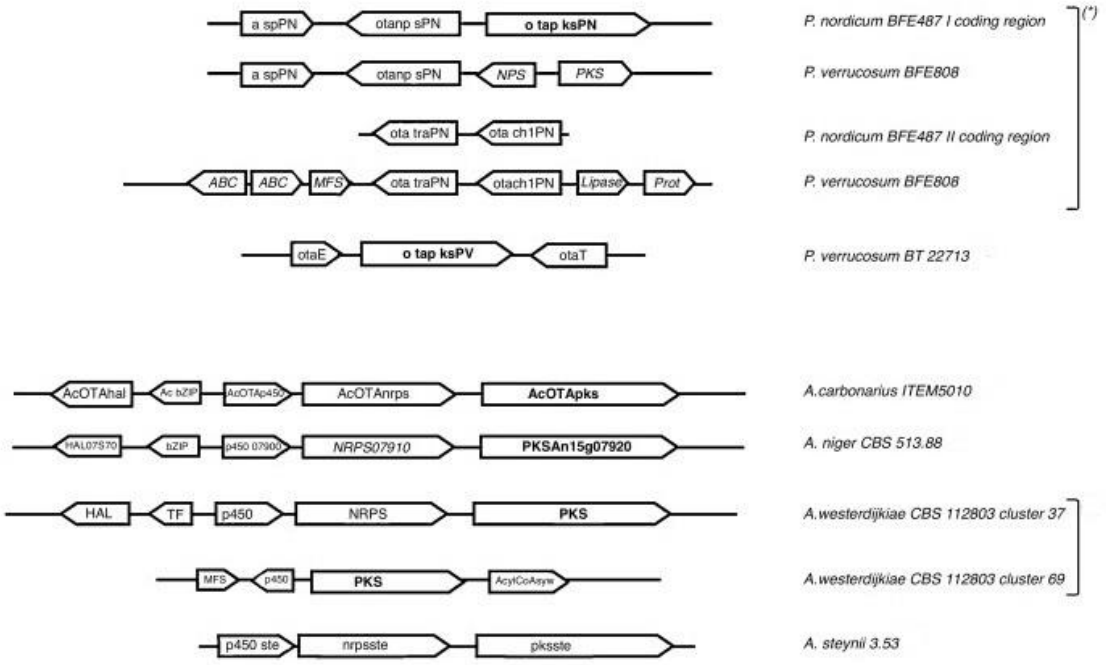

Fig. 2D. Organization of OTA clusters in different *Aspergillus* and *Penicillium* species.. (adapted from Gallo et al., 2017)

Studies concerning the influence of biotic and abiotic stressors on OTA biosynthesis in different fungal species have been often released. However, results are not consistent in all reports, since regulation of secondary metabolism is very complex and acts at different regulatory levels. Nowadays, most of the regulatory aspects underlying OTA production remain still unclear.

2.2.4 Transcriptional regulation

Environmental factors, namely temperature, water activity (a_w), relative humidity, rainfall are crucial for black aspergilli and penicillia to biosynthesize OTA (Battillani et al., 2015). Various ecophysiological studies have been conducted to identify conditions (e.g., temperature, a_w, pH, substrates) favoring the growth, sporulation and toxin production by potential ochratoxigenic species (Spadaro et al., 2010; Delgado et al., 2018).

Due to the fact that mycotoxin biosynthesis is also responsive to environmental factors and given the considerable amount of data generated in the last decade on gene regulatory pathways, there is considerable interest to understand the relationship between environmental factors, fungal interactions and expression of genes involved in toxin production. However, little information is available on the link between physiological factors and induced expression of genes responsible for OTA production.

Geisen et al. (2004) analyzed a key gene of the ochratoxin-A biosynthetic pathway (ota*pks*PN) of *P. nordicum* under the influence of environmental conditions such as temperature, pH, and NaCl. Results highlighted that the induction of this gene coincided with OTA production. Furthermore, this study indicated that the expression of the ota*pks*PN gene was greatly dependent on the media used for fungal growth.

Later, Schmidt-Heydt et al. (2007) tried to elucidate the relationship between food preservatives, environmental factors, OTA and ota*pks*PV gene expression by *P. verrucosum* in a temporal study. Some correlation was demonstrated between regulation of the ota*pks*PV gene expression data and phenotypic quantified OTA under the experimental conditions assayed for water activity (a_w), type of preservative and concentration. Generally, an increase in ota*pks*PV activity was observed after fungal growth in the presence of preservatives, indicating apparently two different regulatory mechanisms of toxin production, one acting under optimal and one under stress conditions.

Moreover, a study on the influence of NaCl rich foods on *P. nordicum* and OTA production was conducted by Schmidt-Heydt et al. (2012). The transcriptional activity of ota*pks*PN paralleled that of toxin production. Specifically, at higher NaCl

concentrations increased OTA production appeared to be in accordance with induction of ota*pks*PN gene.

Meanwhile, the effect of nutritional parameters of carbon and nitrogen sources has been investigated by Abbas et al. (2009) concerning gene expression of ota*pks*AO by *A. ochraceus*. The authors reported low gene induction due to glucose, glucerol, maltose or succinate as well as strong induction by lactose. In addition, the influence of various nitrogen sources on ota*pks*AO expression appeared to correlate well with toxin production. Gene expression analysis revealed important modulator impact at gene transcription level.

The influence of salt on two genes involved in OTA biosynthetic pathway (ota*pks*PN and ota*nps*PN), growth and phenotypic OTA production has been reported (Rodriquez et al., 2014) for three strains of *P. nordicum* on a dry-cured ham-based medium. RT-qPCR revealed that only two strains were able to express the two genes in salt concentrations of 10% and 22%, corresponding to 0.94 and 0.87 water activity (a_w). The relative expression profiles of these two genes differed and the expression levels of ota*pks*PN were much higher compared with ota*nps*PN. Significant differences were also found between transcription levels on different sampling days. Inter-strain differences in the transcription levels of both genes were also observed along with intra-strain differences in the relative expression values of the two strains in both a_w conditions assayed. Expression of these two genes occurred after 3 days of incubation, while phenotypic OTA production was observed only after 6 days. It was finally concluded that there was partial relationship between the temporal relative expression of ota*pks*PN and ota*nps*PN genes and phenotypic OTA production.

Recently, a gene expression study was published for *P. nordicum* during seasoning process of salami (Ferrara et al., 2016). Molecular analysis detected the expression of ota*pks*PN gene after 4 days along with the presence of OTA at a concentration not significantly different from that detected on days 5 and 6. During seasoning, OTA increased in line with up-regulation of ota*pks*PN gene expression, indicating that modulation of ota*pks*PN gene expression was consistent with observed OTA production until day 7. After the expression peak, the level of ota*pks*PN gene transcript was reduced but OTA content did not vary significantly. The reduction of copy number of ota*pks*PN gene could be related to degradation of mRNA transcripts of the target gene (Geisen et al., 2004). These results confirm also the activation of OTA pathway from the early stage of mycelium development.

Studies with gene transcription approaches are limited concering *A. carbonarius*. Ferrara et al. (2014) studied the phenolic components of wine, namely hydroxycinnamic acids (HCAs), on the expression level of AcOTA*pks* and AcOTA*nrps* by RT-qPCR. Germination of conidia was not affected in contrast to growth and toxin production. From gene expression analysis, AcOTA*pks* gene showed a marked reduction of expression level in the presence of *p*-coumaric and ferulic acids. On the contrary, caffeic acid did not seem to influence transcription levels of any gene, implying a different mode of action on the regulation of OTA biosynthesis.

A gene expression analysis was also published concerning ochratoxigenic *Aspergillus* spp. and *Penicillium* spp. species under co-culture with *Kluyveromyces thermotolerans* strains (Ponsone et al., 2013). This yeast species was used as a biocontrol agent in order to investigate if its presence could affect ota*pks* gene expression. However, no direct correlation between the influence of the biocontrol yeast on ota*pks* gene expression and OTA production could be established. Even though the obtained results could indicate

an inhibitory mode of action by the yeast, post-transcriptional regulation was apparently involed.

It must be underlined that despite the important progress achieved on the elucidation of ochratoxin biosynthetic pathway and the confirmation of the role of specific genes, there is a limited number of published studies investigating the influence of OTA related genes by ecophysiological factors, such as environmental conditions, nutrients or bio-antagonism.

2.3 *Aspergillus carbonarius*

2.3.1 Ecophysiology

The most important species in the group of Black *Aspergilli* (*Aspergillus* section *Nigri*) either for its high capacity to produce OTA or for its high percentage of toxigenic strains is *Aspergillus carbonarius* (Battilani et al., 2003). It is a xerophilic filamentous fungus that can be easily distinguished from other biseriate species due to its large conidia and stipes up to several millimeters long (Figs. 2E,F). It is considered to be mainly responsible for OTA contamination in grapes, vines as well as in cocoa and coffee to a lesser extent (Cabanes et al., 2002; Abarca et al., 2004). In addition, it has been recorded as the predominant species in raisins (Abarca et al., 2003; Magnoli et al., 2004).

Greek isolates of *A. carbonarius* have been reported to be high OTA producers (Tjamos et al., 2004). A correlation has been established between the prevalence of *A. carbonarius* from grape berries in Southern Europe and high concentrations of OTA in wines (Mateo et al., 2007). Strains of this fungus have been isolated and identified in several Mediterranean countries, including Spain, Italy, Portugal and Greece (Battilani et al., 2004; Bellí et al., 2004a; Mitchell et al., 2004; Tjamos et al., 2006). The incidence

of *A. carbonarius* has been correlated with latitude indicating that meteorological conditions and especially proximity to the sea can play a major role in OTA presence. Pateraki et al. (2007) reported higher isolation frequencies and OTA levels from sultanas dried near the sea level from those at higher latitudes. Moreover, Batillani et al. (2006) created thermo-wetness maps using temperature and rainfall parameters illustrating the incidence of *A. carbonarius* in the Mediterranean basin.

E. F.

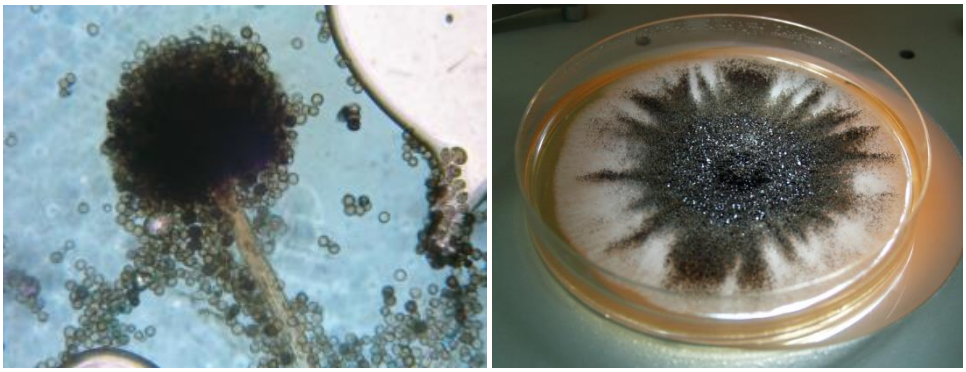

Fig. 2E,F. Illustration of *A. carbonarius* conidiophores (E) (genome.jgi.doe.gov) and growth of the fungus on solid media (F) (personal record)

Factors affecting *A. carbonarius* growth in the vineyard such as temperature, humidity, photoperiod, damaged grapes, are of great importance for fungal proliferation (Hocking et al., 2007). The significance of fungi in the soil lies in the dispersal of spores by air movement from the soil onto berry surfaces. It was observed that the incidence of *A. carbonarius* spores in air samples increased closer to the soil, and a severe dust storm could result in increased presence of fungal spores on grape bunches (Leong et al., 2006a). Moreover, there are other factors affecting *Aspergillus* spp. and ochratoxin A production in grape berries. Black aspergilli prevalence typically increases from berry

set until harvest (Leong et al., 2007). This is probably due to the surface of immature green berries and exposure to UV light that represent a hostile environment for *A. carbonarius* spores (Leong et al., 2006). At veraison, the berry skin softens and sugar content increases; from this stage onwards until harvest, berries are most susceptible to infection by *A. carbonarius* and they are also susceptible to OTA contamination, particularly when damaged (Battilani et al., 2006; Leong et al., 2006a). Consequently, delayed harvest of mature berries has been found to increase the risk of OTA contamination (Gambuti et al., 2005).

The frequent presence of *A. carbonarius* could be attributed to its rapid growth in a wide range of temperature, a_w, and pH conditions. However, the optimal conditions for toxin production are generally different from those for fungal growth (Belli et al., 2005; Passamani et al., 2014). In a previous work (Tassou et al., 2007), the growth boundaries of *A. carbonarius* were reported in the range of 15-37 ^oC and 0.85-0.95 a_w. The respective boundaries for OTA production were 15-35 ^oC and 0.90-0.95 a_w. These ecophysiological parameters have been studied by growing fungal isolates on a synthetic grape juice medium (SGM) designed to simulate the composition of grape berries at early veraison. In this medium, the optimum temperature (T_{opt}) for growth has been observed at 30 °C in many studies (Belli et al., 2005; Battilani et al., 2006d; Leong, 2007; Tassou et al., 2007), even though Mitchell et al. (2004) reported higher optimum temperature (35 °C). In contrast, Esteban et al. (2006) observed optimum tempetature for growth at 15 ^oC and 0.95-0.98 a_w, studying isolates of *A. carbonarius* from different beverages. In another study (Leong et al., 2006b), optimum OTA production was reported at 15 ^oC and 0.95-0.98 a_w, whereas other researchers (Belli et al., 2005; Marin et al., 2005) reported optimum OTA production at 20 ^oC. There are several studies reporting high intra-specific variability of fungal growth and OTA

production when several isolates were studied (Bellí et al., 2004b; Pardo et al 2005; Romero et al., 2007; Tassou et al., 2009; Garcia et al., 2011) as also observed in the present thesis.

The relative importance of temperature and a_w on fungal growth and OTA production in grape berries is difficult to assess. As the sugar content increases from veraison until harvest, the water activity decreases and comes within the optimal range to support OTA production. High temperatures and relative humidities at the time of fungal penetration of grapes results in higher OTA content. Rainfall increases OTA content by increasing relative humidity and causing damage to the grapes; berry splitting and high fungal colonization may occur if rain falls within a month before harvest (Stratakou and van der Fels-Klerx, 2010). Moreover, alternating photoperiods increase fungal growth rate and consequently OTA production (Bellí et al., 2006).

2.3.2 Infection preventive strategies in grapes

Grapes are susceptible to fungal diseases that can reduce the quality of harvested berries and affect the organoleptic attributes of wines. *Aspergillus* bunch rot is a preharvest fungal disease caused by species belonging to *Aspergillus* section *Nigri* (Battilani et al., 2006; Perrone et al., 2006; Tjamos et al., 2006). Ochratoxin A control remains a challenge in the face of continuous efforts to produce quality wine and table grape berries to meet food safety standards. *A. carbonarius* is the dominant cause of OTA production in grapes and their derivatives. The most effective strategy to control OTA contamination in grapes and their products is the prevention of fungal growth before crop harvest (Magan and Aldred, 2007). Inhibition of OTA-producing fungi is the most effective way to diminish the entry of this mycotoxin in the food and feed chain.

There are hardly any data to characterize the behaviour of *A. carbonarius* in relation to fungicide treatments. Effective fungicide applications have been reported (Belli et al., 2007; Valero et al., 2007) even though in some cases treatments resulted in a significant stimulation of OTA production (Medina et al., 2007). Interactions between ecophysiological factors, such as a_w, on fungal growth and OTA production in the presence of fungicides have been reported. Twenty-six fungicides were studied *in vivo* and showed that they could suppress *A. carbonarius* growth and also inhibit OTA synthesis in grapes (Belli et al., 2006). Medina et al. (2007) also examined the efficacy of natamycin to control *A. carbonarius* growth and OTA production under various combinations of a_w and temperature and revealed a strong inhibition effect of both parameters on fungal growth and toxin production.

However, alternative prevention approaches to synthetic fungicides have drawn the attention of scientists in the last years due to the eco-friendly attitude of consumers. Abdollahi et al. (2014), studing the effect of postharvest spraying with essential oils in a storage assay showed that essential oils, especially thyme and fennel, presented good inhibitory effect on fungal decay in Thompson table grapes. In addition, the application of essential oils reduced weight loss, berry and rachis browning and had no considerable adverse effect on the flavour of the fruits.

Biological control has been proposed as a natural method to reduce the impact of ochratoxigenic species in vineyards, since it is able to restrict the proliferation and detrimental effects of plant pathogens on grapes. Control of OTA-producing fungi in grape berries by microbial antagonists is an ongoing attempt (Ponsone et al., 2012; Zhang et al., 2016) gaining increasing attention in the last decade. The application of biological control agents (BCAs) in the field is necessary for the biocontrol of fungi that initiate colonization and pathogenic activity before harvest, which is the case for *A.*

carbonarius in the vineyard, where environmental factors such as humidity and temperature cannot be controlled.

In this context, Ponsone et al. (2011) described two epiphytic yeast strains of *Kluyveromyces thermotolerans* that were able to control the growth and OTA accumulation of *A. carbonarius* and *A. niger* both *in vitro* and *in situ*. The inhibitory effects were depended on ochratoxigenic species, on the yeast strain, a_w and temperature as well as their interactions. The results of the above work are in accordance with other studies presenting significant potential of different yeast species to inhibit *A. carbonarius* growth and OTA production both on grape berries and *in vitro* experiments (Fiori et al., 2014; Zhu et al., 2015). The impact of temperature and relative humidity (RH) on the efficacy of the yeast species *Metschnikowia pulcherrima* and two strains of the yeast-like fungus *Aureobasidium pullulans* against infection by *A. carbonarius* and OTA accumulation was investigated in wine grape berries (De Curtis et al., 2012). The presence of wounds on grape skin enhanced infection of berries by *A. carbonarius*, since unwounded berries showed very low levels of infection regardless of RH and temperature conditions. These results showed that biocontrol treatment positively affected the incidence of infection by *A. carbonarius* on wine grape berries. A positive effect of biocontrol treatment was also recorded on the level of OTA accumulation, even when protection from fungal infection was not satisfactory. It was revealed that the positive effects of biocontrol were dependent on environmental factors, particularly temperature which appeared to be more important than relative humidity. Furthermore, protection efficacy by the biocontrol strains decreases when environmental conditions foster infection by the pathogen.

A limited number of reports have shown the potential use of bacteria as biocontol agents against *A. carbonarius* in grapes. Jiang et al. (2014) investigated the inhibitory

effect of *B. subtilis* liquid culture against *A. carbonarius* contamination in grapes in a storage assay. Fungal contamination was significantly inhibited and the inhibitory effects differed according to grape cultivar and storage temperature. However, bacterial species evaluated in other studies did not prove to be effective inhibitors for *A. carbonarius* (Kapetanakou et al., 2012).

CHAPTER 3

Comparative study of growth responses and screening of intra-specific OTA production kinetics by *A. carbonarius* isolated from grapes

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Abstract

The aim of this work was to assess Ochratoxin A (OTA) production of different *Aspergillus carbonarius* isolates, evaluate their growth profile through different growth measurements, and reveal any underlying correlation between them. Ten different isolates of *A. carbonarius* isolated from Greek vineyards located in different geographical regions were examined *in vitro* for their OTA production potential after an incubation period of up to 11 days. All fungal isolates grew on a synthetic grape juice medium (SGM) similar to grape composition at optimum conditions of temperature and water activity (25°C and 0.98 a_w). Samples for OTA determination were removed at 3, 5, 7, 9 and 11 days of growth and analyzed by HPLC. Based on OTA measurements the isolates were characterized by diverse OTA production ranging from 50-2000 ng/g at day 11. The different fungal growth responses (colony diameter, colony area, biomass, biomass dry weight, and colony density) have been measured and correlated with toxin production by means of principal components analysis (PCA), confirming satisfactory correlation and explained over 99% of data variability. Leudeking-Piret model was also used to study OTA production with time, revealing a mixed-growth associated trend and pointing a fail-safe model with slightly better prediction through colony area. This approach contributes to the assessment of correlation between mycotoxin production and different methods of fungal growth determination in relation to time.

3.1 Introduction

Ochratoxin A (OTA) is a widely detected mycotoxin that was first described as a wine contaminant by Zimmerly and Dick (1996). Abarca et al. (1994) were the first that revealed the role of *Aspergillus* section *Nigri* and especially *Aspergillus carbonarius* in OTA production (Abarca et al., 2001). OTA is now known as a secondary metabolite

produced by fungal species belonging to *Aspergillus* and *Penicillium* genera that are related with nephrotoxic, hepatotoxic, genotoxic, teratogenic, and immunotoxic impact to humans and animals (Castegnaro et al., 1998; IARC, 1993). There is a great food safety concern regarding the presence of OTA in foods and thus the European Union has established maximum OTA levels of 2 μg kg^{-1} for wine, grape juice, grape nectar and grape must intended for direct human consumption and 10 μg kg^{-1} for direct dried wine fruits (European Commission, 2005). Fungal isolates identification around the Mediterranean and other parts of the world have shown the occurrence of OTA-producing *Aspergillus* species in grapes. There is strong evidence of the significance of *A. carbonarius* in OTA production since there is high incidence of ochratoxin-producing isolates within *A. carbonarius* spp. (Stefanaki et al., 2003). Other studies showed that considerable climate differences related to geographical region influenced mould contamination and OTA (Cabañes et al., 2001; Battiliani et al., 2003; Visconti et al., 2008). Batillani et al. (2001) pointed that the major source of OTA in grapes is the skin of berries and considering that grape juices, musts and wines are produced by pressing berries, the diffusion ability of OTA becomes evident (Valero et al., 2006a), making thus OTA contamination a problem originating in the field. Black *Aspergilli* responsible for OTA are already present in vineyards (Tjamos et al., 2004) and the amount of toxin seems to be dependent on the latitude of the production (Battilani et al., 2006). The lower the latitude the more frequent the occurrence and the grater the concentration of the toxin (Rosari et al., 2000; Pietri et al., 2001; Eder et al., 2002; Chiotta et al., 2013).

Although, plenty of studies have examined the ecophysiology of *A. carbonarius* in different environmental conditions (Bellí et al., 2004; Tassou et al., 2009; Spadaro et al., 2010), few studies provide growth response data along with mycotoxin data for the

same sampling time (Marin et al., 2004, Valero et al., 2006a, Taniwaki et al., 2006). However, these studies focused on the examination of growth responses between different fungal species. During the last decade many publications on mycotoxin production over time have been reported on either synthetic media or food substrates, but comparisons between different fungal quantification methods have been rarely reported. However, Marin et al. (2005) correlated different growth responses among them but not with toxin production. Moreover, Garcia et al. (2013) tried to quantify the total amount of aflatoxins from maize and relate it to the amount of mould biomass. Hyphal extension rate is usually reported as radial growth rate (mm h^{-1}) being probably the simplest and most direct measure of fungal growth. However, growth estimation for filamentous fungi from radial extension remains questionable due to differences in the height of mycelium and also its colony density (Taniwaki et al., 2006). In the quest of new methods for fungal quantification, the aim is to observe correlation among growth responses considering that biomass or diameter cannot be directly quantified in food systems (Marin et al., 2005). Garcia et al. (2013) used *Aspergillus flavus* as a model of mycotoxigenic fungus to relate aflatoxin to the amount of fungal biomass. Another attempt from Marin et al. (2007), demonstrated the correlation between colony diameter changes and toxin production in solid medium by means of model development. Fungal growth over time was investigated by Baranyi et al. (1993) who proposed a model that even though it had been developed for bacterial growth it was proved successful at fitting colony diameter increase (Gibson et al., 1994; Ghar et al., 2005). Mould growth was also empirically modeled with the modified Gompertz equation (Zwitering et al., 1990) selected for asymmetrical data (Ghar et al., 2005). Applying existing models to compare commonly employed parameters in growth assessment, as a fungal indicator will probably facilitate the establishment of secondary models. Moreover, correlation of

OTA with different growth responses and fitting data to known models may be a step promoting predictive mycology.

The objectives of this study were to (i) compare different fungal growth responses, (ii) determine the effect of time on OTA production, (iii) correlate toxin with fungal growth under optimum temperature and a_w conditions, and (iv) screen inter-specific kinetics of toxin based on different growth approaches. To our knowledge this is the first attempt to describe multiple ochratoxin kinetics with the existence of growth associated (proportional to growth rate) and no growth associated (proportional to existing biomass/ dry weight) production, and also correlate ochratoxin with a variety of growth responses concerning *A. carbonarius*.

3.2 Materials and Methods

3.2.1 Fungal isolates and growth medium

Nine different wild isolates of *A. carbonarius* (coded as Ac27, Ac28, Ac29, Ac30, Ac31, Ac33, Ac34, Ac43, and Ac47) previously isolated from grapes collected from different geographical areas of Greece and a reference strain of *A. carbonarius* ITEM 5010 (Institute of Science of Food Production -ISPA, Bari, Italy) were used throughout this study. Isolates belonged to the fungal culture collection of the Laboratory of Food Microbiology and Biotechnology (LFMB) of the Agricultural University of Athens (stored in glycerol at -20 ^{o}C). All isolates were tested for their potential for OTA production on Czapek yeast extract agar (CYA), after incubation at 25 °C for 7 days as described by Kizis et al. (2014). OTA is classified as a possible human carcinogen within the 2B Group by IARC (IARC, 1993) and the related precautions were taken into account during laboratory work. The experiment was performed on Synthetic Grape juice Medium (SGM), a culture medium that simulates grape composition between

véraison and ripeness (Delfini, 1982). Media were prepared by adding D(+) glucose, 70 g; D(–) fructose, 30 g; L(–) tartaric acid, 7 g; L(–) malic acid, 10 g; $(NH_4)H_2PO_4$, 0.67 g; KH_2PO_4, 0.67 g; $MgSO_4 \cdot 7H_2O$, 1.5 g; NaCl, 0.15 g; $CaCl_2$, 0.15 g; $CuCl_2$, 0.0015 g; $FeSO_4 \cdot 7H_2O$, 0.021 g; $ZnSO_4 \cdot 7H_2O$, 0.0075 g; (+) Catechin hydrate, 0.05 g; agar, 25 g, to 1000 ml distilled water. The a_w of this basal medium was 0.98, measured by an AquaLab LITE (Degacon, USA) water activity meter at 25 °C. The pH of the SGM was adjusted to 3.8 with KOH (2 M).

3.2.2 Inoculation and incubation

Spore suspensions of each *A. carbonarius* isolate were prepared by collecting spores from 7-day old colonies grown on Malt Extract Agar at 25 ^oC. Conidia were harvested from sub-cultures in an aqueous solution of 0.05% Tween 80 by scraping the surface of the mycelium. The final concentration of spores was assessed by a Neubauer counting chamber (Brand, Wertheim, Germany) and adjusted by appropriate dilutions to 10^6 spores/mL. Sterilized cellophane membranes were placed on the top of SGM agar plates in order to help biomass assessment. It has been shown that membrane allows fungus to obtain nutrients from the substrate and grow very similarly as with no cellophane layer (Ramos et al., 1999). Petri dishes were centrally single spotted with 10^3 spores on the surface of the membrane. Incubation was performed at 25 ^oC and a_w 0.98, which is optimum for *A. carbonarius* growth (Bellí et al., 2004; Garcia et al., 2010; Kapetanakou et al., 2011). Plates were sampled over time for the determination of biomass dry weight, colony radius, colony area and OTA production, for a period of 11 days. All the assays were replicated in triplicate.

3.2.3 Growth assessment

Colony diameter (mm), colony area (mm^2) and biomass (mg dry weight) were measured at the same time in days 3, 5, 7, 9 and 11. Colony radius was observed on a daily basis and recorded at right angles by the aid of a ruler. Colony area was calculated by estimating the surface of the circle (πR^2) formed by each fungal colony. The mycelium remained intact and collected from the cellophane membrane to monitor biomass. Fungal dry mass was determined by drying the mycelium at 105 °C (Passanen et al., 1999), and measured after cooling at room temperature using desiccators (Taniwaki et al., 2006). Measurements were carried out periodically until weight was stabilized. Fungal biomass (mg) was recorded before drying as well. Finally, colony density was calculated by dividing mycelium dry weight by colony area (Marin et al., 2005; Garcia et al., 2013).

3.2.4 Extraction and detection of Ochratoxin A

The whole content of each plate was used for OTA extraction at 3, 5, 7, 9, and 11 days of incubation. Studies have indicated that OTA can be diffused throughout the culture medium, so taking into account the content of the Petri dish would ensure determination of the whole amount of OTA produced by the fungus (Valero et al., 2006b). Each sample was weighted and mixed with a 4-fold quantity of extraction solution (80% methanol: 20% water) using the Ultra Turrax (Heidolph Instruments, Schwabach, Germany) for 2 min at the highest speed (26 x 10^3 rpm) (Kapetanakou et al., 2009). Extracts were filtered through a Whatman No2 filter paper, then through a 0.2 μm syringe-driven filter unit (Millex, Millipore Co., Bedford, Mass.) and stored at 4°C until HPLC analysis.

OTA was detected using an HPLC system equipped with a JASCO LC-Net II/ADC system controller, a JASCO AS-2055 Plus auto sampler, with a Model PU-980 Intelligent pump, a Model LG-980-02 ternary gradient unit pump and an FP-2020 Plus fluorescent detector (JASCO Inc., Easton, USA). The analysis was performed under isocratic conditions at a flow rate of 1 mL/min of the mobile phase (water/acetonitrile/acetic acid; 49.5/49.5/1) through a Waters spherisorb C18 analytical column, 5μm ODS2 (4.6 x 250 mm) (Resteck Co., Pinnacle II, Bellefonte, USA). Injection volume was 10 μL and run time for samples was 20 min with OTA detected at about 11 min. The detection limit of the analysis was 1 ppb.

3.2.5 Statistical analysis

Analysis of variance (ANOVA) was performed allowing an overview of all the results and establishing correlations among the diverse growth parameters and OTA concentration of the different fungal isolates. The data set was analyzed by the statistical package JMP8 (SAS Institute Inc., Cary, NC, USA). Pearson's correlation matrix and descriptive statistics (means, standard deviations and coefficients of variance, CV%) were also computed by JMP8. Multivariate statistical analysis (Principal component analysis, PCA) was also employed to investigate any underlying relationship among the different variables through Statistica software ver. 8.0 (Statfoft, Tulsa, Oklahoma).

For ochratoxin modeling, the Leudeking-Piret mixed-growth associated model was used for product (OTA) formation as detailed previously by Garcia et al. (2013). In this work, fungal growth and OTA production were expressed by different equations depending on the fungal growth assessment parameter employed. Thus, when fungal

growth was expressed as changes in diameter (X) versus time, then growth and OTA production were fitted by the following equations:

If $t < \lambda, \quad X = 0;\ P = 0$

If $\lambda < t < t_{Xmax}, \quad X = at + b;\ P = (a\alpha + b\beta)t + a\beta\frac{t^2}{2}$

Where P is product concentration (g/L), α is the growth-associated coefficient for P production (gP/gX), β is the non-growth-associated coefficient for P production (gP/gX h) and t_{Xmax} is the time point where the linear model reaches its maximum value

For colony area (X), the respective equations are the following:

If $t < \lambda, \quad X = 0;\ P = 0$

If $\lambda < t < t_{Xmax}, \quad X = \pi(at + b)^2; P = \pi\beta a^2\frac{t^3}{3} + (\pi\alpha a^2 + \pi\beta ba)t^2 + (2\pi\alpha ba + \pi\beta b^2)t$

Finally, for biomass dry weight (X):

If $t < \lambda, \quad X = 0;\ P = 0$

If $\lambda < t < t_{Xmax}, \quad X = ct^2 + dt + e;\ P = \beta c\frac{t^3}{3} + \left(\alpha c + \frac{\beta d}{2}\right)t^2 + (\alpha d + \beta e)t$

Product formation (P) as well as fungal growth expressed as changes in diameter, colony area, and biomass dry weight were estimated by means of nonlinear regression based upon the Marquardt algorithm using Statgraphics® Centurion XV, version 15.1.02 (Statpoint, Inc., Maryland, USA).

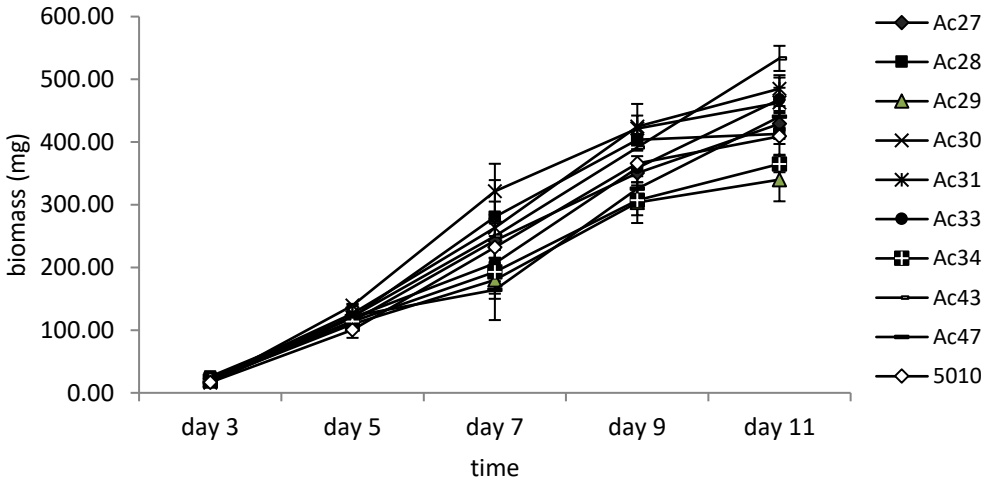
3.3 Results and Discussion

3.3.1 Growth responses

Differences in fungal growth variables concerning colony diameter, biomass, mycelium dry weight, colony area, and colony density versus time are presented in Fig. 3.1. The influence of colony age on the data obtained by the five different methods used to

measure fungal growth is also shown in this Fig., where the actual values presented an increasing trend with incubation time (Fig. 3.1A,B,D,E) with the exception of colony density (Fig. 3.1C) where the opposite effect was evident. All five growth responses were found to be highly positively correlated among them, except colony density that was negatively correlated with the remaining growth parameters (Table 3.1). Similar significant positive correlations between dry weight and diameter of A. *carbonarius* were also confirmed by Marin et al. (2004) and of *Aspergillus flavus* by Garcia et al. (2013). Biomass dry weight correlation (R^2 = 0.96-0.98) with diameter indicated that colony diameter in single cultures is an easy-to-use and acceptable choice for fungal growth estimation for research purposes even though it is difficult to be applied on food substrates.

. **A.**



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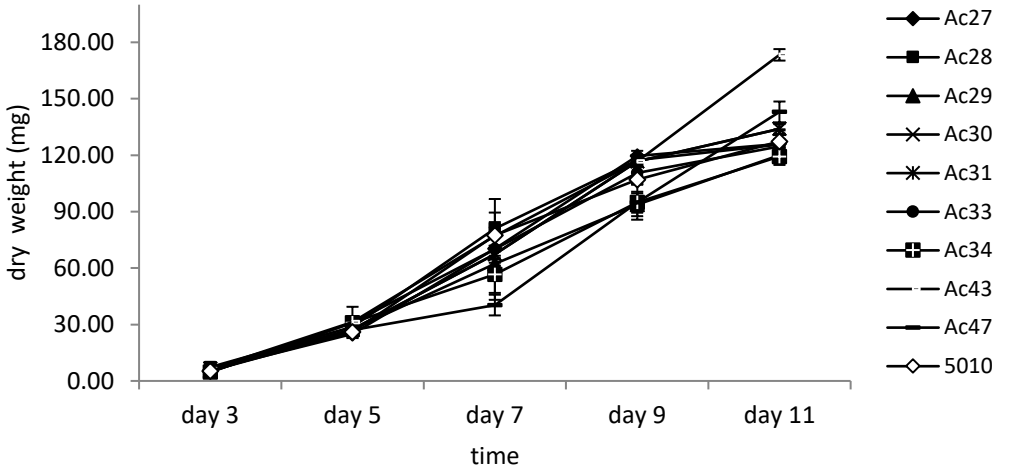
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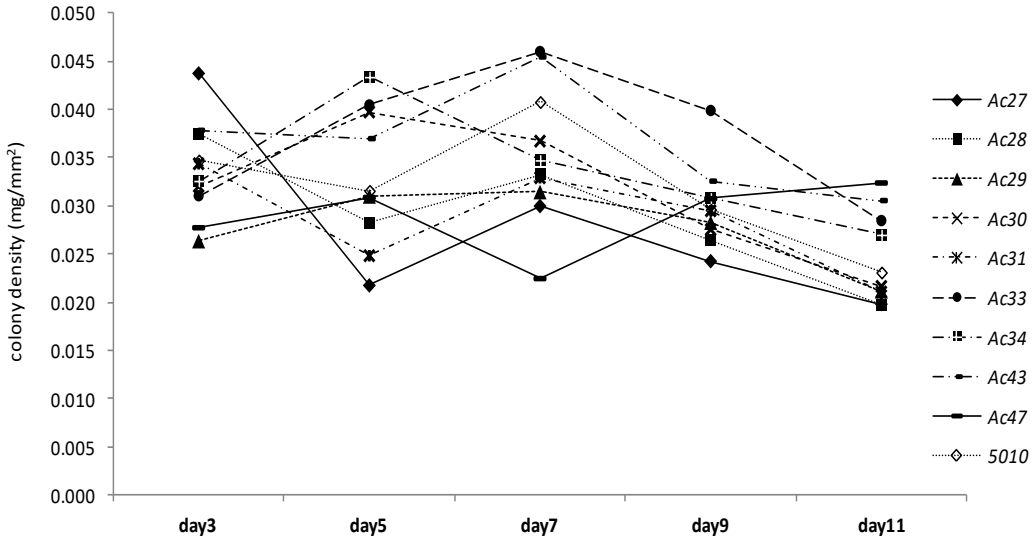
150.00
120.00
90.00
60.00
30.00
0.00
day 3
day 5
day 7
day 9
day 11
time
Ac27
Ac28
Ac29
Ac30
Ac31
Ac33
Ac34
Ac43
Ac47
5010

C.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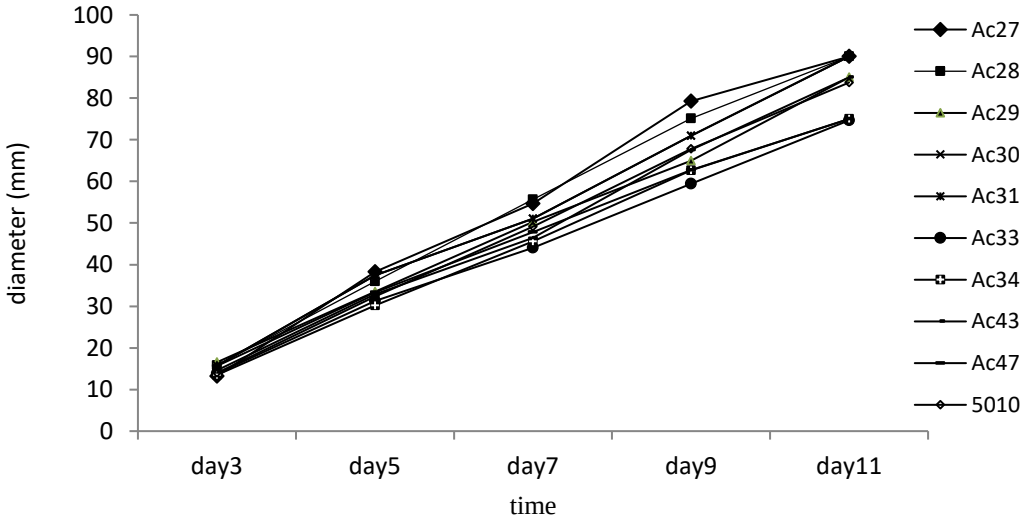
0.045
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
day3
day5
day7
day9
day11
Ac27
Ac28
Ac29
Ac30
Ac31
Ac33
Ac34
Ac43
Ac47
5010

D.


diameter (mm)
100
90
80
70
60
50
40
30
20
10
0
day3
day5
day7
day9
day11
time
Ac27
Ac28
Ac29
Ac30
Ac31
Ac33
Ac34
Ac43
Ac47
5010

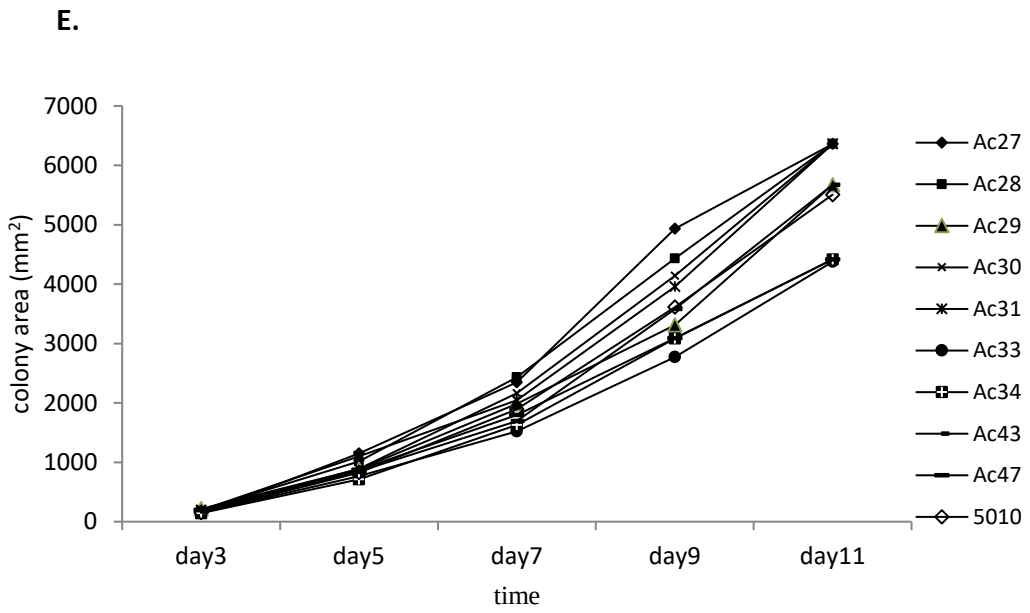


Fig. 3.1: Changes in growth responses of 10 isolates of *A. carbonarius* plotted against incubation time (A: changes in biomass; B: changes in dry weight; C: changes in colony density, D: changes in diameter, E: changes in colony area; data points indicate mean values ± standard deviation of 3 replicates; for clarity of the Fig. no error bars were included when standard deviation was less than 5%).

There are studies suggesting that colony diameter is not an acceptable indirect measure of fungal biomass since colonies seem to become denser with thicker morphologies in older areas (Watt et al., 1995; Taniwaki et al., 2006). Coefficients of variation (CV%) among growth responses were also estimated revealing higher dispersion for colony area and lower for mycelium diameter (Table 3.2). Moreover the values of the CV index indicated that day 3, corresponding to the early stage of fungal development, presented the highest variation compared to day 11, where all fungal isolates seemed to have similar growth responses (Table 3.2).

Table 3.1 Correlation among growth responses (Pearson coefficients and corresponding P-values in parentheses)

	diameter	dry weight	biomass	colony area	colony density
diameter	1	0.9528	0.9552	0.9763	-0.4784
		(<.0001)	(<.0001)	(<.0001)	(<.0001)
dry weight	0.9528	1	0.9858	0.9234	-0.2716
	(<.0001)	(<.0001)	(<.0001)	(<.0001)	0.0008
biomass	0.9552	0.9858	1	0.9201	-0.2975
	(<.0001)	(<.0001)	(<.0001)	(<.0001)	0.0002
colony area	0.9763	0.9234	0.9201	1	-0.5203
	(<.0001)	(<.0001)	(<.0001)	(<.0001)	(<.0001)
colony density	-0.4784	-0.2716	-0.2975	-0.5203	1
	(<.0001)	0.0008	0.0002	(<.0001)	

Table 3.2 Coefficients of variation (CV%) of growth responses along time

Time (days)	biomass CV%	dry weight CV%	diameter CV%	colony area CV%
3	17.55	18.28	12.44	24.76
5	9.40	14.05	10.85	22.07
7	21.55	17.11	10.06	20.11
9	11.93	9.52	10.58	21.08
11	13.02	12.93	9.56	17.98

3.3.2 Ochratoxin A data kinetics

Ochratoxin A was detected after 3 days of incubation and reached a maximum at 9-11 days (Fig. 3.2). Regarding OTA production, fungal isolates could be discriminated into three broad classes containing low (<100 ng/g), medium (between 100-1000 ng/g) and high (>1000 ng/g) OTA producers at 11 days of incubation. Observation of OTA concentration in agar plates showed that the toxin increased with time reaching a plateau at the end of incubation period. However, for some strains maximum OTA levels were attained earlier followed by a decrease thereafter (Fig. 3.2).

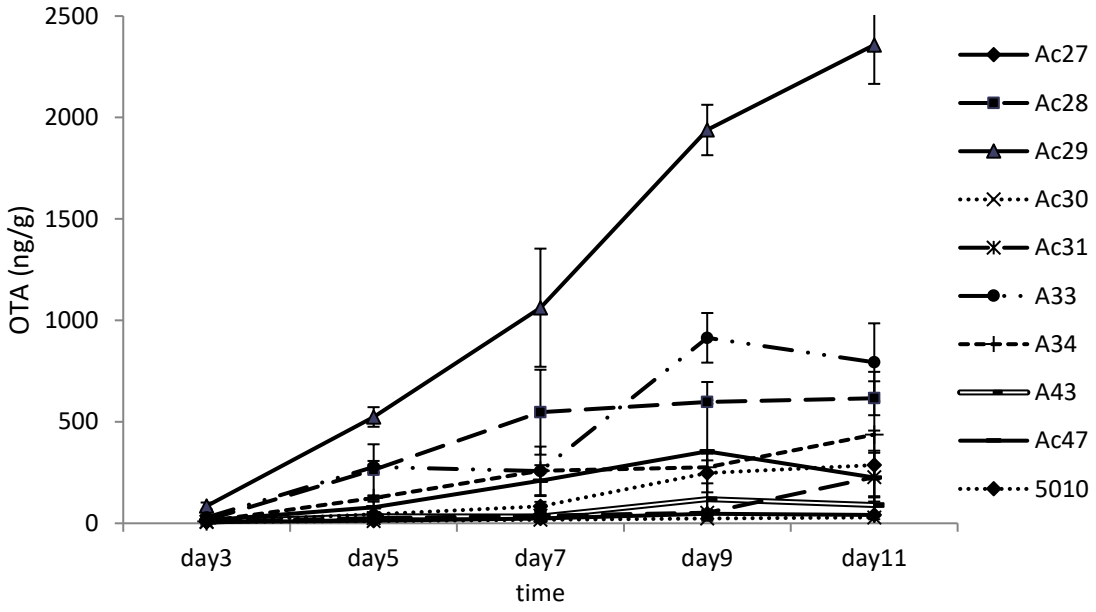


Fig. 3.2 Changes in OTA production over time for *A. carbonarius* isolates on SGM medium.

This decrease was observed after the 9th day of mycelium growth could be attributed to toxin degradation by the fungus in an attempt to find an alternative carbon source to maintain its metabolic activity (Valero et al., 2006a). Analysis of variance for OTA production revealed that all single factors and their 2-way interactions were statistically significant at $p<0.001$. The significance of the factor 'isolate' was obviously biased by the differences in the OTA producing capacity of the different isolates studied. Analysis of variance for OTA between the different sampling times pointed an almost 2-fold higher effect of day 11 on OTA production than all the other days (data not shown). Moreover Coefficients of variation (CV%) revealed a wider dispersion of detected OTA production among isolates than among the five sampling days for each isolate (Table 3.3).

Table 3.3 OTA dispersion and CV% values.

Isolates	Day 3	Day 5	Day 7	Day 9	Day 11	Mean
Ac30	106.01	111.81	132.62	5.40	32.07	77.58
Ac27	113.37	86.09	109.03	24.77	13.92	69.43
Ac43	74.54	1.80	90.8	65.42	49.54	56.42
Ac31	56.48	8.50	68.37	24.01	70.81	45.63
5010	94.44	24.71	27.22	38.47	24.73	41.91
Ac47	27.58	34.92	36.18	94.49	102.98	59.23
Ac34	78.42	7.20	46.32	12.02	70.81	42.95
Ac33	45.34	10.62	3.60	13.38	24.16	19.42
Ac28	31.81	47.81	38.24	1.54	13.57	26.59
Ac29	27.12	29.05	33.16	38.18	22.49	32.00

This observation points the significance of inter-specificity of the *A. carbonarius* species. The analysis of variance pointed an almost 2-fold higher effect of day 11 on OTA production than all the other days. Few studies (Marin et al., 2004; Taniwaki et al., 2006) have reported on the effect of incubation time on the amount of OTA produced. Contrary to long incubation periods employed by many authors, in the present study the focus was given on the early stage of fungal infection. As inferred by the analysis of variance, the significance of time on OTA production was extremely high, confirmed by the fact that day 11 seemed to have in most cases the highest effect. Moreover, observations of the CV index among isolates showed that decreasing the mean OTA producing ability resulted in increasing OTA dispersion, as illustrated by the CV values for isolates Ac27 and Ac30, with higher values observed at early days of production (Table 3). With regard to the isolates used in the current work, they were originated from the areas of Crete and Attica, corresponding both to a geographical localization of low altitude and hot and dry regions. As mentioned before, several reports point also the

impact of region and climate (Visconti et al., 2008; Perrone et al., 2013) in *A. carbonarious* presence and OTA production in grapes. In addition to that, there are similar works indicating the importance between different grape cultivation practices (Bau et al., 2005; Tjamos et al., 2014). In the present work one isolate presented significantly higher toxin production, even from the early stage of growth, among all isolates. This particular isolate, Ac29, was originated from the same vineyard as isolate Ac27, which presented a 40-fold lower OTA production. So the variability in toxin potential between these specific isolates cannot be explained exclusively in terms of geographical location and cultivation practices and hence further investigation is necessary in terms of OTA related genetic factors. The focus of the present work was the *in vitro* investigation of the growth pattern of *A. carbonarius* in a growth medium resembling grape juice between véraison and ripeness, since this fungus has been isolated more frequently during this period in Greece (Tjamos et al., 2006; Meletis et al., 2007). Since grapes are considered one of the greatest sources of OTA contamination by these fungi, it is crucial to know their growth behavior and correlation with toxin production. R^2

3.3.3 Correlation between growth and OTA production

A statistically significant positive correlation ($p < 0.05$) was observed between most isolates' OTA production and growth responses on SGM, as derived by the Pearson correlation coefficients (Table 3.4), with the exception of colony density where a negative correlation was noticeable. OTA production was found to be correlated with each growth response parameter for ten of the isolates examined. The higher OTA producers (isolates Ac28, Ac29, and Ac33) seemed to be better correlated with the measured growth parameters ($p < 0.001$) compared to the rest of the isolates.

Table 3.4 Correlation among OTA production and growth responses (Pearson coefficients)

Isolate	Diameter mm	Dry weight mg	Biomass mg	Area mm^2
Ac27	0.7370*	0.7847*	0.7409*	0.722*
Ac28	0.8725**	0.8923**	0.8949**	0.7994*
Ac29	0.8932**	0.9015**	0.8942**	0.8658**
Ac30	0.5913*	0.5771*	0.5870*	0.5782*
Ac31	0.7213*	0.6642*	0.6817*	0.7896*
Ac33	0.8776**	0.8932**	0.8930**	0.8572**
Ac34	0.6963*	0.7640*	0.7497*	0.6508*
Ac43	0.6312*	0.6295*	0.6577*	0.5812*
Ac47	0.3092	0.5221	0.4831	0.1815

** Significance $P<0.001$, * $P<0.05$

Principal component analysis (PCA) was performed taking as variables all 10 fungal isolates, the different growth measurements, OTA levels, and sampling times, confirming the correlation reported above (Fig. 3). PC1 explained 70.6% of the variability in the dataset and it was positively correlated with all growth variables except colony density. PC2 explained the second larger variation in the dataset (14.4%) and it was associated with OTA production, including mainly the group of high OTA producer isolates. Finally, PC3 explained 13.91% of the variability and it was related to colony density. From the plot of scores (Fig. 3.3), it can be inferred that Ac29 was highly correlated with OTA presenting an increasing trend with time. The first PC was related to time as there was a gradual transition of the growth assessment parameters from the left to the right quadrant of the plot, corresponding from low to high incubation times. Valero et al. (2006a) also showed correlation between colony radius and OTA production using also an isolate of *A. carbonarius*, however this research highlights the great impact of diverse fungal isolates (inter-specific variability) on OTA production.

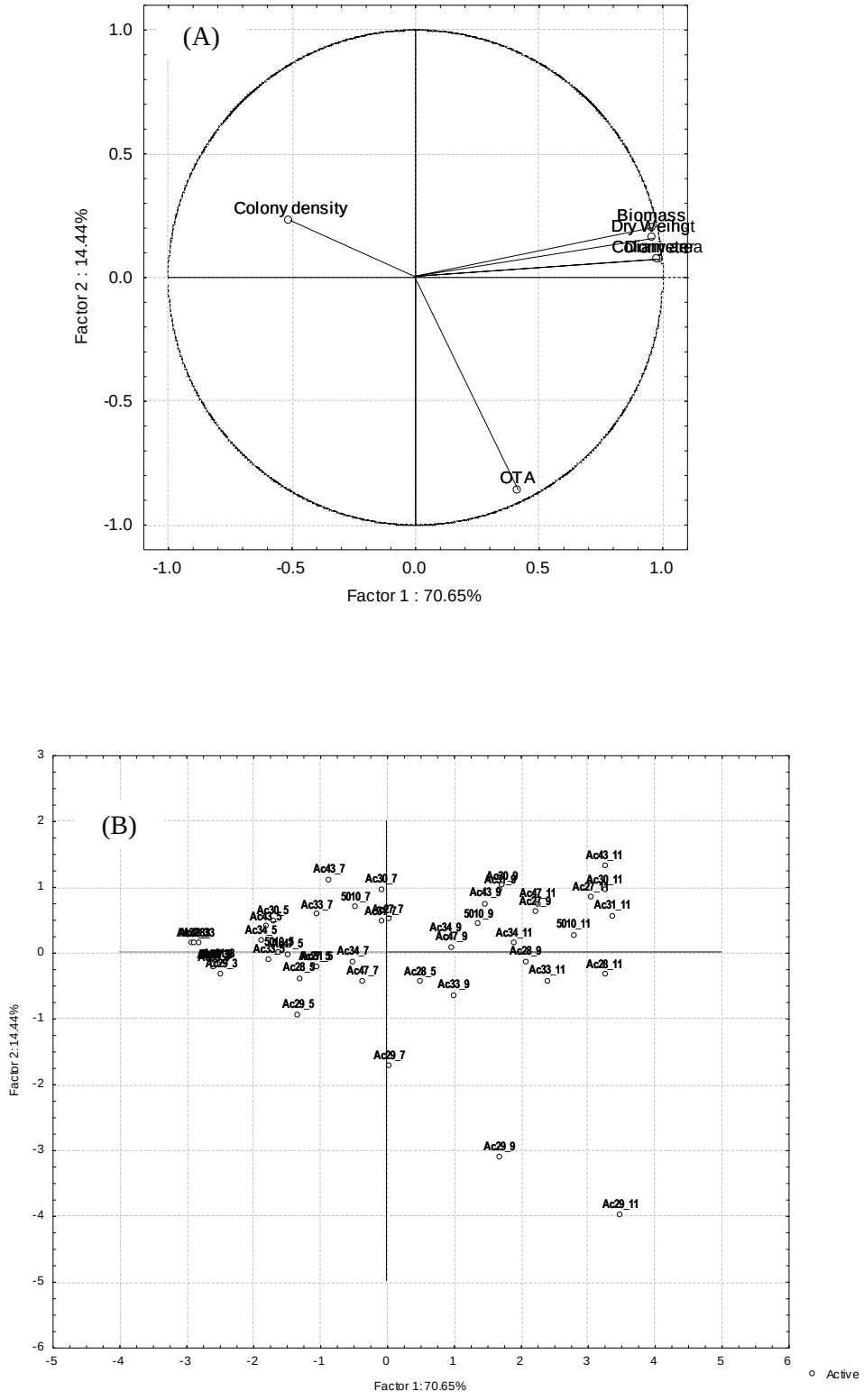


Fig. 3.3 Plot of loadings (A) and scores (B) resulting from PCA analysis for growth and OTA production of *A. carbonarius* isolates considering time of 5 incubation days.

3.3.4 Modeling Ochratoxin A data

Based on correlation results (Table 3.4), a selection in the initially employed fungal growth parameters was made and only three of them were finally taken into consideration, namely colony diameter, colony area and biomass dry weight that presented the highest correlation with OTA. The selected growth parameters were further modeled using the Leudeking-Piret mixed-growth associated model to predict the amount of OTA produced in relation to incubation time.

Table 3.5 shows the estimated model parameters and Fig. 4 provides a graphical illustration of the fitting results for Ac27, Ac29, Ac34, and 5010. Generally, predicted OTA concentration through diameter, colony area or biomass dry weight, led to a mixed-growth associated model, since no specific trend of toxin formation was predicted.

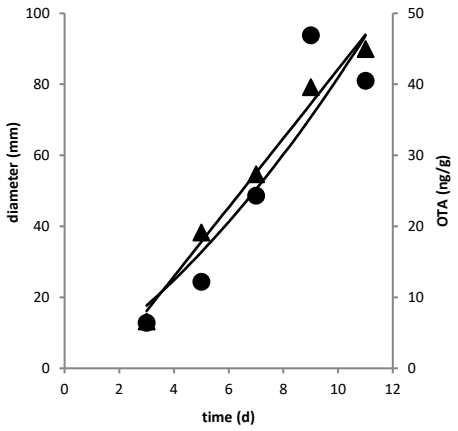
Moreover, a low A_f mean value of 1.385 suggested small differences between predicted and observed data as presented in Fig. 3.5. Also, B_f mean values as 1.24 for colony diameter, 1.04 for colony area and 1.22 for biomass suggested a fail-safe model (Table 3.6). For isolates Ac31, Ac43, and Ac47 the Leudeking-Piret model could not predict OTA at day 3 for some of the growth parameters measured, so B_f and A_f values could not be estimated.

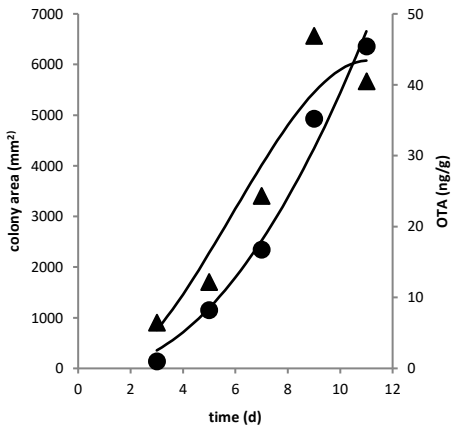
Table 3.5 Parameters and standard errors concerning OTA estimated by the Leudeking-Piret model

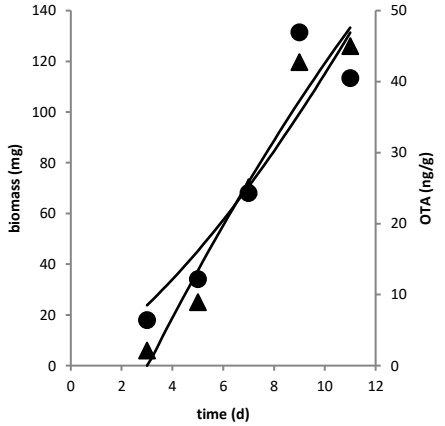
	Diameter			Area			Dry weight		
	α (ng/g mm)	β (ng/g mm d)	r^2	α (ng/g mm^2)	β (ng/g mm^2 d)	r^2	α (ng/g mg)	β (ng/g mg d)	r^2
Ac27	0.80 ± 0.30*	0.40 ± 0.31*	83.85	0.01 ± 0.004	0.003±0.001*	91.10	0.17 ± 0.03	0.02 ± 0.02*	84.33
Ac28	6.01 ± 2.52*	0.14 ± 0.72*	82.11	0.38 ± 0.04	-0.08±0.01	96.61	2.63 ± 0.53	0.31 ± 0.31*	83.10
Ac29	6.78 ± 4.11*	4.54 ± 1.14	97.30	0.94 ± 0.09	-0.15 ± 0.02	99.36	8.74 ± 1.46	2.52 ± 0.69	97.19
Ac30	0.24 ± 0.01	0.008 ± 0.003*	99.64	0.01 ± 0.004	0.003±0.001*	82.28	0.09 ± 0.002	0.0003 ± 0.001	99.64
Ac31	-0.74±0.85*	0.61 ± 0.24*	83.70	-0.02 ± 0.02*	0.01 ± 0.008*	93.00	0.16 ± 0.25*	0.16 ± 0.25*	83.70
Ac33	3.91 ± 5.18*	1.61 ± 1.43*	78.90	0.48 ± 0.22*	0.08 ± 0.07*	82.90	2.75 ± 1.00*	0.84 ± 0.57*	79.46
Ac34	2.17 ± 1.08*	0.67 ± 0.31*	94.14	0.19 ± 0.04*	0.01±0.008*	95.16	1.86 ± 0.38	0.32 ± 0.17*	94.04
Ac43	0.62 ± 0.57*	0.11 ± 0.17*	70.45	0.06 ± 0.02*	-0.01±0.009*	77.09	0.17 ± 1.11*	-0.46±0.69*	52.14
Ac47	3.26 ± 2.64*	0.84 ± 0.66*	64.16	0.23 ± 0.07	0.04 ± 0.02*	80.92	12.20 ± 7.45*	-2.78±2.15*	52.14
5010	0.27 ± 0.72*	0.70 ± 0.21	93.92	0.07 ± 0.03*	0.007 ± 0.03*	94.55	0.66 ± 0.17	0.38 ± 0.09	94.25

*=not significant

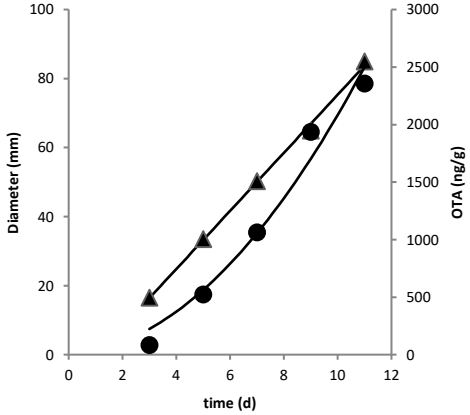
Ac27

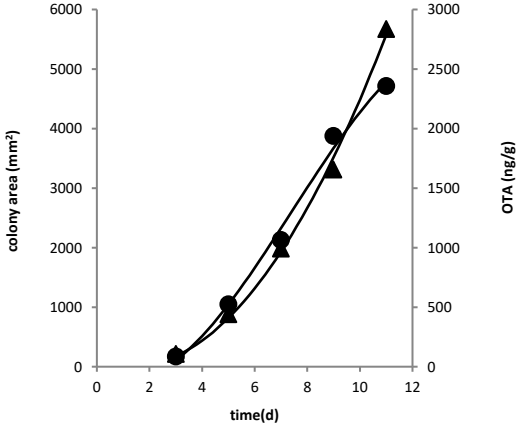

diameter (mm)
OTA (ng/g)
time (d)

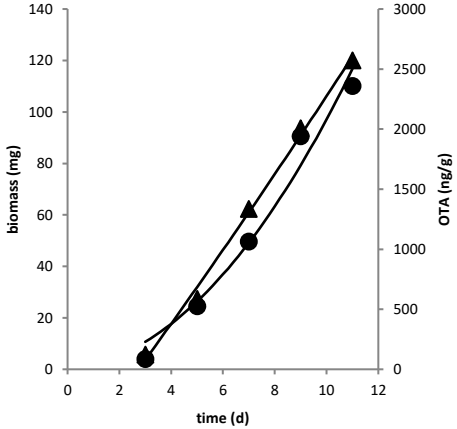

colony area (mm²)
OTA (ng/g)
time (d)


biomass (mg)
OTA (ng/g)
time (d)

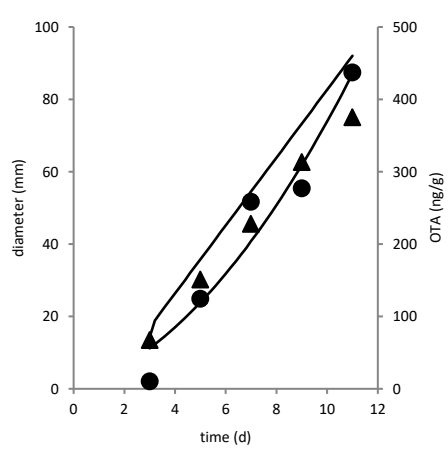
Ac29

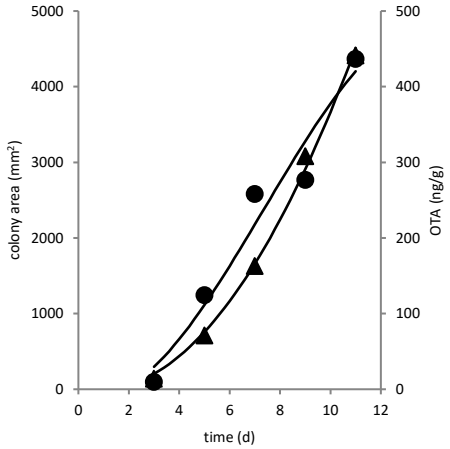

Diameter (mm)
OTA (ng/g)
time (d)

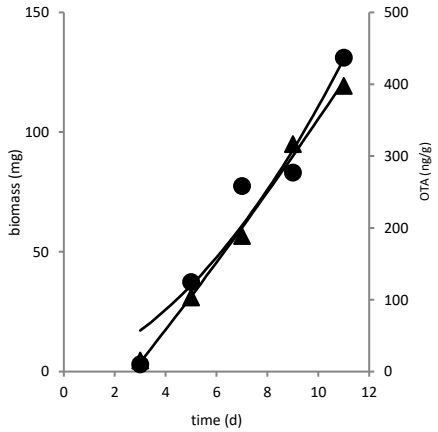

colony area (mm²)
OTA (ng/g)
time(d)


biomass (mg)
OTA (ng/g)
time (d)

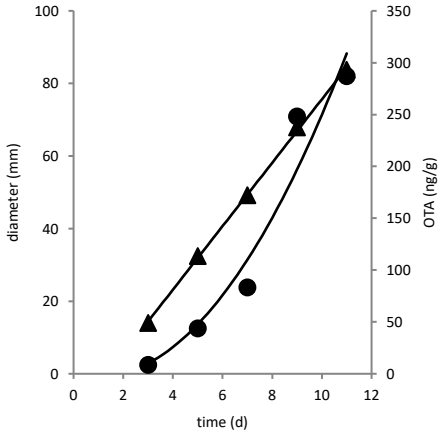
Ac34

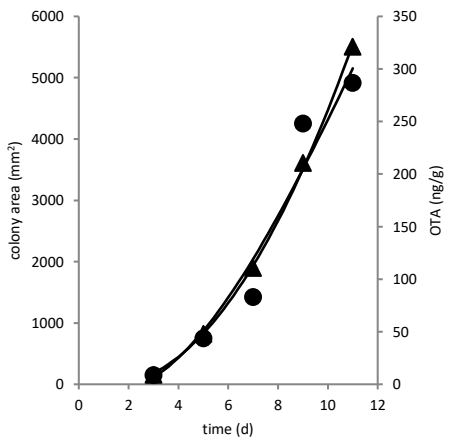






5010





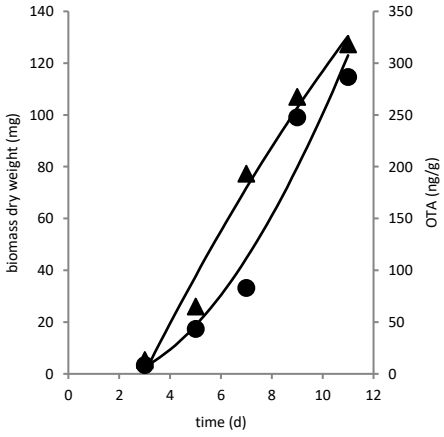


Fig. 3.4 Fitting of OTA (▲) concentration data to Leudeking-Piret models based on the different growth assessments (●).

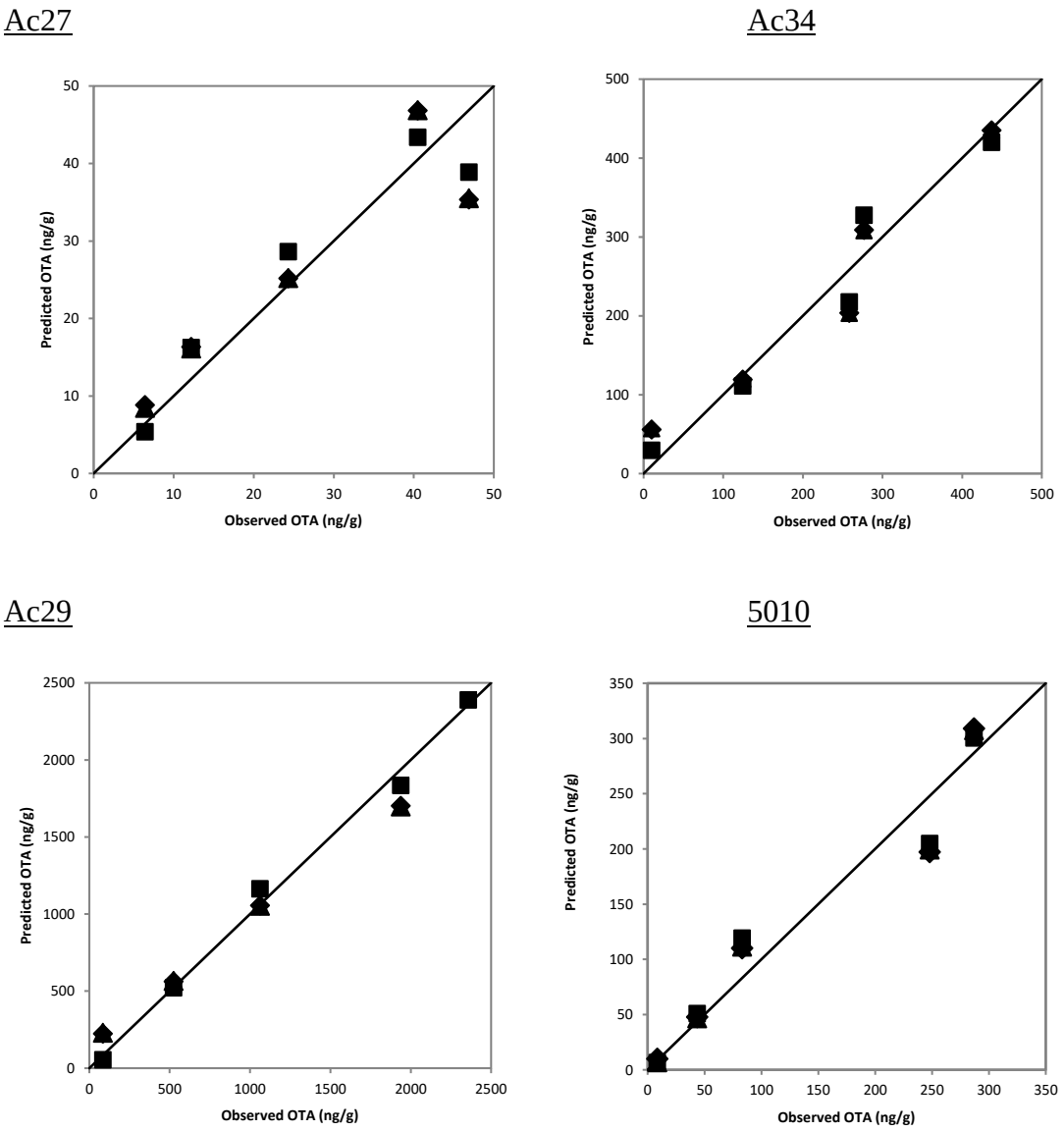


Fig. 3.5 Indicative diagrams of observed versus predicted values of OTA through diameter (■), colony area (▲), biomass (◆).

Table 3.6 Accuracy (A_f) and Bias (B_f) factors derived from OTA data

	diameter				colony area				biomass dry weight			
	B_f	A_f	y	r^2	B_f	A_f	y	r^2	B_f	A_f	y	r^2
Ac27	1.11	1.24	0.9573x	0.79	1.03	1.19	0.9765x	0.89	1.10	1.23	0.9585x	0.80
Ac28	1.56	1.80	0.955x	0.71	1.39	1.46	0.991x	0.95	1.54	1.77	0.958x	0.74
Ac29	1.21	1.28	0.9908x	0.97	0.92	1.13	0.9979x	0.99	1.22	1.29	0.9906x	0.96
Ac30	1.01	1.02	0.9994x	0.99	0.54	1.93	0.9703x	0.90	1.01	1.02	0.9995x	0.99
Ac33	1.32	1.63	0.9243x	0.73	1.18	1.40	0.9386x	0.78	1.29	1.60	0.9263x	0.74
Ac34	1.36	1.52	0.9824x	0.93	1.20	1.37	0.9855x	0.94	1.37	1.53	0.9822x	0.93
5010	1.08	1.18	0.975x	0.93	1.02	1.22	0.9776x	0.94	1.03	1.21	0.9763x	0.93

In our study of OTA production in tandem with growth assessments, observations showed a decrease in concentration without any sign of decrease in mycelium growth. It must be stressed that as new plates were analyzed at each time period, an increase in the already recognized intrinsic variability in mycotoxin production was expected. Overall, modeling OTA concentration along time and taking inter-specificity into consideration, pointed a slightly better prediction through colony area.

Conclusions

In conclusion, all growth responses studied were found to be correlated with each other. However, higher dispersion expressed as CV% was observed at the early stage of fungal development. Regarding OTA, dispersion was higher among isolates than between the sampling days, and also among isolates of lower ochratoxigenic potential. Multivariate statistical analysis showed that PCA explained more than 99% of the data-set variability in the 3 first PC with component 1 corresponding to the sampling time for all isolates.

In relation to Leudeking-Piret mixed-growth associated model, OTA production in the present study followed a rather mixed growth associated trend among the *A. carbonarius* isolates. Statistical indices of A_f and B_f for model performance suggested that the model is a safe approach for OTA prediction. The present work highlights that the dependence of the results from each method assayed lays not only at species level but also among species isolates. Due to the variability of *A. carbonarius* to diverse environmental conditions further research is needed to validate our results with additional experimental data. Understanding, and even more, predicting fungal growth, could become an important step in the evaluation and prediction of OTA production of *A. carbonarius*, since toxin formation was highly correlated with growth parameters. So if growth could be limited, OTA presence could also be limited.

A deeper understanding of isolates' diversity of this species may trigger a better intervention for toxin prevention in field, while the primary modeling approach could serve as a tool for generating secondary models, promoting predictions for a better toxin control.

CHAPTER **4**

Monitoring the Temporal Expression of Genes Involved in Ochratoxin A Production of *Aspergillus carbonarius* under the Influence of Temperature and Water Activity

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Abstract

The objective of this study was to investigate the effect of environmental factors, namely temperature and water activity, on genes involved in the regulation of ochratoxin A (OTA) production over time. For this purpose, the previously characterized toxigenic *Aspergillus carbonarius* Ac29 isolate from Greek vineyards and the *A. carbonarius* ITEM 5010 reference strain were subjected to combined temperature and water activity (a_w) treatments to study OTA production and relative gene expression. The fungal isolates were grown on a synthetic grape juice liquid medium (SGM) under different temperature (20 °C, 25 °C and 30 °C) and a_w (0.94 and 0.98) regimes. The expression of the AcOTA*pks*, AcOTA*nrps*, and *laeA* OTA related genes was investigated using real time PCR. Gene expression was monitored at the same time points, along with fungal biomass and OTA accumulation at three, six and nine days of incubation. In gene expression analysis, stimulation of the biosynthetic genes was observed a few days before any toxin could be detected. This fact may underline a possible early indicator of potential toxin contamination of grapes. However, the transcript levels varied with respect to the different combinations of ecophysiological conditions and time, highlighting a complex regulation of OTA related gene expression of *A. carbonarius* in the specific medium.

4.1 Introduction

Ochratoxin A (OTA) is the most important mycotoxin encountered in grapes and grape products, which is widely distributed as a natural contaminant. It is categorized as group 2B carcinogen by the World Health Organization (WHO) (WHO, 1990) as it displays nephrotoxic, hepatotoxic, teratogenic and immunosuppressive properties affecting seriously human health. In general, ochratoxins (OTs) are secondary metabolites

produced by several species of filamentous fungi belonging to the *Aspergillus* or *Penicillium* genera. *Aspergillus carbonarius* is considered the most important OTA producer in grapes, especially for those cultivated in warm climates (Battilani et al., 2006).

The basic chemical structure of OTs, concerning OTA, OTB, and OTC, consists of an isocumarin nucleus bonded to a L-phenylalanine unit by an amide bond. OTA is the chlorinated form of the toxin, which is most prevalent, whereas B and C, the non-chlorinated and esterified forms, respectively (Bayman and Baker, 2006). Its structure indicates that enzymatic reactions are needed for metabolite biosynthesis. Even though this part has not been fully explored, a number of putative pathways have been recently revealed and genes related to OTA biosynthetic pathway and regulations have been suggested.

More specifically, according to OTA molecular structure and proposed biosynthetic pathways, its synthesis requires several proteins, including a polyketide dihydroisocoumarin synthase (PKS), a non-ribosomal peptide synthase (NRPS) for ligation of the amino acid phenylalanine and the polyketide, and a halogenase for chlorination (Gallo et al., 2012). As Hertweket (2009) reported, most of the known mycotoxins produced by fungi consist of a polyketide or peptide catalyzed by PKS or NRPS large multimodular enzymes. The functional role of *pks* and *nrps* genes has been revealed in studies concerning different fungal genera such as *Penicillium nordicum*, *A. carbonarius, A.ochraceus, A. westerdijkiae* and they have been established to date as OTA biosynthetic key enzymes (Geisen et al., 2006; Gallo et al., 2014). Since both genes have been demonstrated to be necessary in toxin biosynthesis, they have been used as targets to detect and quantify OTA producing molds by molecular techniques. Furthermore, among mycotoxin gene clusters, regulatory genes also exist to control the

expression of these biosynthetic genes. LaeA is a protein known as secondary metabolite regulator of various filamentous fungi, being also involved in the regulatory pathway for OTA biosynthesis as a global transcriptional factor (Crespo-Sempere et al., 2013).

The presence of OTA in food products could be the result of direct contamination by molds and its accumulation may be affected by different factors such as temperature, a_w, pH and substrate composition such as carbon and nitrogen sources (Mohamnadreza and Neda, 2011). From the food safety perspective, evaluation of induction of these key genes could become an indicator tool for risk assessment of toxigenic species since gene transcription usually precedes phenotypic production.

The purpose of this study was to employ reverse transcription quantitative PCR (RT-qPCR) to explore the influence of temperature, a_w and time on OTA related gene transcripts expression pattern. While plenty of data exist on the effect of the above factors on growth and OTA production of *Aspergillus carbonarius*, there are no data on the impact of these factors at gene expression level. To our knowledge this work is the first attempt to investigate the effect of ecophysiological factors such as temperature and a_w on *Aspergillus carbonarius* gene expression along with OTA production in relation to time.

4.2 Materials and Methods

4.2.1 Fungal Isolates and Culture Media

A wild isolate of *A. carbonarius* from grapes of Greek vineyards was used throughout this study. The isolate, coded Ac29, has been previously characterized by molecular methods (Kizis et al., 2014) and belongs to the fungal culture collection of the Laboratory of Food Microbiology and Biotechnology (LFMB) of the Agricultural

University of Athens (AUA). In addition, *A. carbonarius* ITEM 5010 was kindly provided by Prof. Tsitsigiannis from the AUA Phytopathology Department and used as reference strain. It is a genome sequencing ochratoxigenic strain of *A. carbonarius*, isolated from grape berries (Apulia, Italy) and it has been already used as reference strain in similar gene expression studies (Gallo et al., 2012; Ferrara et al., 2016).

Spore suspensions of each fungus were prepared using 7-day-old colonies grown on Malt Extract Agar at 25 °C. Conidia were harvested from sub-cultures in an aqueous solution of 0.01% Tween 80 (Merck, Schuchardt, Hohenbrunn, Germany) by scraping the surface of the mycelium with a sterile glass rod. The concentration of the final spore suspension was assessed using a haemocytometer (Brand, Wertheim, Germany) and adjusted by appropriate dilutions to approximately 10^6 spores/mL.

The study was performed on a Synthetic Grape Juice Liquid Medium (SGM) with pH adjusted to 3.8 using 2M KOH, according to Lappa et al. (2015). SGM was used in Erlenmeyer flasks (Fig. 4A) containing 50 mL of medium inoculated with fungal spore suspension to provide an initial count of 10^6 conidia/mL in the flask. The a_w of this basal medium was 0.98, as measured by an AquaLab LITE (Decagon Devices Inc., Pullman, WA, USA) water activity meter at 25 °C, and modified to the required level of 0.94 by adding glycerol (Mitchell et al., 2004). Incubation was carried out at 20 °C, 25 °C and 30 °C for 9 days in a rotating shaker at 100 rpm. Rotation helped mycelia to be submerged into the liquid medium and suppress sporulation in order to avoid melanin production which would disturb RNA extraction procedure (Lappa et al., 2017).

Fig 4A. Liquid shaking flask cultures of *A. carbonarius.*

4.2.2 OTA Determination and Growth

For toxin determination, the whole content of each flask was analyzed at Days 3, 6 and 9 as reported elsewhere (Lappa et al., 2017). Specifically, each sample of 50 mL was homogenized using an Ultra Turax homogenizer (Heidolph Instruments, Schwabach, Germany) for 1 min at the highest speed. Subsequently, 5 mL of each homogenate were mixed with 5 mL of 100% methanol and left still for 30 min for OTA extraction. Furthermore, extracts were filtered through a Whatman No 2 filter paper in order to remove any suspended solids and filtered again through a 0.2 μm syringe-driven filter unit (Millex, Millipore Co., Bedford, MA, USA). All extracts were stored at −20 °C until analysis. OTA was determined by HPLC analysis as detailed elsewhere (Lappa et al., 2015). Briefly, a Model PU-980 Intelligent pump and an FP-2020 Plus fluorescent detector (JASCO Inc., Easton, PA, USA) were used. The analysis was performed under isocratic conditions at a flow rate of 1 mL/min of the mobile phase (water/acetonitrile/acetic acid; 49.5/49.5/1) through a Waters spherisorb C18 analytical

column, 5µm ODS2 (4.6 × 250 mm) (Resteck Co., Pinnacle II, Bellefonte, PA, USA). Injection volume was 10 µL and run time for samples was 20 min with OTA detected at about 11 min. Additionally known concentrations of OTA were spiked on SGM and a recovery assay for the HPLC method was assessed (Lappa et al., 2017). The detection limit of the analysis was 1 ng/mL of the measurement solution. For growth determination, fungal mycelium was harvested from the flasks, and biomass wet weight was determined at Days 3, 6, and 9. Both toxin and growth determination were conducted in triplicate.

4.2.3 RNA Isolation and cDNA Synthesis

RNA was extracted as described by Lappa et al. (2017). Briefly, 3 biological samples of grown mycelia were collected from SGM, washed with double distilled H_2O (ddH_2O), dried in Whatman No 2 paper, flash frozen in liquid nitrogen for stabilization of expression, and stored at −80 °C until extraction. Frozen tissues were lyophilized and approximately 10 mg of fungal mycelium were grounded to powder and used for nucleic acid extraction. Invitrogen, PureLink RNA mini kit (Ambion, Carlsbad, CA, USA) was used for total RNA isolation according to manufacturer's protocol and Trizol (Ambion, Carlsbad, CA, USA) was employed in RNA extraction. Moreover, to remove genomic DNA contamination, samples were treated with Turbo DNase (Ambion, Carlsbad, CA, USA) according to kit instructions. The quality of RNA samples was checked through an RT-qPCR amplification to ensure absence of genomic DNA. RNA concentration and purity of each sample were assessed spectophotometricaly using a NanoDrop spectrophotometer (IMPLEN GmbH, Munchen, Germany). RNA integrity was also verified on 3% agarose ethidium–bromide staining gel (Fig. 4B). Extended preliminary experiments were conducted evaluating several isolation protocols, in order

to select the most appropriate extraction method for A. carbonarius samples concerning RNA concentration, purity, and genomic DNA absence.

First-strand cDNA was synthesized using PrimeScript Reverse Transcriptase (Takara, Kukatsu, Japan) according to manufacturer's instructions. A concentration of 500 ng of total RNA was used for each sample in a 20 μL final reaction volume. Reaction mixture was incubated for 5 min at 65 °C and the specific set up program was maintained at 30 °C for 10 min, 42 °C for 60 min, and 70 °C for 15 min.

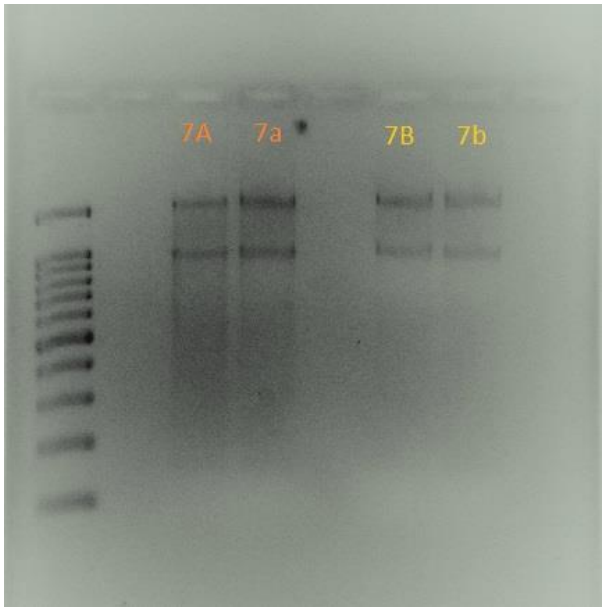


Fig. 4B. Indicative 3% agarose jel of *A. carbonarius* RNA

4.2.4 Real Time PCR

For gene expression assay, Real Time PCR was used to amplify the *Ac*OTA*pks*, *Ac*OTA*nrps* and *laeA* target genes. Constitutively expressed β-*tubulin* gene served as internal reference for gene expression normalization as this is a widely used housekeeping gene reported in relevant studies (Gallo et al., 2012; Crespo-Sempere et al., 2013). Nucleotide sequences of primers used in the qPCR assays are shown in Table 4.1.

The Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used to carry out Real time PCR experiments which were conducted in 96-well plates. To monitor cDNA amplification, qPCRBioSyGreen Mix High-Rox (PCR BIOSYSTEMS, London, UK) was used in a 10 μL reaction. Cycling conditions according to the specific SYBRGreen protocol were: 95 °C for 0.5 min, 60 °C for 0.25 min, and 72 °C for 0.25 min (40 cycles). Melting curve analysis of the PCR products was performed by heating to 95 °C for 0.15 min and 60 °C for 1 min, and continuous measurement of the fluorescence to verify the PCR product. Template-free negative controls were also used at every run. Data analysis was assessed by StepOnePlus RT-PCR System Software v2.1 and transcription levels of the target genes were determined after normalization with the reference gene (tub-β). An indicative amplification plot is presented in Fig. 4C. In all cases, transcript values were expressed as normalized individual data points, based on the formula $E^{\Delta Ct}$ (Schmittgen and Livak, 2008), according to each primer pair efficiency, where ΔC_t = C_t gene of interest and C_t internal control. Gene expression measurements were considered to be comparable since equal amounts of RNA were used as template and also reaction conditions were the same in all qPCR assays. Gene expression was assayed in triplicate for each individual biological sample.

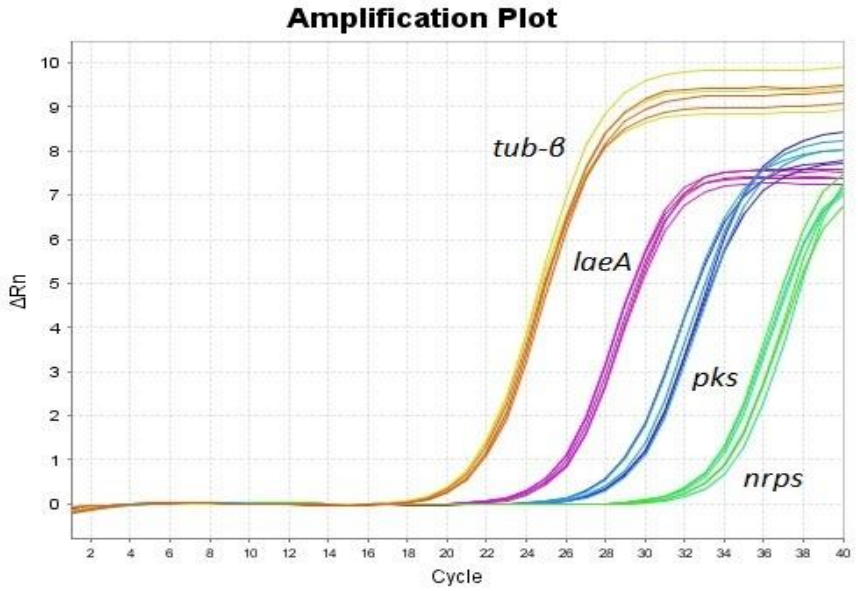


Fig 4C. Indicative amplification of targeted genes of *A. carbonarius* from liquid cultures.

Table 4.1 Nucleotide sequences of primers for qPCR assay

Primer pair	Gene	Nucleotide sequences 5'—3'	Reference
F-pks	*AcOTApks*	GTC AAG GTC GGG TGC TAC AA	Lappa et al. (2017)
R-pks		TCG GAA TGA TAC GCG ACT TT	
F-nrps	*AcOTAnrps*	CTC CAC CCA TCC TCC CGT TC	Crespo Sempere et al. (2013)
R-nrps		AAT CCA TGT CCT CAC CAT CGC	
F-laea	*laeA*	CAC CTA TAC AAC CTC CGA ACC AC	Crespo Sempere et al. (2013)
R-laea		GGT TCG GCC AAC CGA CGA CGC TG	
F-tubβ	*β-tubulin*	CGC ATG AAC GTC TAC TTC AAC GAG	Crespo Sempere et al. (2013)
R-tubβ		AGT TGT TAC CAG CAC CGG ACT	

qPCR efficiency tests for each primer were performed under the specific experimental conditions and evaluated as 96% for *AcOTApks*, 101% for *AcOTAnrps*, 98% for *laeA* and 94% for β-*tubulin* genes, respectively.

4.2.5 Data Analysis

Data concerning toxin production and gene expression levels were analyzed to investigate how these responses were distributed across the experiment treatments, determining also any intra-species differences. Data were subjected to general linear model (GLM) analysis to investigate the effect of time, temperature and a_w on measured fungal responses. Moreover, effect test was employed pointing the significant effects among treatments and biological responses. Statistical analysis was performed using JMP software (SAS, Institute INV, Cary, NC, USA). The statistical significance was set at $p \leq 0.05$. Furthermore, two-dimensional (2D) surface response contour plots were generated using Minitab 14 (Minitab Inc., State College, PA, USA) in relation to the combinations of temperature × time for biomass and OTA production for each considered fungus and a_w level. Finally, Principal Component Analysis (PCA) was performed on the correlation matrix of the variables as described elsewhere (Ringener et al., 2008). The explanatory variables used in the analysis were biomass and OTA production, a_w levels, incubation temperature and time, fungal strain and the expression of the three selected genes. For the selection of the optimum number of Principal Components (PCs), factors with eigenvalues greater than 1.0 were selected. The results of the PCA analysis were graphically presented by the plots of loadings and scores for the first two principal components indicating a condensed representation of the correlation between the original variables and the distribution of the samples. PCA analysis was implemented with XLStat software version 2006.06 using a (Addinsoft, Paris, France) varimax rotation.

4.3 Results and Discussion

4.3.1 Impact of ecophysiological factors on growth and toxin production over time

Two-dimensional (2D) contour plots representing biomass production (g) of *A. carbonarius* Ac29 and *A. carbonarius* ITEM 5010 in relation to temperature and incubation time for each a_w level assayed are shown in Fig. 4. Higher biomass was produced at 0.98 a_w (Fig. 4.1 B,D) for both fungi irrespective of temperature and incubation time compared to 0.94 a_w (Fig. 4.1 A,C).

Moreover, an intra-strain difference was observed since the Ac29 isolate produced more biomass compared to the reference strain ITEM 5010 in all combinations of temperature × time for the same a_w level. In general, biomass production increased proportionally with time for both fungi, and reached a maximum at 25 °C and 0.94 a_w, whereas for 0.98 a_w maximum biomass production has shifted to 26 °C. It needs to be noted that at day 3 no growth was observed for both strains at 0.94 a_w regardless of temperature and at 0.98 a_w at 20 °C. The above results are consistent with other ecophysiological studies for *A. carbonarius* regarding optimum growth temperature (Mateo et al., 2009; Kapetanakou et al., 2009). These results indicate that the combination of specific temperature and water activity has an important effect on fungal growth. There are reports investigating a number of *A. carbonarius* isolates, showing that low a_w (ca. 0.94) is restrictive not only to growth, but also to OTA production, especially when they act in parallel with other environmental parameters (Romero et al., 2005).

Fig. 4.5 presents 2D contour plots for OTA production of the two fungal strains in relation to temperature and time for each a_w level separately. OTA accumulation levels varied reaching a maximum value of 423 ng/mL, with the isolate Ac29 being a higher OTA producer compared to the reference strain ITEM 5010, enforcing thus the finding of a previous study in which the selected fungal strains present high OTA potential

(Lappa et al., 2015). In general, a similar trend was observed in both strains as OTA gradually increased throughout incubation time. It has already been reported (Belli et al., 2004) that reduction of a_w in grapes results in reduction of OTA accumulation. On the other hand, high a_w levels (in the range of 0.98) enhance OTA production as reported by other researchers (Mitchell et al., 2003). The optimum temperature for OTA production was different for the two strains at 0.98 a_w, since Ac29 presented the highest toxin concentration at 28–30 °C in contrast to ITEM 5010 where maximum toxin accumulation was observed at ca. 26 °C (Fig. 4.2 B,D). However, at 0.94 a_w, both fungi presented maximum OTA production at ca. 25–26 °C (Fig. 4.2 A,C). Our results are consistent with previous studies which have reported 25 °C as optimum temperature for OTA production by *A. carbonarius* (Mitchell et al., 2003), and also agree with Belli et al. (2005) who reported 30 °C as the temperature of maximum OTA production observed. However, other studies report 20 °C as the optimum temperature for toxin production (Marin et al., 2006; Tassou et al., 2009). Such differences are possibly associated with the use of different isolates (Esteban et al., 2006), growth media (Pardo et al., 2006) and experimental conditions such as solid or liquid growth cultures (Lappa et al., 2017). Furthermore, even thought it has been demonstrated previously that toxin production is well associated with biomass for both strains (Lappa et al., 2015), the fact that Ac29 produced higher amounts of OTA at 30 °C could suggest that this strain is adapted to high temperatures, since it has been isolated from vineyards in Crete, a warm climate region in Southern Greece.

Finally, it needs to be noted that isolate Ac29 did not produce any OTA at 20 °C and 30 °C/0.94 a_w, whereas no toxin was detected for strain ITEM 5010 at 20 °C/0.94 a_w. In the cases where no toxin was detected, growth was macroscopically visible, pointing that ecophysiological conditions that allow fungal growth are broader than those required for

OTA production (Tassou et al., 2009). This highlights the significance of the combination of the above ecological factors on toxin production and also the importance of intra strain studies. Furthermore, analysis of variance performed on OTA revealed highly statistical significance ($p < 0.0001$) for all single factors assayed (strain, temperature, a_w and time) (Table 4.2). Among the parameters tested, a_w had the strongest effect followed by strain, time and finally temperature. Moreover, ANOVA assessed the impact of cross effect among the parameters assayed and the combination of strain x a_w presented the highest impact. Furthermore, comparison of the mean values for OTA revealed differences between the two strains at each incubation time at 25 °C and 30 °C/0.98 a_w which is also graphically confirmed by the 2D contour plots (Fig. 4.2 B,D). In addition statistically significant differences were observed between the two strains at 20 °C/0.94 a_w after nine days of incubation. From the intra-species perspective, temperature had the same impact on both strains in terms of toxin production. However, a_w had greater impact on strain Ac29 ($p < 0.0001$) in contrast to reference strain ($p < 0.0004$) which appeared to be more sensitive to the parameter of time. Biomass and OTA raw data are also provided in Fig. 4.3.

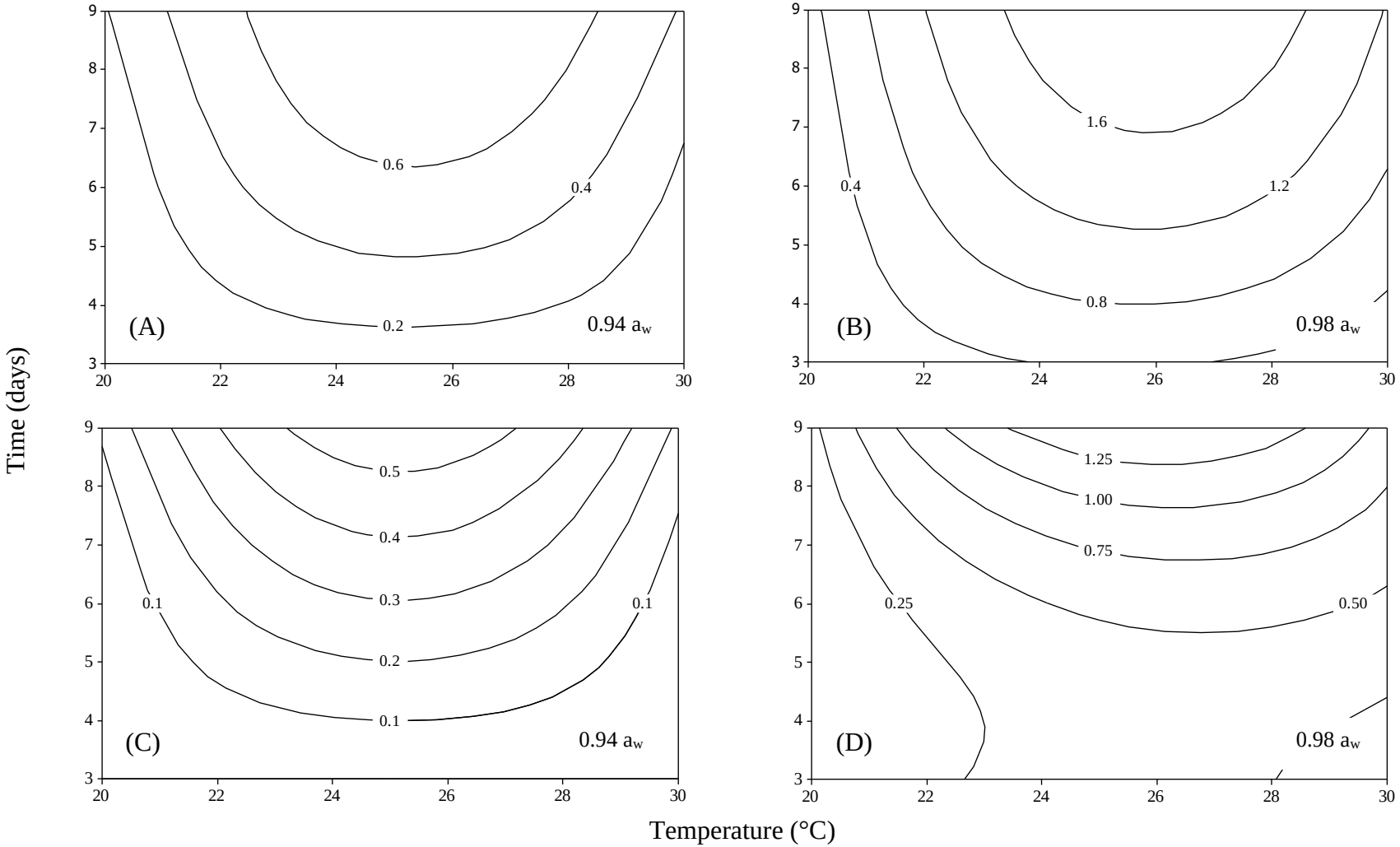


Fig. 4.1 Two-dimensional contour plots showing the effect of temperature and incubation time on biomass production (g) for: *Aspergillus carbonarius* Ac29 (A, B); and *A. carbonarius* 5010 (C, D).

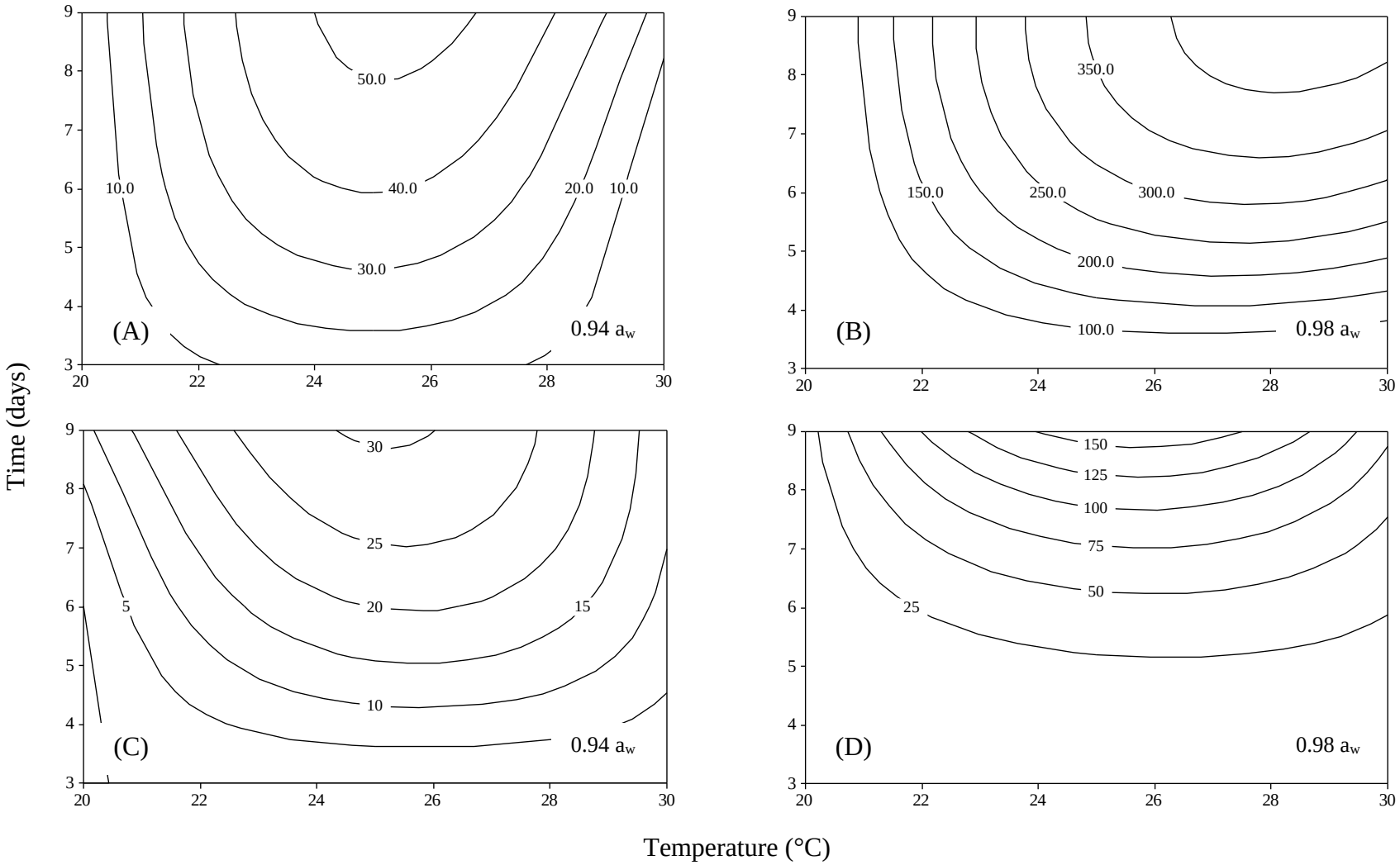


Fig. 4.2 Two-dimensional contour plots showing the effect of temperature and incubation time on OTA production (ng/mL) for: *Aspergillus carbonarius* Ac29 (A, B); and *A. carbonarius* 5010 (C, D).

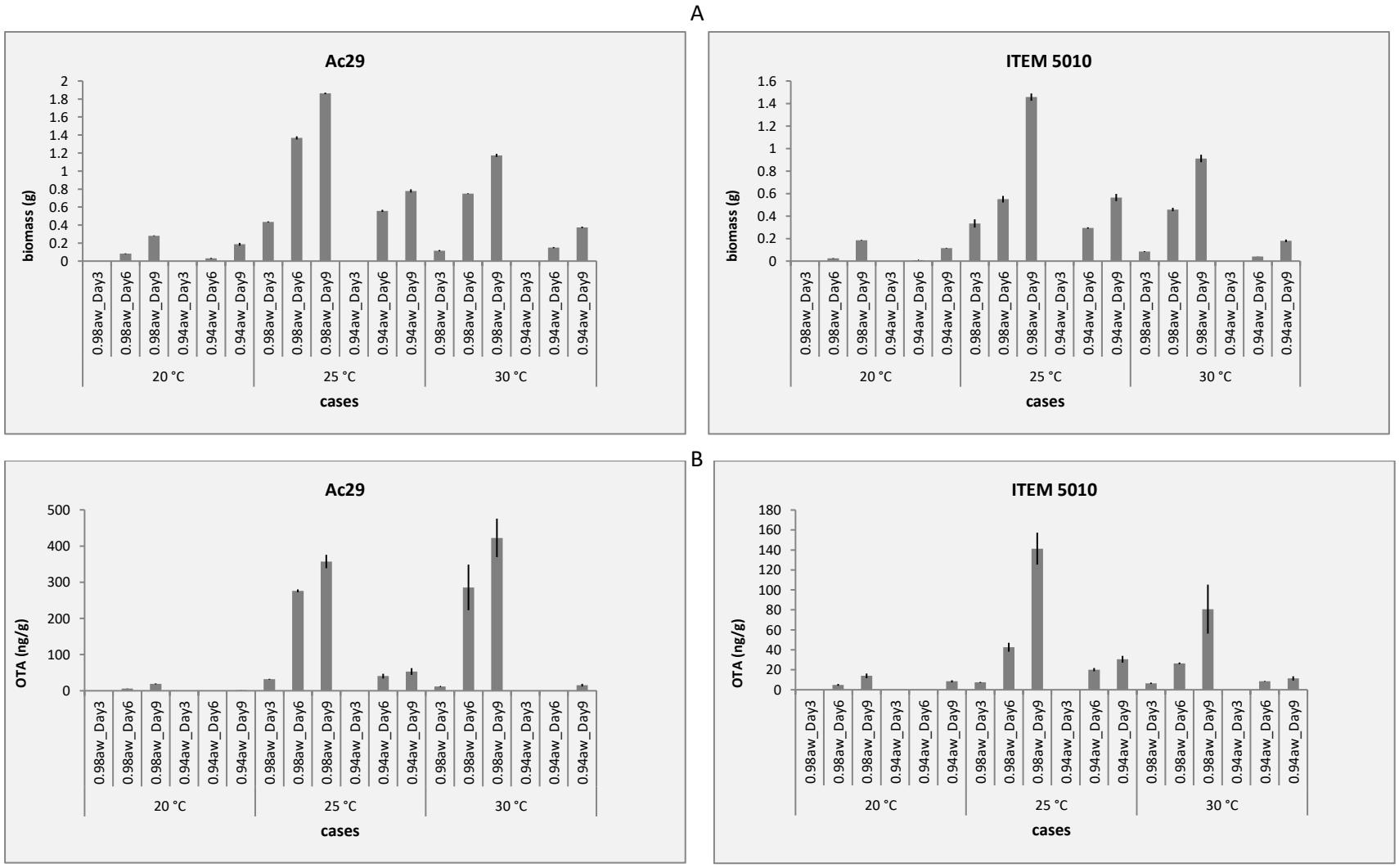


Fig. 4.3.: Effect of temperature, a_w and time on (A) biomass and (B) OTA production of *Aspergillus carbonarius* strains on SGM (Synthetic grape medium). Error bars when visible represent the standard error of the mean value of 3 replicates.

Table 4.2 Analysis of variance for the effect of ecophysiological factors on OTA production.

Factor	DF	Sum of Squares	F Ratio	Prob > F
Strain (S)	1	102,931.82	152.7185	<0.0001
Temperature (T)	2	137,844.54	102.259	<0.0001
a_w	1	200,240.91	297.0947	<0.0001
Time (*t*)	2	153,981.37	114.23	<0.0001
T × a_w	2	88,487.9	65.6441	<0.0001
t × a_w	2	100,385.72	74.4705	<0.0001
T × t	4	60,879.31	22.5814	<0.0001
T × a_w × t	4	43,630.88	16.1836	<0.0001
S × T	2	52,627.61	39.0414	<0.0001
S × a_w	1	86,572.75	128.4468	<0.0001
S × T × aw	2	45,901.93	34.052	<0.0001
S × T	2	40,530.5	30.0673	<0.0001
S × T × t	4	25,880.57	9.5997	<0.0001
S × T × t × a_w	4	23,840.98	8.8431	<0.0001
S × t × a_w	2	39,596.13	29.3741	<0.0001

4.3.2 *Impact of ecophysiological factors upon gene regulation over time*

The effect of incubation temperature, a_w and time on the expression of AcOTA*nrps*, AcOTA*pks* and *laeA* genes during *A. carbonarius* growth in SGM are presented in Fig.s 4.4 and 4.5 for Ac29 and ITEM 5010, respectively. In general, with regard to the expression of the two biosynthetic genes and the global regulator, both strains showed regulation in all cases studied and different expression patterns were observed. Statistical analysis showed that gene expression of all three genes was significantly affected by the parameters used in the study (Table 4.3A). Analysis of variance for the

whole response of the selected genes revealed high statistical significance for every single factor (Table 4.3B). The effect of temperature on gene expression was analyzed at different incubation times. A *t*-test revealed that AcOTA*pks* ($p < 0.0225$) and *laeA* ($p < 0.0328$) were the genes mostly affected by the different temperature regimes, especially at Day 6 where the highest gene expression was observed.

Table 4.3 Analyses of variance for the effect of ecophysiological factors on: inter-strain differences (**A**); and intra-strain differences (**B**) in transcriptional profiles

A.

Genes	Factor	P value	F ratio
AcOTA*nrps* (p<0.0103)	temperature	0.0541	2.10
	a_w	0.6840	0.0005
	time	0.2600	0.53
	strain	0.0016**	10.40
AcOTA*pks* (p<0.0001)	temperature	<.00021***	3.97
	a_w	0.2339	0.63
	time	0.0476*	2.79
	strain	<.0001***	37.27
laeA (p<0.0028)	temperature	0.0158*	3.56
	a_w	0.0368*	4.11
	time	0.9761	0.11
	strain	0.0045**	8.58

B.

Strain	Cross effect	P value	F ratio
Ac29	**AcOTA*nrps***		
	time x temperature	0.0061**	6.14
	AcOTA*pks*		
	time x temperature	0.0011**	8.78
	laeA		
	time x temperarture	0.0047**	6.54
	temperature x aw	0.0023**	11.27
	time x temperature x aw	0.0018**	7.96
5010	**AcOTA*pks***		
	time x temperature x aw	<.0001***	13.72
	laeA		
	time x temperarture	<.0001***	20.86
	time x aw	0.042*	4.53
	time x temperature x aw	0.0023**	7.61

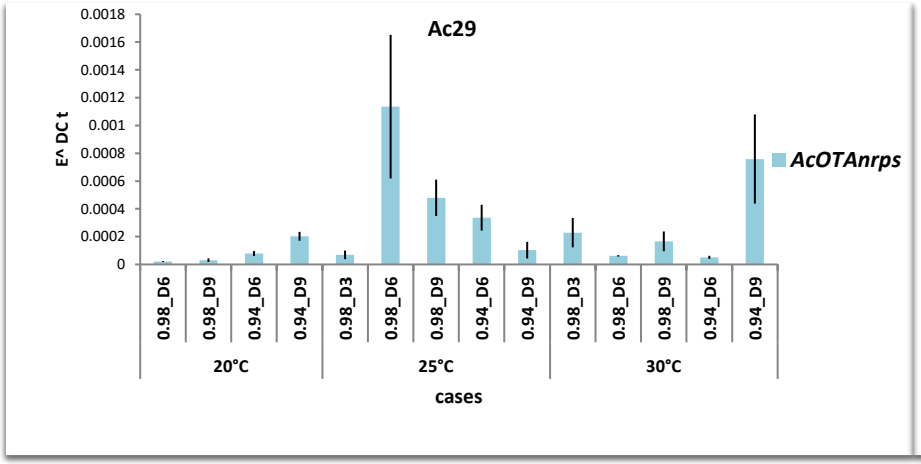
Moreover, a_w effects were evaluated at different sampling times indicating that gene expression at Day 6 was highly affected by a_w levels. Specifically, AcOTA*nrps* expression of Ac29 was higher at 25 °C/Day 6 at both a_w levels assayed. In addition, at 20 °C and 30 °C, gene expression levels for the same fungus increased with time at 0.94 a_w (Fig. 4.4A). AcOTA*pks* expression did not present any difference at 20 °C, but up-regulation was observed at 25 °C and 30 °C (Fig. 4.4 B). The levels of *laeA* expression increased from 20 to 25 °C followed by a decrease at 30 °C (Fig. 4.4 C). On the other hand, the general trend of AcOTA*nrps* expression for the strain 5010 followed an increase with temperature and the same holds for AcOTA*pks* transcripts (Fig. 4.5 A, B). Finally, the expression of *laeA* gene decreased with increasing temperature (Fig. 4.5 C).

Differences among transcript levels were also observed at different sampling times when *A. carbonarius* grew at constant temperature. Increase of incubation time resulted in a strong trend of turning up transcription of AcOTA*nrps* for strain ITEM 5010 at all three temperatures assayed and for Ac29 at 20 °C and 30 °C. Furthermore, for the reference strain, expression of *laeA* followed an increasing trend with time at 20 °C and 30 °C/0.98 a_w (Fig. 4.5 C). Hence, sampling time caused greater transcriptional changes than growing conditions. The influence of time on AcOTA*pks* at different sampling times did not provide a clear trend in both fungi.

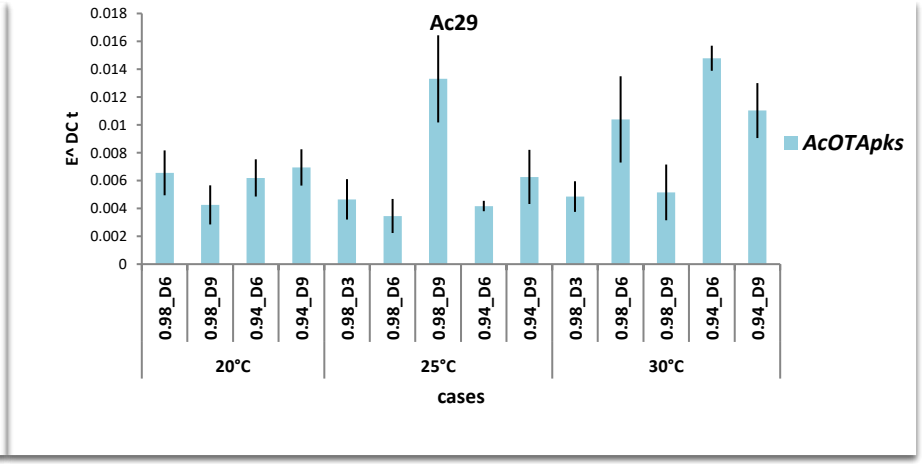
According to Table 4.3A, fungal strain caused the highest variation in gene response for the ecophysiological parameters studied. Moreover, results showed that AcOTA*pks* ($p < 0.0001$) gene was most highly affected by fungal strain. Further, analysis of interactions by ANOVA showed the impact of the combined effect of the ecophysiological parameters studied on gene expression at intra-strain level (Table 4.3B). The highest impact is presented for each strain underlying the complexity of interactions of all parameters on gene expression results.

Principal Components Analysis (PCA) undertaken on the samples corresponding to different combinations of temperature, a_w, time, and fungal strain resulted in the identification of three Principal Components (PCs) with eigenvalues higher than 1.0 which explained 67.3% of the total variance. Moreover, the results of the analysis are graphically illustrated in Fig. 4.6 where the original variables are projected onto the plane formed by the selected two first PCs. Specifically, Fig. 4.6A can be used to establish relationships among variables. Thus, a strong positive correlation was observed for OTA and biomass production, indicating that the higher the biomass the higher the amount of OTA (Lappa et al., 2013). A close relationship was also evident for temperature and AcOTA*pks* and AcOTA*nrps* gene expression, indicating that higher temperatures resulted in higher expression of these genes, whereas the opposite was observed for temperature and *laeA* gene expression. According to this graph, there is no relationship between OTA and *laeA* gene expression as the two vectors corresponding to these variables form an angle close to 90 °C (Rodriquez-Gomez et al., 2012).

A.



B.



C.

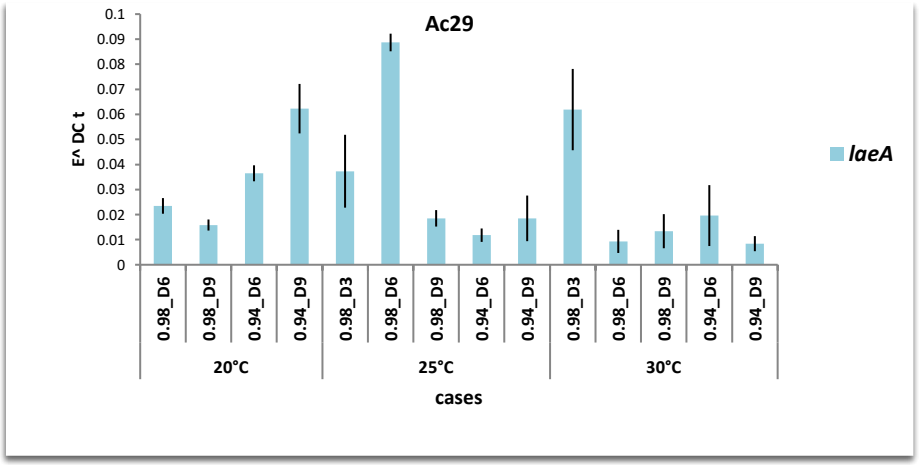
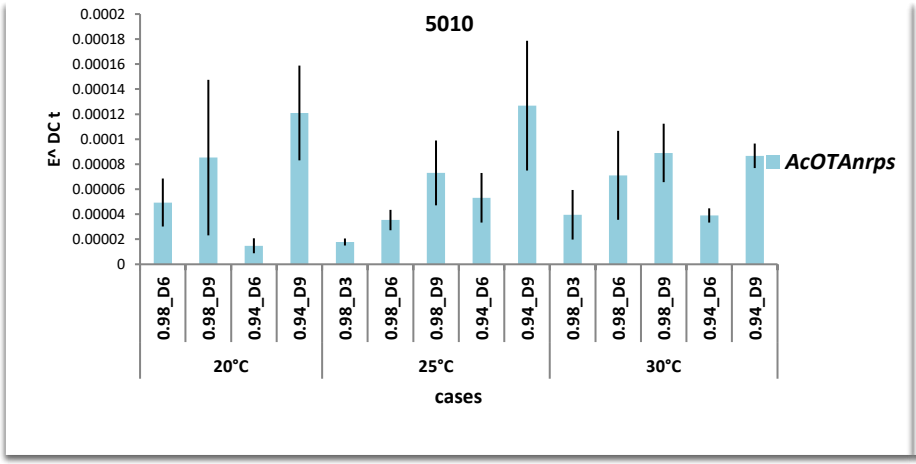
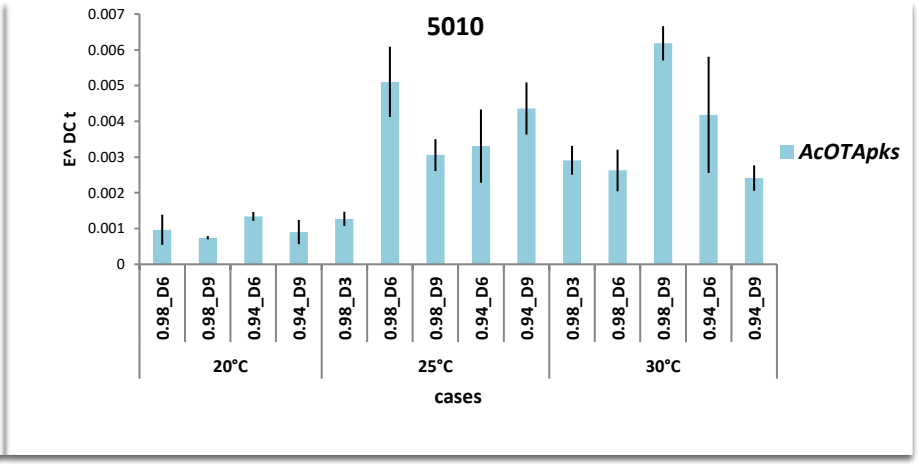


Fig. 4.4 Effect of temperature and a_w on transcript levels of: AcOTA*nrps* (**A**); AcOTA*pks* (**B**); and *laeA* (**C**) genes of *Aspergillus carbonarius* Ac29 through time. Error bars indicate standard error of three replicates. In the case of absence of fungal growth at Day 3, no values are displayed.

A.



B.



C.

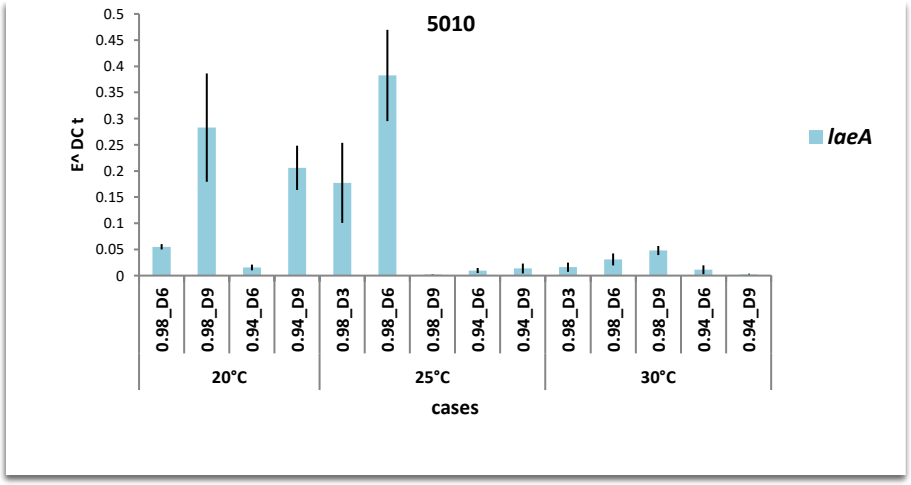


Fig. 4.5 Effect of temperature and a_w on transcript levels of: AcOTA*nrps* (**A**); AcOTA*pks* (**B**); and *laeA* (**C**) of reference strain *Aspergillus carbonarius* ITEM 5010 through time. Error bars indicate standard error of three replicates. In the case of absence of fungal growth at Day 3, no values are displayed.

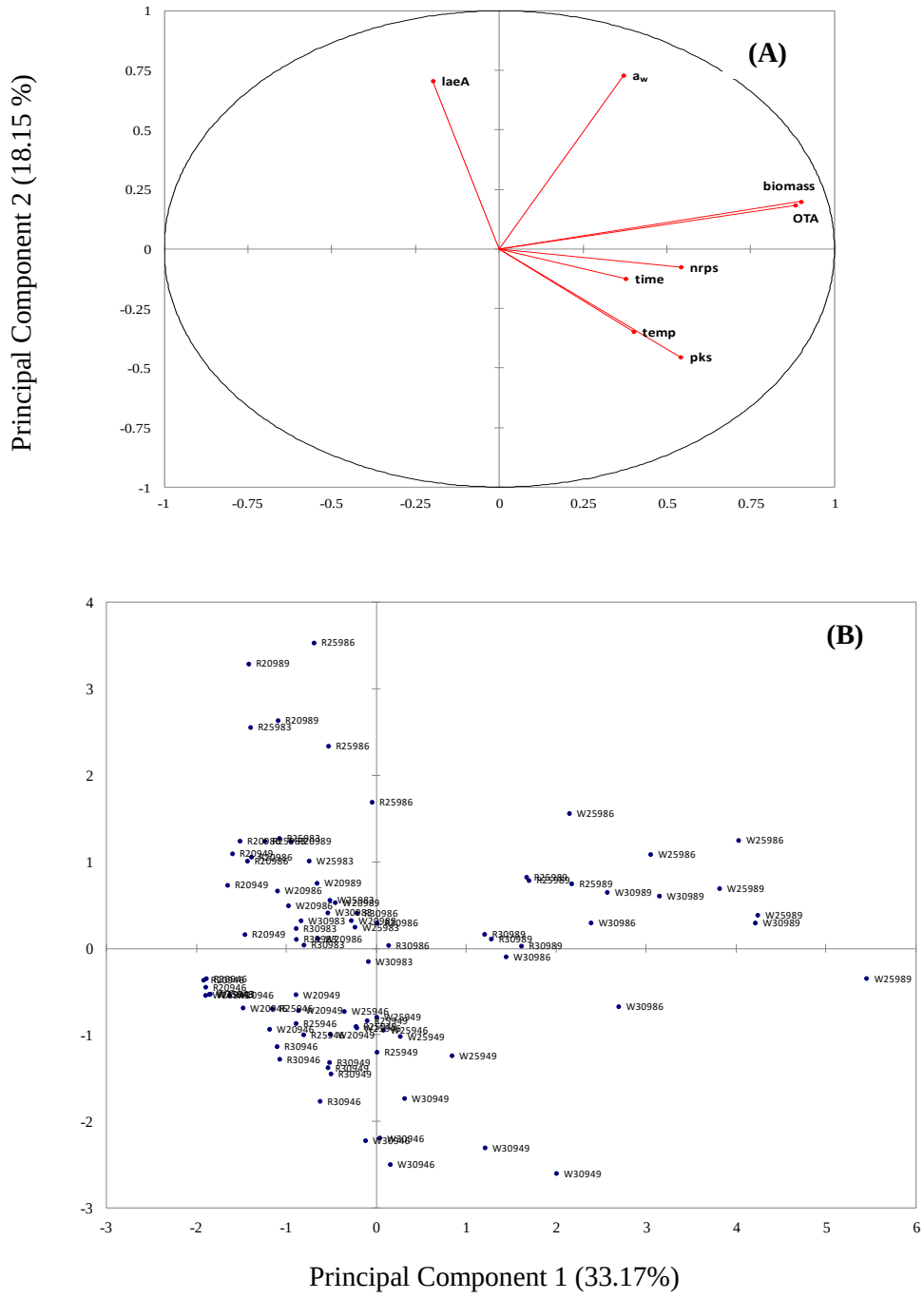


Fig. 4.6 Plot of: variables (**A**); and cases (**B**) of the principal components analysis illustrating the correlation between a_w, temperature, fungal biomass, OTA production and temporal expression of *Aspergillus carbonarius* OTA related genes by Ac29 and reference strain ITEM 5010, grown on SGM. (Cases are coded as follows: W and R correspond to wild and reference fungal strain, respectively; the following two numbers correspond to incubation temperature; the next two digits indicate a_w level; and the last digit incubation time).

It is also notable that the vectors for AcOTA*nrps* and AcOTA*pks* gene expression are located in diagonally opposed quadrants in relation to *laeA* gene expression, meaning that, when the former two genes are up regulated, the latter gene is down regulated. Finally, the vector for time is correlated to AcOTA*nrps*, and AcOTA*pks* genes, indicating higher expression of these genes with the course of time.

The distribution of the samples on the plane formed by the first two PCs is illustrated in Fig. 4.6B. The wild strain Ac29 is mainly located in the right part of the plot, especially for the cases characterized by high a_w (0.98) and high incubation time (nine days) at 25 and 30 °C. Moreover, by taking into account the plot of variables (Fig. 4.6A), it is clear that the fungus Ac29 grown at these conditions is associated with higher biomass and OTA production and higher expression of the AcOTA*pks* and AcOTA*nrps* genes. The remaining cases for Ac29 corresponding to low a_w (0.94) regardless of temperature and time as well as those cases associated with 20 °C irrespective of a_w and time are located in the left part of the plot together with the reference strain without presenting a clear pattern. Certain cases of the ITEM 5010 located in the upper left quadrant of the plot, corresponding to high a_w (0.98), 20 °C and 25 °C and diverse incubation times, are associated with the expression of the *laeA* gene that seems to present high expression at these conditions.

As reported above, AcOTA*nrps*, AcOTA*pks* and *laeA* have been systematically correlated with OTA production. However, it should be highlighted that there are no previous studies investigating the combined effect of different temperature regimes, a_w levels and time on the transcriptional responses of *A. carbonarius*. In the current work, the focus was given on two structural genes localized in the ochratoxigenic biosynthetic cluster, and one global regulator. In general, there are limited studies elucidating the expression of toxigenic related genes with different ecophysiological parameters.

Specifically, the association between temperature, a_w and transcript profiles concerning mycotoxin production has already been published (Magan and Medina, 2016; Medina et al., 2013). In the present work, even though a_w was the key factor affecting OTA production, this was not observed with expression patterns, where temperature seemed to act as the key factor influencing transcript levels. These results are in accordance with Yu et al. (2011) who reported that temperature is a modulator of mycotoxin production, such as aflatoxins, indicating that high temperature negatively affects aflatoxin production by turning down transcription of the two key transcriptional regulators. However, mixed responses of different regulation between evaluated parameters occurred elsewhere. Recently, Gallo et al. (2016) also observed that temperature was correlated to the induction of expression of structural biosynthesis genes, but not to that of aflatoxin regulatory genes. Rocha et al. (2015) also reported weak association between fumonisin production by *Fusarium verticillioides* and some of FUM genes expression levels. In another work, it was found that FUM2 and FUM21 gene expression levels were slightly affected by modification of a_w whereas temperature was the main controlling factor in fumonisin B_1 production by two strains of *Fusarium verticillioides* (Lazzaro et al., 2012). Furthermore, the profiles of gene expression of both toxigenic strains analyzed were quite different. These differences on gene regulation indicate the importance of strain variability in these experiments, since the majority of studies usually investigate the behavior of one fungal strain.

The regulatory mechanism underlying OTA biosynthesis is not completely understood yet, possibly due to its complexity through different levels of regulation. This mechanism could act within the biosynthetic cluster or external to it and its phenotypic expression is likely to subordinate to other regulatory processes acting at post-transcriptional level. It is also notable that translational or post translational control may

also occur in a co-regulation mechanism of the genes studied (Sonenberg et al., 2009). Growth conditions that favor the expression of OTA biosynthetic genes do not always result in OTA biosynthesis by fungi. This could suggest that the influence of abiotic factors is mediated via induction of transcription of ochratoxin A biosynthetic genes. In fact, production of fungal secondary metabolites could be affected by numerous signals of inhibitory or promotional effect on the regulatory systems. Patterns in transcript biosynthesis are not necessarily correlated with phenotypic metabolites produced.

Finally, gene expression was assayed by RT-qPCR in relation to abiotic factors known to influence ochratoxin A biosynthesis. Molecular measurements are more sensitive compared to the analytic quantification of OTA, indicating the successful implementation of analysis of transcriptome response. Black aspergilli are normally present in vineyards, but OTA is not always detected even though it can be quantified at very low concentrations (Battilani et al., 2003). In this regard, RT-qPCR provides very useful information to relate molecular changes under different ecological conditions in a rapid and convenient way.

Conclusions

The influence of the environmental factors of temperature and a_w has been studied in this work and different profiles were observed in response to time concerning OTA and gene expression levels by *Aspergillus carbonarius.* The expression of OTA key biosynthetic genes during growth on SGM at different environmental conditions was successfully assessed, since transcripts were detected in all cases. Early activation of both OTA biosynthetic key genes confirmed the predictive nature of RT-qPCR analysis, since molecular indicator genes were expressed a few days before any OTA could be detected. High variation between the wild fungal isolate Ac29 and the reference strain

ITEM 5010 has been observed underlying the significance of intra-species variability. Temperature and a_w were involved in the transcription process of the specific genes for mycotoxin production. Stimulation of genes and the observed transcript levels may suggest a different regulation action evolved. This indicates that expression is possibly closely related with control at the post-transcriptional level and requires further investigation. Even though OTA and biomass appeared to have a constant trend with the environmental parameters under study, a determined pattern could not be clearly established between transcript levels and toxin production. Results constitute a first indication on the responses in different ecophysiological environments, highlighting the complexity of regulation mechanisms at gene expression level of *A. carbonarius* and suggesting further research on the fine regulation of gene expression.

CHAPTER 5

In vitro evaluation of essential oils against *Aspergillus carbonarius* isolates and their effects on Ochratoxin A related gene expression in synthetic grape medium.

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Abstract

Clove, mandarin, cinnamon and lemongrass essential oils (EOs) were tested for their efficacy against 2 wild isolates (Ac29, Ac28) and a reference strain (5010) of *Aspergillus carbonarius*. Two different assays were performed on Malt Extract Agar (MEA) and Czapek Yeast Extract Agar (CYA) solid cultures, as well as on synthetic grape medium (SGM) liquid cultures. The impact of EOs on fungal growth and toxin production was measured after 7 days of incubation at 25 ºC. Furthermore, the effect of clove, lemongrass and mandarin EOs on *A. carbonarius* OTA related genes *Ac*OTA*nrps*, *Ac*OTA*pks* and *laeA* was investigated by relative expression using Real Time PCR on SGM cultures. Results showed that in solid cultures, complete fungal inhibition was obtained at 100-300 μL/L of EOs revealing a dose dependant effect of clove, cinnamon and lemongrass, whereas mandarin was proved to be less effective. EOs could not completely inhibit OTA production but reduce it by 15-98% depending on the type and concentration of EO, even though in certain cases of low concentrations, EOs showed to enhance toxin production. In liquid cultures, a major effect on growth inhibition and OTA reduction was observed for all EOs. Regarding gene expression, down regulation of *Ac*OTA*nrps* was observed in the majority of treatments that could be associated with toxin reduction. Moreover, *Ac*OTA*pks* exhibited some differential expression but *laeA* did not show any differences in the transcriptional profile. The above gene expression results may indicate a possible mode of action of EOs in OTA biosynthetic pathway.

5.1 Introduction

The ability of different filamentous fungal species to excrete mycotoxins has drawn the attention of the scientific community as a serious potential health issue. Their presence as natural contaminants of agricultural products is still a major food safety problem.

Research on mycotoxin contamination is a major priority for scientists worldwide, since the specific toxins are stable and resistant to heat or acidic environment and could remain in the food chain during distribution and storage for a long period causing serious safety issues. *Aspergillus* genera belong to the most important fungal group, as they can biosynthesize toxic secondary metabolites. *Aspergillus carbonarius* has been defined as one of the most important opportunistic pathogen in grapes, since it has the highest incidence of ochratoxin-producing isolates within the *Aspergillus* group (Stefanaki et al., 2003) and high potential of toxin production. Ochratoxin A (OTA) is considered to be a prevalent and most toxic mycotoxin of the ochratoxins group, since it is rated as potential carcinogen (group 2B) by the International Agency for Research on Cancer (IARC, 1993), nephrotoxic (JECFA, 2008) with immunotoxic and hepatotoxic impact to humans and animals. Mycotoxins may reach consumers by direct contamination of plant material or carry over their metabolites to food products. The European Union has established strict legislation for many products, including grapes, concerning the use of chemicals and maximum residue levels of pesticides (MRLs). Therefore, over the last few decades the interest of scientists has been focused on alternative applications of natural antimicrobials to control fungal growth on grapes and grape products.

It has been demonstrated that essential oils (EOs) contain diverse bioactive components that prevent moulds and their toxic metabolites, having at the same time the advantage of non-phytotoxicity and biodegradation. The Federal Regulation Code has recognized, EOs of clove, cinnamon, lemongrass, etc., as safe (GRAS) in the United States (U.S. Code of Federal Regulation, 2012). Several experiments with EOs have reported that clove, cinnamon and lemongrass are promising candidates for the inhibition of foodborn pathogens and spoilage microorganisms (Soliman and Badeaa, 2002; Tian et al., 2012). There are also reports on the inhibition of spore formation in *Aspergillus* species by

lemongrass, cinnamon and clove (Paranagama et al., 2003; Pawar and Thaker, 2006), spore germination (Tzortzakis and Economakis, 2007), or interaction with the cells of hyphae as reported for *Citrus sinensis* on *A. niger* (Sharma and Tripathi, 2006). Low molecular weight and highly lipophilic compounds of EOs pass easily through cell membranes and disrupt the fungal cell. On the other hand, regarding the effect of EOs on mycotoxin biosynthesis there are studies indicating actions like inhibition of lipid peroxidation during aflatoxin B1 formation (Bluma et al., 2008). Moreover, a gene expression study has reported that a-terpinen affected genes involved in ergosterol biosynthesis in *Saccharomyces cerevisiae* (Parveen et al., 2004).

OTA is a polyketide mycotoxin linked to the amino acid phenylalanine. This molecular structure indicates that enzymatic reactions are needed for metabolite biosynthesis and even today this pathway has not been completely elucidated. However, a number of putative pathways have been proposed and recently the role of two genes has been revealed, namely NRPSs and PKSs that have been confirmed to be key enzymes of OTA biosynthesis gene cluster (Gallo at al., 2009, 2012, 2014). Moreover, LaeA protein has been reported to govern secondary metabolites production on *Aspergillus* genome (Bow and Keller, 2004) and recently *laeA* gene has been correlated with the regulation of OTA biosynthesis in *A. carbonarius* (Crespo-Sempere et al., 2013).

During the last decade many studies have examined the ecophysiology of *A. carbonarius*, however very few deal with agents for growth inhibition and toxin control such as gallic acid, *Matricaria chamomilla* essential oil, natamycin and pine resin (Romero et al., 2010; Tolouee et al., 2010; Kogkaki et al., 2015). Moreover, there are few publications on the use of EOs as anti-ochratoxigenic agents (Santos et al., 2010; Hua et al., 2014) and even less on their impact on the growth and toxin production of *A. carbonarius* (Garcia-Cela et al., 2011; Passone et al., 2012).

Thus, the objective of this work was to determine the efficacy of the EO of clove, mandarin, cinnamon and lemongrass in the control of *A. carbonarius* growth in different media and investigate any potential influence on the expression of OTA related genes. To our knowledge this is the first report on EOs which along with intra-specific OTA production investigates also their effect on *A. carbonarius* gene expression.

5.2 Materials and methods

5.2.1 Essential Oils

Four EOs were evaluated in the present study, namely *Eygenia cariophyllus* (clove oil, 87% eugenol), *Citrus reticulata* (mandarin, 89% limonene), *Cinnanomum cassia* (cinnamon, 78% e-cinnamaldehyde) and *Cymbopogon citratus* (lemongrass, 45% geranial, 25% neral) purchased from Pranarôm Intenational S.A. (Ghislenghien, Belgium). The concentration of the main active compounds for each EO was provided by the manufacturer.

5.2.2 Fungal isolates and sample preparation

Two different wild isolates of *A. carbonarius* (Ac28 and Ac29) from the fungal collection of the Laboratory of Microbiology and Biotechnology of Foods (LMBF) of the Agricultural University of Athens were used in this work. The fungi have been previously isolated from grapes, characterized by molecular methods (Kizis et al., 2014) and their OTA potential has been reported elsewhere (Lappa et al., 2015a). In addition, a strain of *A. carbonarius* ITEM 5010 kindly provided by Prof. Tsitsigiannis from the Laboratory of Phytopathology of the same University was used as reference. The study was performed on 3 different growth media: (a) Malt extract agar (MEA, malt extract, 20 g; peptone, 1 g; glucose, 20 g; bacteriological agar, 20 g; distilled water, *c.* 1000 ml) which favors fungal

sporulation, (b) Czapek Yeast extract Agar (CYA, SD, Oxoid) which is recommended for the determination of OTA in *A. carbonarius* (Bragulat et al., 2001), and (c) Synthetic Grape Juice Medium (SGM) that simulates grape composition between véraison and ripeness (Delfini, 1982). The pH of the SGM was adjusted to 3.8 with 2M KOH. MEA and CYA were used for the preparation of solid media whereas SGM for liquid media. Spore suspensions of each *A. carbonarius* isolate were prepared by collecting spores from 7-day old fungal colonies grown on MEA at 25 ^{o}C. Conidia were harvested from sub-cultures in an aqueous solution of 0.01% Tween 80 (Merck, Schuchardt, Germany) by scraping the surface of the mycelium with a sterile glass rod. The final spore suspensions were assessed using a haemocytometer (Brand, Wertheim, Germany) and adjusted by appropriate dilutions to approximately 10^6 spores/mL. EOs were initially dissolved in sterile Tween 80 (0.01% aqueous solution) that was used as a nonionic emulsifier to facilitate dispersion into the media (Fu et al., 2007). Further on, EOs were individually diluted in autoclaved media under constant agitation, dispersed into petri dishes, and allowed to solidify within 5 min. Plates containing 50 - 1000 μL/L of EOs (Table 5.1) were prepared by adding the respective amount of EO directly into the autoclaved medium that was further centrally single spotted with 10^3 spores on the surface. All plates were sealed using Parafilm to minimize desiccation of the medium and loss of the EO. For gene expression studies, SGM was used in shaking flask cultures. Specifically, conidia were inoculated into 100 ml Erlenmeyer flasks containing 50 mL of the medium supplemented with 50-100 μL/L of EOs and sealed with Parafilm (Table 5.1). Incubation was carried out for 7 days at 25 ^{o}C in a rotating shaker at 100 rpm. Control samples (i.e., cultures without EOs) were also used. Cultures showing no growth were incubated for 7 more days and then transferred to plates without EO to determine fungicidal or fungistatic effects.

Table 5.1: Concentrations of essential oils employed in the experimental work.

Essential Oils	Concentrations (µL/L)	
	SOLID MEDIA	LIQUID MEDIA
Lemongrass	50, 100, 200, 300	50, 75, 100
Clove	50, 100, 200, 300	50, 75, 100
Cinnamon	50, 75, 100, 150	50, 75, 100
Mandarin	400, 700, 1000	400, 700, 1000

For kinetic studies in solid media cultures, colony radius was observed on a daily basis and recorded at right angles with the aid of a ruler, while for liquid cultures mycelium was harvested from broth and fungal biomass weighed. The growth of fungal cultures containing different concentrations of EOs was compared with the control culture. All the assays for fungal growth and OTA production were replicated in triplicate.

5.2.3 Growth assessment

At the beginning of this work, preliminary tests were undertaken to select the appropriate concentration range for each EO in solid and liquid media. Specifically, isolate *Ac*28 was employed in establishing the concentration range of EOs according to growth inhibition results. The interaction between fungal mycelia and EOs was observed through growth changes over time and inhibition was recorded as mycelial diameter reduction over time. It must be noted that mandarin essential oil did not show any inhibition effect at 300 µL/L as the other EOs and thus higher concentrations (400, 700, and 1000 µL/L) were evaluated. Moreover no growth inhibition was observed for this EO at CYA therefore no further experiments were undertaken for OTA production in this medium. Percentage of inhibition (%) was calculated on day 7 for growth and OTA production as follows:

$$Inhibition\ (\%) = (1 - \frac{T}{C}) \cdot 100 \quad (1)$$

where T is the diameter of fungal mycelium treated with EO or OTA concentration at day 7 and C is the respective value in the control sample (i.e., treatment without EO).

Based on the antifungal effects observed in solid media from a previous work (Lappa et al., 2015b), it was decided to investigate further the anti-ochratoxigenic action of EOs at gene expression level. For this assay, a specific medium which simulates grape juice was used along with the two wild isolates of *A. carbonarius* (Ac28 and Ac29) from grapes. For this task, preliminary experiments showed that a good yield of RNA could not be achieved in solid cultures, due to conidial melanin produced after the mycelium started to sporulate (approximately on day 3 of incubation). On the contrary, evaluation of shaking flask cultures showed limited conidiation, achieving thus high yield of RNA for gene expression studies.

5.2.4 Ochratoxin A detection

For solid cultures, OTA production was assessed according to the method of Bragulat et al., (2001). Specifically, six agar plugs (6 mm diameter each) were removed across the diameter of the mycelium by means of a sterile cork borer, weighed into a 1.5 mL eppendorf tube and extracted with 1 mL of 100% methanol. After shaking the tubes for 5 min in a rotating shaker, they were left still for 1 h and then filtered through a 0.2 μm syringe-driven filter unit (Millex, Millipore Co., Bedford, Mass.). For liquid cultures, the whole content of each flask was analyzed for toxin determination. Specifically, each sample of 50 mL was homogenized using an Ultra Turax homogenizer (Heidolph Instruments, Schwabach, Germany) for 1 min at the highest speed ($23x10^3$ rpm) and 5 mL of each homogenate was mixed with 5 mL of 100% methanol for 30 min. Extracts were filtered through a Whatman No 2 filter paper to remove any suspended solids and

then through a 0.2 μm syringe-driven filter unit (Millex, Millipore Co., Bedford, Mass.). All extracts were stored at -20 °C until HPLC analysis.

OTA was detected using an HPLC system equipped with a JASCO LC-Net II/ADC system controller, a JASCO AS-2055 Plus auto sampler, with a Model PU-980 Intelligent pump, a Model LG-980-02 ternary gradient unit pump and an FP-2020 Plus fluorescent detector (JASCO Inc., Easton, USA). The analysis was performed under isocratic conditions at a flow rate of 1 mL/min of the mobile phase (water/acetonitrile/acetic acid; 49.5/49.5/1) through a Waters spherisorb C18 analytical column, 5μm ODS2 (4.6 x 250 mm) (Resteck Co., Pinnacle II, Bellefonte, USA). Injection volume was 10 μL and run time for samples was 20 min with OTA detected at about 11 min. The detection limit of the analysis was 1 ng/g. Recovery assay for OTA extraction method was performed by spiking known concentrations of OTA (50 and 500 ng/mL) on media with each EOs providing satisfactory results (80-110%).

5.2.5 Gene expression assay

Mycelia were collected from liquid SGM, washed with ddH_2O, dried in Whatman No 2 paper, flash freezed in liquid Nitrogen (lN_2) for stabilization of expression, and stored at -80 °C until extraction. Frozen tissues were lyophilized for 24h and approximately 10 mg of fungal mycelium were used for nucleic acid extraction. Invitrogen, PureLink RNA mini kit was used for RNA isolation according to manufacturer's protocol and Trizol (Ambion, USA) was used as the phenol for RNA extraction. Samples were also treated with Turbo DNase (Ambion, USA) to eliminate possible trace amounts of contaminating genomic DNA according to kit instructions. Further on, the quality of RNA samples was checked through a qPCR amplification to ensure absence of genomic DNA. Nucleic acid quantification was assessed spectophotometricaly by measuring the

absorbance at 260 nm. Moreover, the purity of samples was evaluated using a NanoDrop spectrophotometer (IMPLEN, Germany) through the $A_{260\ nm}$/ $A_{280\ nm}$ ratio and RNA integrity was verified on 3% agarose ethidium-bromide staining gel. First-strand cDNA was synthesized using PrimeScript Reverse Transcriptase (Takara, Japan) according to manufacturer's instructions in a 20 μL final reaction volume. RNA used was 0.5 μg for each sample. For priming Random Hexamers (Invitrogen, USA) was used. Reaction mixture was incubated for 5 min at 65 ^{o}C and the specific set up program was 30 ^{o}C for 10 min, 42 ^{o}C for 60 min, and 70 ^{o}C for 15 min.

For the relative quantification assay, Real Time PCR was used to amplify AcOTApks (polyketide synthase) and AcOTAnrps (non ribosomal peptide synthase) genes as target genes, and also *laeA* gene as a global regulator for secondary metabolites in Aspergillus spp. Constitutive expressed β-tubulin gene served as internal reference for gene expression normalization because this is a widely used housekeeping gene in relevant studies (Gallio et al., 2012; Crespo-Sempere et al., 2013). New primers were designed for the gene pks on the basis of a recently published work (Gallio et al., 2014) referring to A. carbonarius (Gene Bank Accession no. GW327951). Homologous genes have been searched through the genome database of the genus *Aspergillus* (http://www.aspgd.org) for *Acar5010-173482* using the BLAST algorithm. The sequence of the fungal genes was recovered by the Joint Genome Database Institute. OligoPerfect Designer Software ™ (Invitrogen, USA) and AspGDweb-primer were employed to determine possible pairs of oligonucleotides following standard primer design criteria for use in Real Time PCR reactions. Nucleotide sequences of primers used in the qPCR assays are shown in Table 5.2.

Table 5.2: Nucleotide sequences of primers for qPCR assay

Primer pair	Gene	Nucleotide sequences 5'—3'	Reference
F-pks	*AcOTApks*	GTC AAG GTC GGG TGC TAC AA	Present study
R-pks		TCG GAA TGA TAC GCG ACT TT	
F-nrps	*AcOTAnrps*	CTC CAC CCA TCC TCC CGT TC	Crespo Sempere et al. (2013)
R-nrps		AAT CCA TGT CCT CAC CAT CGC	
F-laea	*laeA*	CAC CTA TAC AAC CTC CGA ACC AC	Crespo Sempere et al (2013)
R-laea		GGT TCG GCC AAC CGA CGA CGC TG	
F-tubβ	*β-tubulin*	CGC ATG AAC GTC TAC TTC AAC GAG	Crespo Sempere et al. (2013)
R-tubβ		AGT TGT TAC CAG CAC CGG ACT	

qPCR efficiency tests for each primer were performed under the specific experimental conditions and evaluated as 96% for *AcOTApks*, 101% for *AcOTAnrps*, 98% for *laeA* and 94% for β-*tubulin* genes, respectively.

Real time PCR experiments were conducted in 96-well plates in an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, USA) using the SYBR Green chemistry to monitor cDNA amplification. qPCRBioSyGreen Mix High-RoxRox (PCR BIOSYSTEMS, UK) was used in a 10 μL reaction. Optimization of the amount of primers and template was also performed. Cycling conditions according to the specific SYBR Green protocol were: 95 ^{o}C for 0.5 min, 60 ^{o}C for 0.25 min, and 72 ^{o}C for 0.25 min [40 cycles]. Melting curve analysis of the PCR products was performed by heating to 95 ^{o}C for 0.15 sec and 60 ^{o}C for 1 min, with continuous measurements of the fluorescence to verify the PCR product. Template-free negative controls were also used at every run. Data analysis was assessed by StepOnePlus RT-PCR System Software v2.1 and expression levels were determined according to Pfaffl method (Pfaffl, 2001, 2004) using the equation:

$R = \mathbf{E}_{\mathbf{target}}^{\Delta \mathbf{Cttarget\ (control\text{-}sample)}} / \mathbf{E}_{\mathbf{ref}}^{\Delta \mathbf{CtrefC\ (control\text{-}sample)}}$. Data were converted to relative expression and presented in log2 values as fold changes (Kubista et al., 2007). The above method calculates the expression ratio of a target gene between a treated with EO sample

and a control sample ('relative calibrator'). Gene expression measurements were considered to be comparable since equal amounts of RNA were used as template and also reaction conditions were the same in all qPCR assays. Gene expression assays were replicated in duplicate.

5.2.6 Data analysis

Analysis of variance (ANOVA) was performed allowing an overview of all the results, determining differences and establishing significant effects between transcription levels and EOs treatments for fungal isolates. The data set was analyzed by the statistical package JMP8 (SAS Institute INV., Cary, NC, USA). Moreover, multiple linear regression (MLR) was employed to investigate any underlying relationship among the different variables and treatment effects through XLSTAT ver. 2014.2.03 software (Addinsoft, Paris, France). The model for multiple linear regression is given by the following formula:

$$Y = a_0 + a_1 \cdot X_1 + a_2 \cdot X_2 + \cdots + a_i \cdot X_i \qquad (2)$$

where Y is the dependent variable, X are the independent or predictor variables, and $\alpha_0 \ldots \alpha_i$ are parameters to be estimated. In our case, the dependent variable (Y) was the diameter of the fungal mycelium or OTA production after 7 days of incubation for the different EOs assayed, and the independent variables (X) were the type of the EO (clove, cinnamon, lemongrass, mandarin), the concentration of the EO (100-300 μL/L), the growth medium (MEA or CYA), and the fungal strain (Ac28, Ac29, and 5010). In the case of liquid cultures in SGM the growth was expressed as fungal biomass after 7 days of incubation. The relative contribution of the independent variables on fungal growth and OTA production was graphically illustrated by taking into account the standardized (beta) coefficients of the linear model.

5.3 Results

5.3.1 Inhibitory effect of EOs on A. carbonarius growth

The growth of fungal isolates on different solid media in the presence of EOs is shown in Fig. 5.1. Antifungal effects differed with the type and concentration of EO, growth medium and fungal isolate. The statistical analysis revealed significant effects ($p<0.0001$) of treatments in terms of fungal growth. EOs and concentration factors presented the highest impact ($p<0.0001$) followed by the growth medium ($p=0.005$). As shown in Fig. 5.2A, cinnamon proved to have the greatest effect ($p<0.001$) among the EOs tested. Clove, cinnamon and lemongrass EOs could significantly inhibit the growth of *A. carbonarius* in both MEA and CYA at 50-300 μL/L compared to the control (absence of EOs), whereas the EO of mandarin was proved to be the least effective. In both MEA and CYA media, *A. carbonarius* growth was totally inhibited by clove and lemongrass at 300μL/L for all fungal isolates. Clove EO presented very high dose-related impact on fungal growth. Specifically, growth inhibition in MEA for Ac29 was estimated to 21%, 40%, and 72% at 50, 100, and 200 μL/L, respectively. For the same concentrations of clove, strains Ac28 and 5010 showed growth inhibition of 15%, 34%, and 76%, as well as 8%, 35%, and 73%, respectively. This gradual decrease was also observed in CYA cultures and the effect of growth inhibition was more pronounced in CYA compared to MEA for all concentrations of EOs assayed. The highest effect of clove EO was observed for isolates 5010 and Ac28 where total inhibition occurred at 100 and 200 μL/L, respectively, whereas Ac29 was not inhibited even at 300 μL/L. In MEA, lemongrass EO presented inhibition for Ac29 of 1%, 10% and 58% at 50, 100 and 200 μL/L, respectively. Moreover, Ac28 was also inhibited at 3%, 8%, and 72% as the concentration of lemongrass EO progressively increased, similarly with 5010, where the respective inhibition rates were 3%, 13%, and 55%. In the case of CYA medium higher

percentages of fungal growth inhibition were observed in all concentrations of lemongrass EO compared to MEA. The effect of selected EOs on *A. carbonarius* growth in solid cultures is exemplified in Fig.s 5A,B.

A

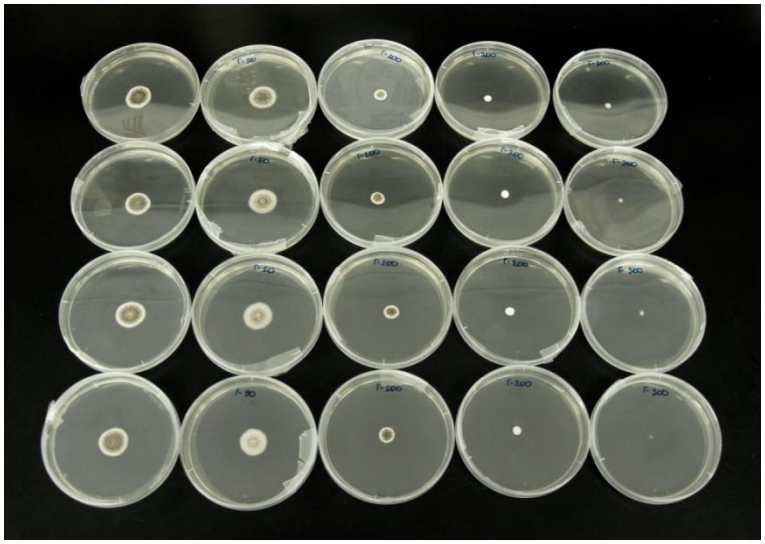


Fig. 5A. Inhibition of *A.carbonarius* growth by clove oil in CYAmedium in the 3rd day of incubation.

B

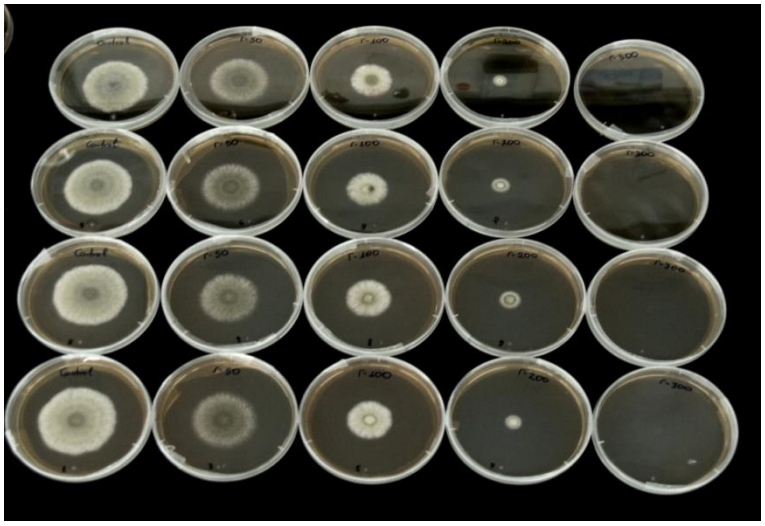
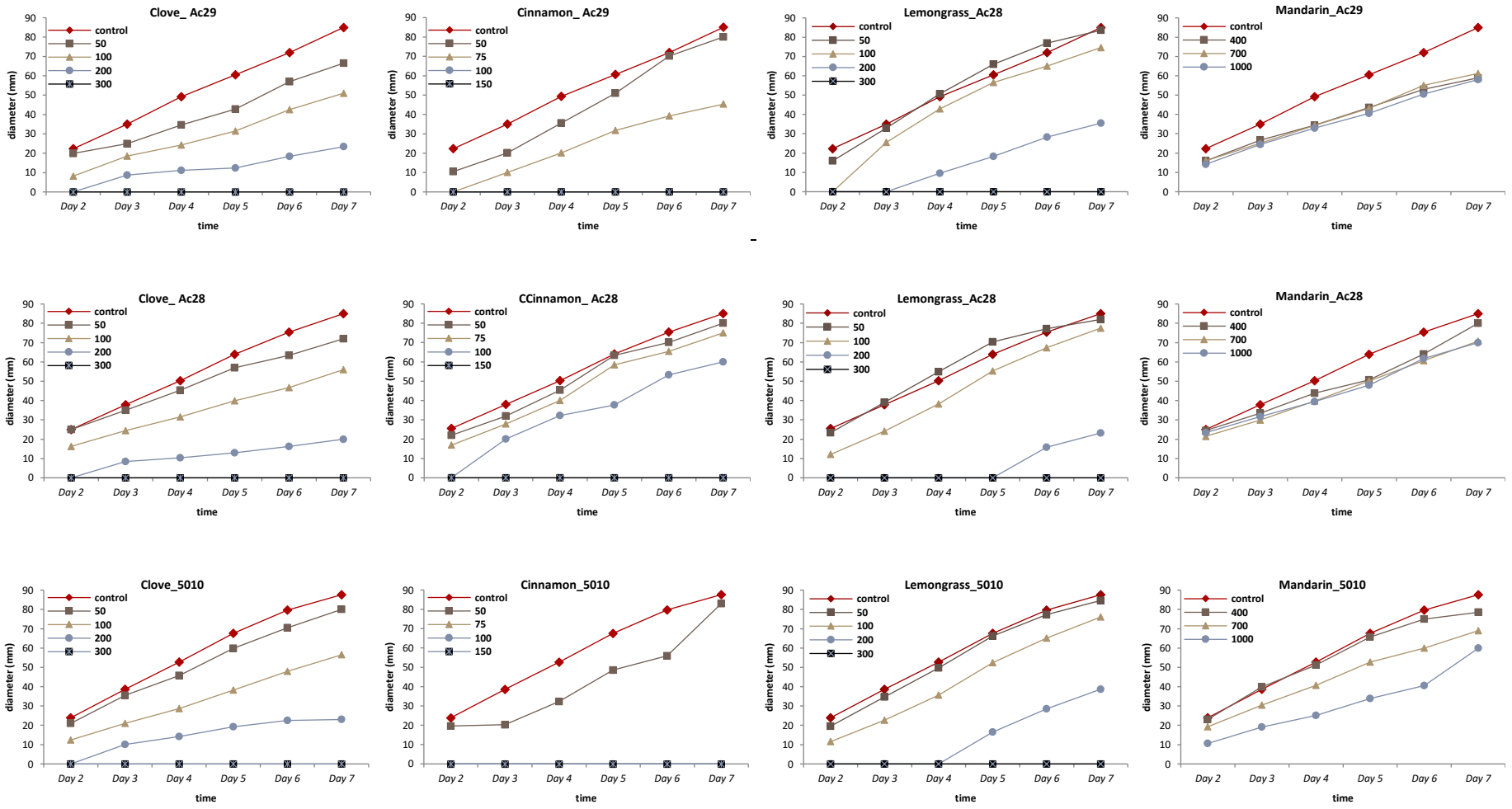


Fig. 5B . Inhibition of *A.carbonarius* growth by clove oil in MEA medium at the 5th day of incubation

A.


Clove_ Ac29
control
50
100
200
300
Cinnamon_ Ac29
control
50
75
100
150
Lemongrass_Ac28
control
50
100
200
300
Mandarin_Ac29
control
400
700
1000
Clove_ Ac28
control
50
100
200
300
CCinnamon_ Ac28
control
50
75
100
150
Lemongrass_Ac28
control
50
100
200
300
Mandarin_Ac28
control
400
700
1000
Clove_5010
control
50
100
200
300
Cinnamon_5010
control
50
75
100
150
Lemongrass_5010
control
50
100
200
300
Mandarin_5010
control
400
700
1000
diameter (mm)
time
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7

B.

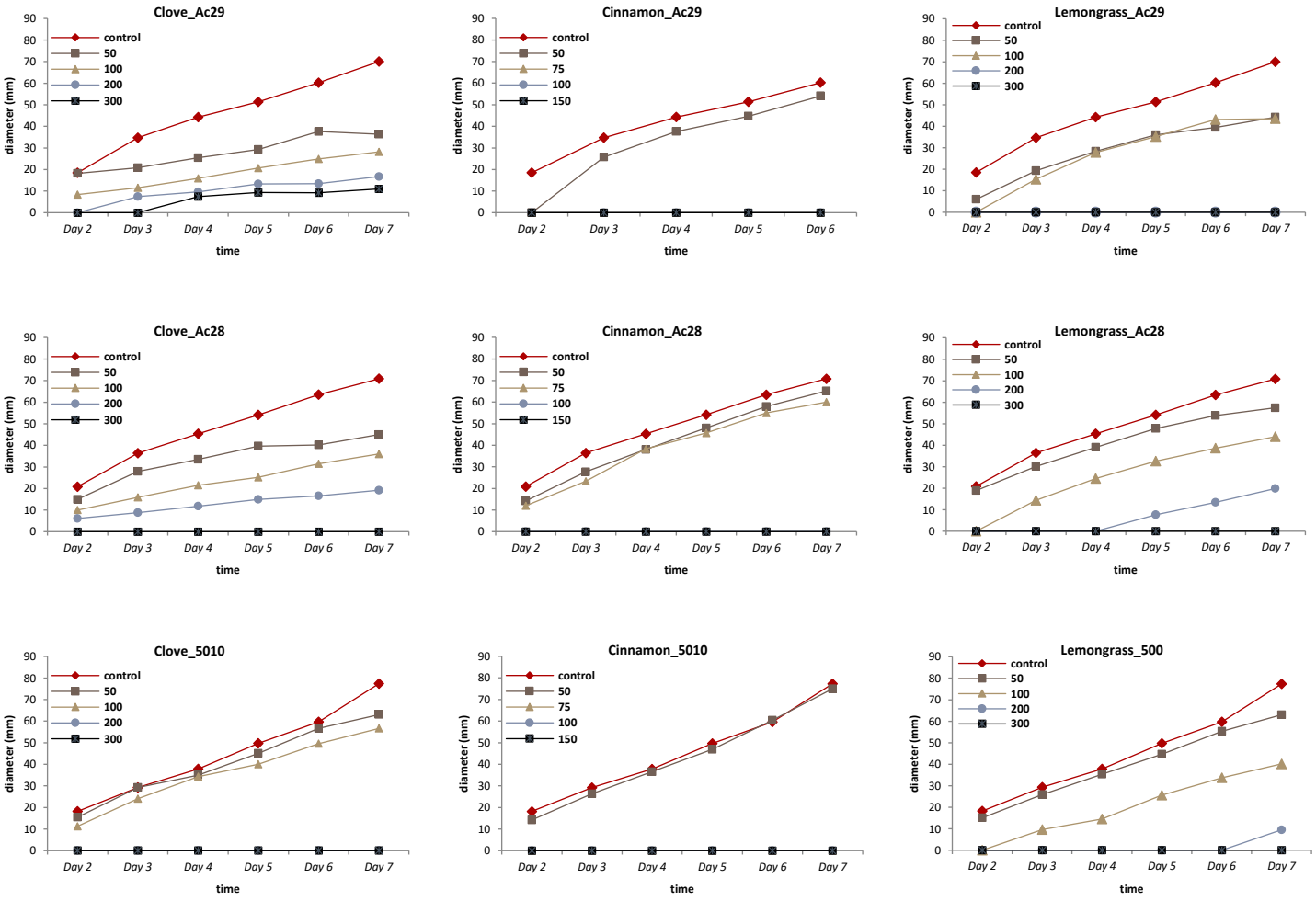


Fig. 5.1 Changes of growth of *Aspergillus carbonarius* isolates in solid cultures of MEA (A) and CYA (B) under the influence of EOs against incubation time. (standard deviation was < 5% of the respective value)

Cinnamon presented the highest antifungal effect among all EOs tested, since 100, 75, and 50 μL/L totally inhibited the growth of Ac28, 5010, and Ac29, respectively. Cinnamon EO showed also different effects between the two media employed to assess fungal growth, as in CYA inhibition occurred at lower concentrations for Ac29 and Ac28, but the opposite was observed for 5010 where lower concentrations resulted in very low inhibition of fungal growth. More specifically, in MEA 50 μL/L of cinnamon EO reduced diametric growth by *ca.* 5%, whereas at 75 μL/L the respective inhibition was 43% and 11% for Ac29 and Ac28, respectively. In CYA low levels of inhibition occurred at 50 μL/L concentration of EO resulting in 10%, 8% and 3% growth reduction for Ac29, Ac28 and 5010, respectively. However higher concentrations totally inhibited growth. These results may suggest a stronger effect of cinnamon EO in spore germination. Finally, mandarin EO, even though it was used in higher concentrations compared to other EOs, could not completely inhibit fungal growth even at 1000 μL/L. For strains Ac29 and Ac28 inhibition was observed at 30% and 17%, respectively, whereas strain 5010 presented a dose dependent effect since inhibition of 10%, 21%, and 30% was observed at 400, 700, and 1000 μL/L, respectively.

EOs of clove, lemongrass and mandarin were also evaluated in SGM liquid cultures and proved highly effective in fungal growth inhibition as expressed by changes in biomass (Fig. 5.3A). Statistical analysis revealed that clove and lemongrass EOs had the greatest impact (p = 0.005 and p = 0.001, respectively) on biomass reduction. Specifically, 50 μL/L lemongrass EO reduced fungal biomass by 64% and 48% in Ac29 and Ac28, respectively, whereas no growth was observed in higher concentrations. Clove and mandarin EOs showed a high dose dependent effect for both fungal isolates.

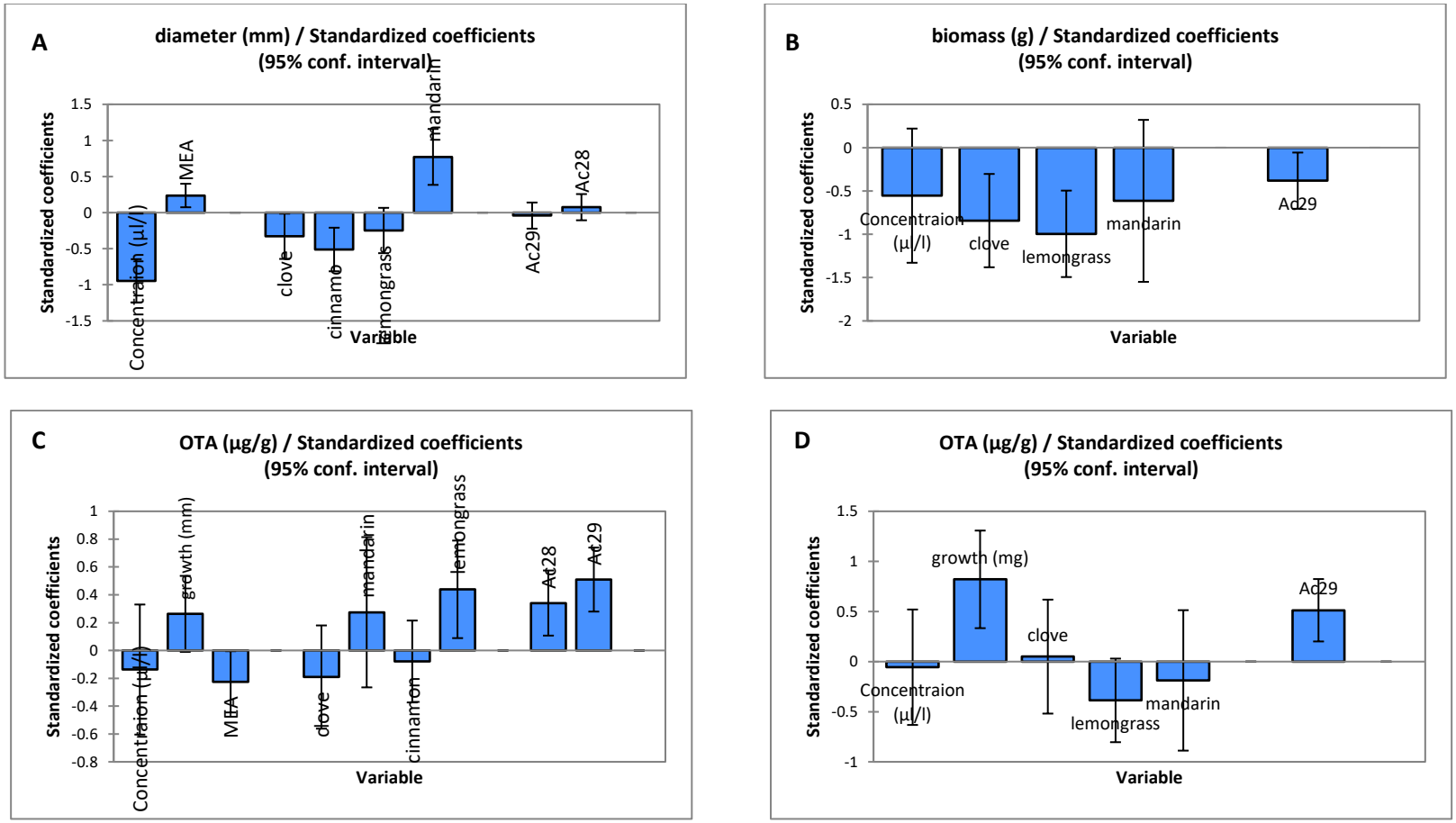


Fig. 5.2 Relative contribution (standardized coefficients) of different EOs, EO concentration, medium (MEA, CYA), and fungal strain on growth (A, B) and OTA production (C, D) as derived from multiple linear regression analysis. The asterisks indicate the level of *p*-value (*=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001).

Specifically, growth inhibition of Ac29 by clove EO was 39%, 76%, and 97% at 50, 75, and 100 μL/L, respectively, whereas for the same concentrations Ac28 presented a decrease in biomass of 22%, 46%, and 77%.Mandarin EO showed gradual inhibition in biomass growth of 75%, 86%, and 89% at 400, 700 and 1000 μL/L, respectively, for Ac29. The respective decrease for Ac28 was 47%, 60%, 76%. Moreover, evaluation of all concentrations of EOs showed an isolate dependent inhibition effect, indicating that Ac29 seemed to be most affected by the presence of all EOs (Fig. 5.2B).

5.3.2 Inhibitory effect of EOs on A. carbonarius OTA production

The effect of the different EOs and solid growth media on OTA production by the isolates of *A. carbonarius* is presented in Fig. 5.2. EOs showed the greatest impact regarding OTA ($p < 0.0001$) and Ac28 was the most affected isolate ($p < 0.0001$). Specifically, in MEA clove EO showed the highest OTA reduction even at low concentrations (Fig. 5.4A), namely 89%, 98%, and 98% for Ac29 and 82%, 59%, 72% for 5010 at 50, 100, and 200 μL/L, respectively. Clove EO was less effective but more dose dependent against Ac28, inhibiting OTA by 27%, 59%, and 72% as the concentration of EO progressively increased. In CYA growth medium (Fig. 5.1B) clove EO was more effective in isolates Ac29 and Ac28 decreasing OTA levels to 91% and 84% at 300 and 200 μL/L, respectively. On the other hand, stimulation of OTA production was observed for lemongrass EO in most cases, with the exception of the highest concentration (200 μL/L) where toxin reduction of 77% and 90% was observed on MEA for isolates Ac28 and 5010, respectively. In addition, for the same concentration of lemongrass EO, *A. carbonarius* 5010 showed an OTA reduction of 97% in CYA growth medium. Results from cinnamon EO treatments were also differential.

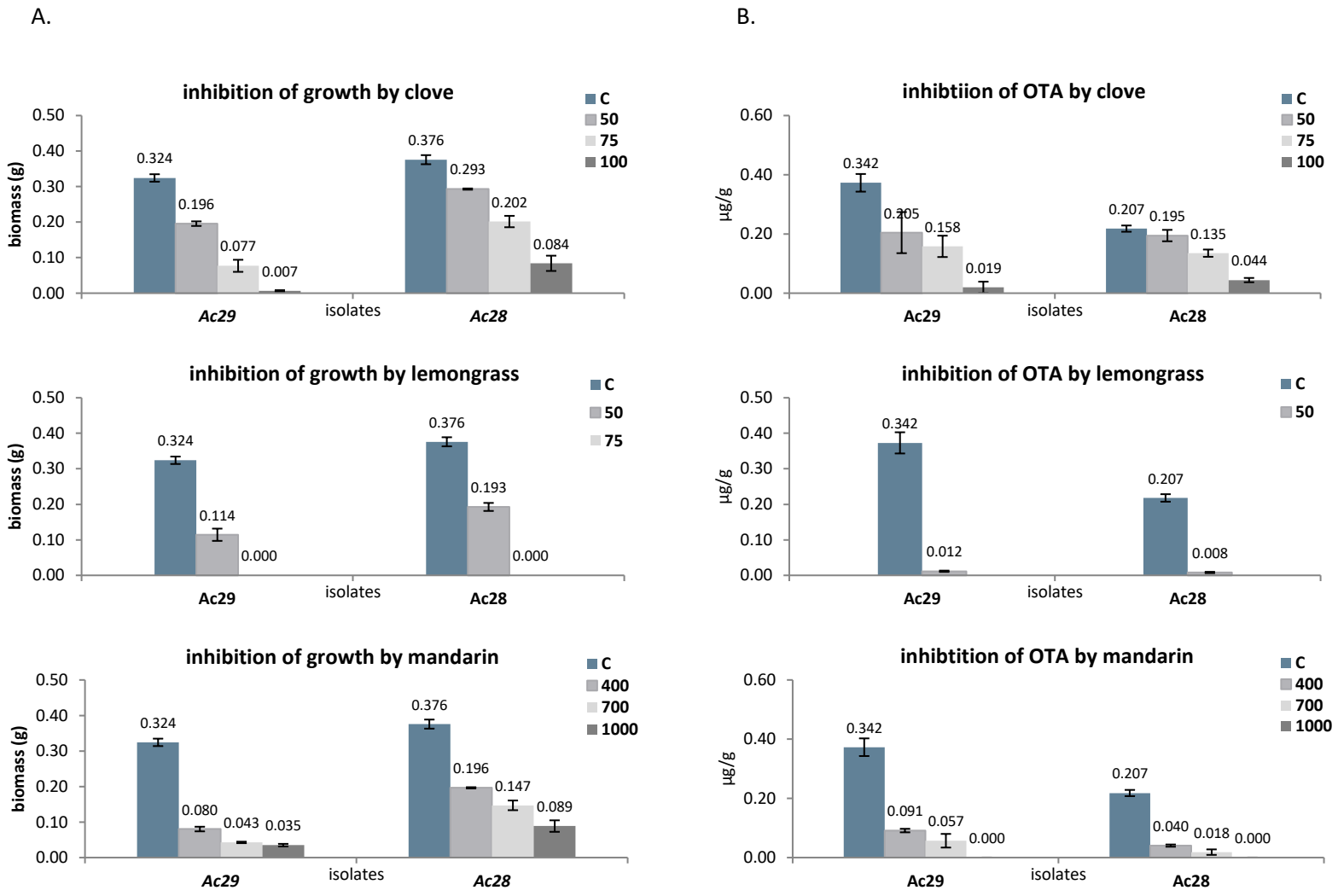


Fig. 5.3 Effects of EOs on *Aspergillus carbonarius* biomass (A) and OTA (B) on SGM liquid cultures. Error bars indicate standard error of 3 replicates.

Cinnamon anti-ochratoxingenic effect was evident for Ac29 in both growth media since 58% and 82% OTA reduction was observed at 50 and 75 μL/L, respectively, on MEA and 90% reduction at 50 μL/L in CYA. However, isolate Ac28 presented a different profile in relation to the growth media. Specifically, in MEA all concentrations of cinnamon EO resulted in increased toxin production, with the exception of the lowest concentration (50 μL/L) on CYA where OTA production was enhanced. Finally, mandarin EO affected mostly isolate Ac29 inhibiting OTA production by *ca.* 74% regardless of concentration. The same EO was less effective against the other two isolates enhancing toxin production at low concentrations but reducing OTA by 15% and 45% for Ac28 and 5010, respectively, at 1000 μL/L in MEA.

In the case of liquid cultures, the decrease in OTA production was proportional to the concentration of EOs (Fig. 5.3B). In addition, *A. carbonarius* appeared to be more sensitive to the various EOs assayed in liquid compared to solid cultures. Lemongrass EO was very effective as it reduced toxin by 96% at the lowest concentration assayed (50 μL/L) for Ac28 and Ac29. Regarding clove EO the behavior of the 2 ochratoxigenic isolates was similar since both showed a gradual decrease in OTA production with increasing concentration of EOs. Clove EO showed a milder effect (Fig. 5.3A) at the same concentration compared to lemongrass EO, however its inhibition effect increased gradually to 39%, 76% and 97% at 50, 75 and 100 μL/L, respectively, for Ac29 and 22%, 46%, and 77% for *Ac*28. The anti-ochratoxigenic effect of mandarin increased proportionally with concentration resulting in OTA reduction ranging from 73%, 83%, and 100% at 400, 700, and 1000 μL/L, respectively, for Ac29 and 80%, 91%, and 100% for Ac28. It needs to be noted that even though higher levels of toxin reduction were observed for clove and lemongrass EOs, only mandarin treatment at 1000 μL/L completely inhibited toxin production for both ochratoxigenic isolates.

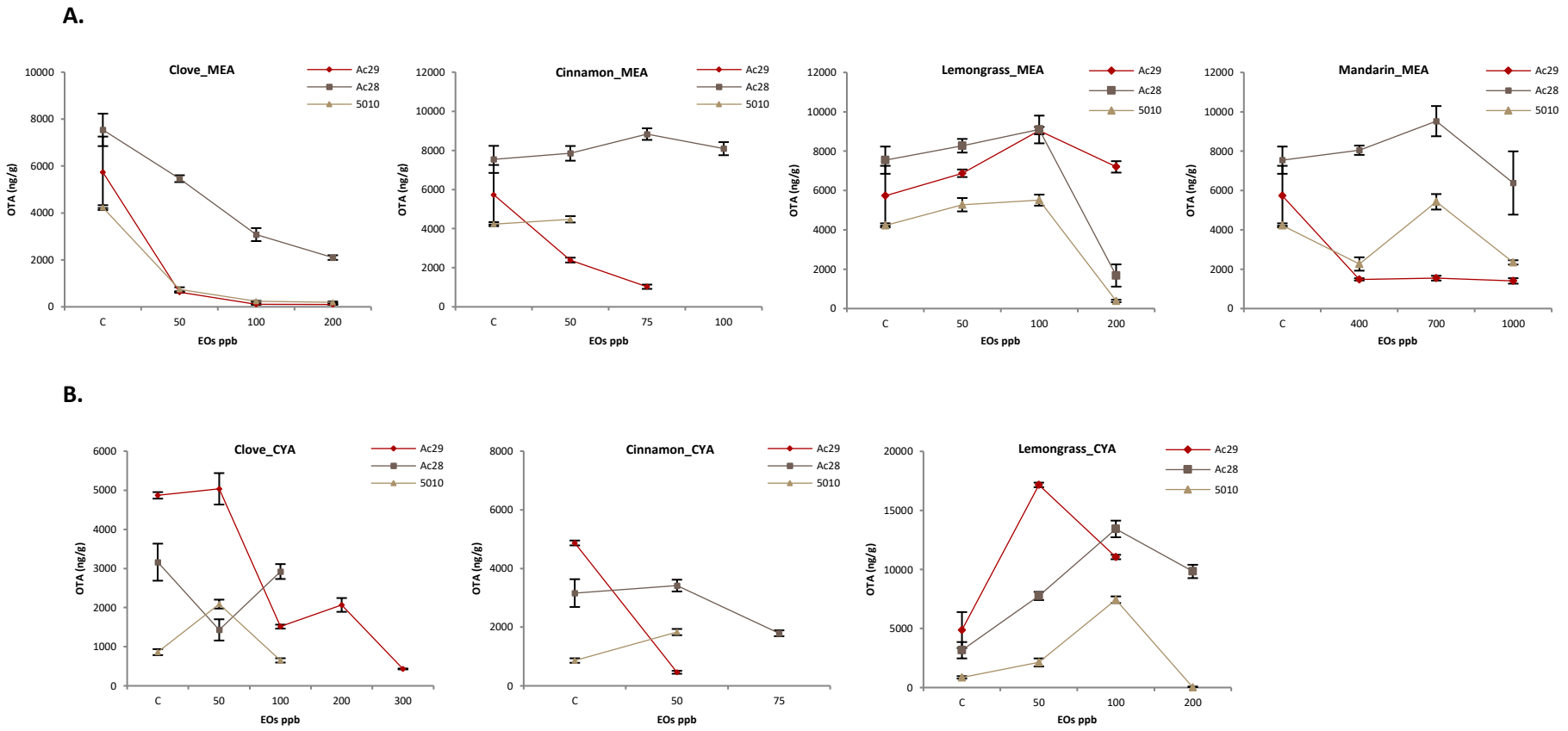


Fig. 5.4 Changes in OTA production of *Aspergillus carbonarius* isolates by the use of EOs in MEA (A) and CYA (B) growth media. Data points indicate mean values ± standard error of 3 replicates.

The statistical analysis showed that OTA decrease was affected by growth inhibition (p = 0.004) due to the presence of EOs. Furthermore, Ac28 isolate was most affected with regard to OTA production (Fig. 5.2D). Cinnamon results are not presented in liquid cultures due to the fact that inconsistent data were obtained during this treatment. However complete inhibition by cinnamon EO was observed for both Ac28 and Ac29 isolates at 100 μL/L.

5.3.3 Effect of EOs on A. carbonarius gene expression

The influence of EOs in the gene expression assay of *A. carbonarius* was evaluated and compared with the control samples. Both isolates (Ac28 and Ac29) used in this study showed expression of the biosynthetic cluster genes *Ac*OTA*nrps* and *Ac*OTA*pks*, whereas *laeA* also exhibited regulation in all control samples and treatments. The different relative expression profiles of these specific three genes related to each isolate are presented in Fig. 5. Furthermore, the interaction between different EOs and concentration levels was apparent in fungal isolates affecting expression levels. Regarding *Ac*OTA*nrps,* transcriptional levels were down regulated in a response to the majority of treatments. More specifically, Ac29 expression was affected at 50 μL/L lemongrass EO, 75 μL/L clove EO, and 1000 μL/L mandarin EO. On the other hand, Ac28 expression was mostly affected by mandarin, since gene expression decreased proportionally as EO concentration increased. Moreover, *Ac*OTA*pks* seemed to be influenced only in Ac29 where almost the same level of down regulation was observed in all treatments. In the case of *laeA* (Fig. 5.5C), no effect in expression levels was observed between treatments and control samples. Statistical analysis confirmed the interactions between treatments and gene regulation. Table 5.3 presents intra-strain differences in the transcription profile of both genes and also inter-strain differences in

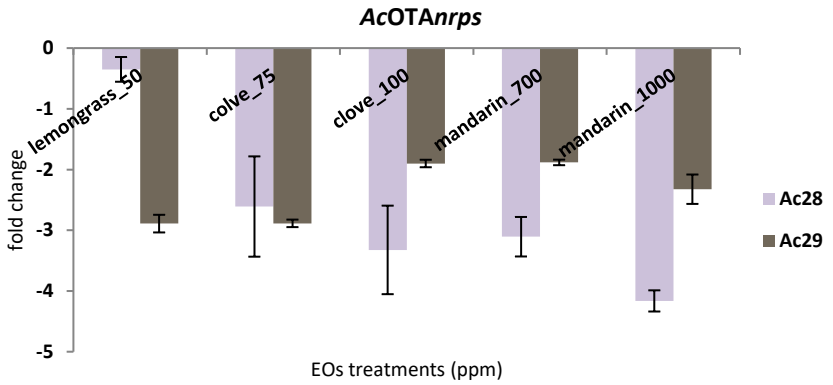
relative expression values between the two isolates in the treatments studied. Analysis of variance for the whole response of genes revealed highly statistical significance ($p < 0.0001$) of *Ac*OTA*pks* for all single factors assayed (fungal isolates and EOs). Two-way ANOVA also assessed the impact of combined effect of fungal isolate and EO on gene expression ($p = 0.005$). Moreover, it was observed that the impact of EOs on both OTA biosynthetic genes was proved statistically significant only for *Ac*28.

Table 5.3: Significance of EOs impact on inter- and intra-strain differences in relative gene expression profile.

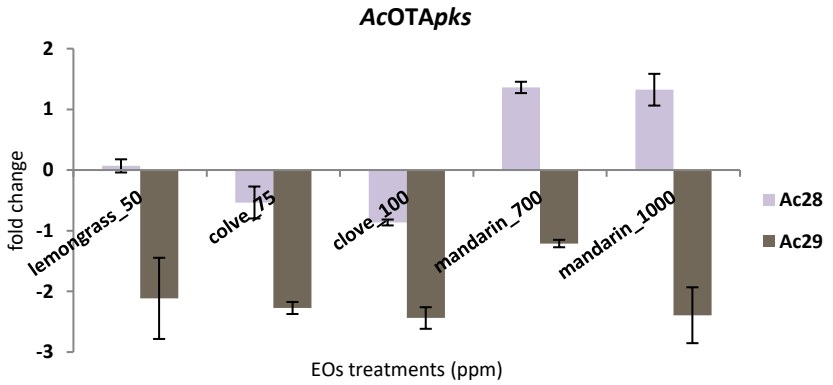
Factor	**Parameter**	**P value**	**F ratio**
Inter-strain differences			
Ac*OTA*nrps	EOs	0.1501	2.12
	Isolate	0.5031	0.46
Ac*OTA*pks	EOs	0.0007***	11.56
	Isolate	0.0185**	6.70
Intra-strain differences			
***Ac*28**	*Ac*OTA*nrps*	0.005**	11.76
	*Ac*OTA*pks*	0.0002***	34.70
***Ac*29**	*Ac*OTA*nrps*	0.1984	2.00
	*Ac*OTA*pks*	0.0021**	16.81

The asterisks concentrated on the bars indicate the relative level of *p*-value (*=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001).

A.



B.



C.

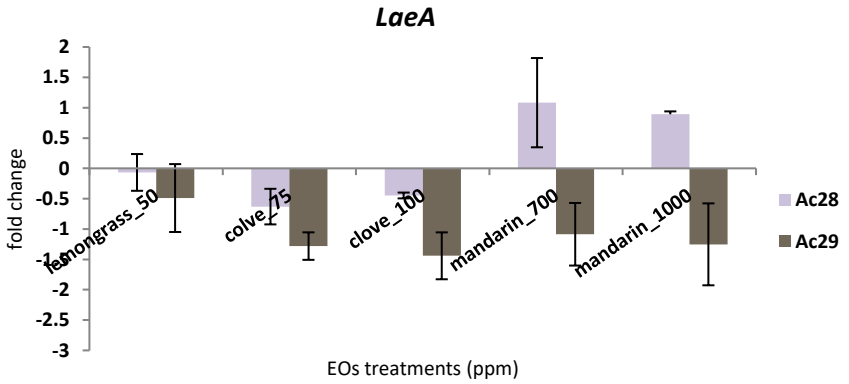


Fig. 5.5 Effect of EOs on the relative expression of *Ac*OTA*nrps* (A), *Ac*OTA*pks* (B) and *laeA* (C) during the growth of *Aspergillus carbonarius* isolates on SGM. Error bars indicate standard error of 2 replicates.

5.4 Discussion

The EOs evaluated in this study revealed antifungal activity against *A. carbonarius* in all treatments. In solid media, the inhibitory effect of clove EO on fungal growth increased proportionally with concentration. The results indicated also increasing anti-ochratoxigenic activity in all concentrations assayed on MEA but differential effects on CYA. The determination of anti-toxigenic effect of clove EO observed in this study was also consistent with previous reports that evaluated EOs for mycotoxin control. Pereira et al., (2006) also observed that clove EO was able to inhibit totally the growth of *Aspergillus ochraceus* but no significant effect in toxin prevention occurred. In another study (Passone et al., 2012) complete inhibition of OTA accumulation in peanut meal extract agar by *Aspergillus niger* was reported using clove EO at the concentration of 3000 μL/L. More recently, Hua et al. (2014) evaluated clove oil for OTA production by *Aspergillus ochraceus* and reported similar results with the present work, since complete fungal growth inhibition was observed at 500 μL/L of clove EO on MEA. The major component in clove EO is eugenol, which is a phenolic compound extracted from buds and leaves of clove (*Eugenia cariophyllus*). The effect of the principle compound of clove EO on growth and mycotoxin production has already been reported against toxigenic fungal genera (Amiri et al., 2006). The mode of action of eugenol could be attributed to the ability of this compound to permeabilize the cell membrane and interact with enzymes and proteins (Walsh et al., 2003; Omidbeygi et al., 2007), as well as its ability to inhibit enzymatic activity (Gill and Holley, 2006).

Cinnamon was the most effective EO since lower concentrations were needed for complete fungal growth inhibition in both solid media assayed. Moreover it was especially effective in CYA since a higher decrease of OTA production was observed in this medium, even though no complete inhibition of toxin was feasible. These results

emphasize the significance of different OTA conducive culture media in fungal responses and metabolite production. Our findings are in line with a previous study (Hua et al., 2014) where cinnamon EO was reported to be effective against *A. ochraceus* growth due to the antifungal activity of cinnamaldehyde, the main compound of cinnamon. In another work (Soliman et al., 2002) it was reported that 500 μL/L of cinnamon EO inhibited *A. flavus, A. paraciticus, A. ochraceus,* and *Fusarium moniliforne* on PDA medium. Significant inhibition of cinnamon EO was also reported by Sumalan, Alexa and Poiana (2013) against *Fusarium* spp. even though the level of concentrations used was higher compared to the concentrations applied in the present work. However, in a similar study (Hope et al., 2005) toxin production was enhanced even though fungal growth was inhibited. The additional stress imposed by fungicidal agents could possibly stimulate mycotoxin production as a defense response especially in suboptimal levels. Regarding the phenolic origin of EOs such as cinnamon, it has been suggested (dos Santos Oliveira et al., 2008) that phenolic structure is more important than concentration. Reports that phenolic compounds have greater antimicrobial impact compared with monoterpens and hydrocarbons (Burt, 2004) appeared to be in accordance with the results of the present study. Nevertheless as mentioned before, in liquid culture treatments, results among replicates were inconsistent. Specifically, it was observed that most fungi either did not manage to germinate or a small mycelium appeared after several days of incubation. However, there were also few cases where the observed fungal growth was similar to the control. Such inconsistency has been reported previously (Palumbo et al., 2007) indicating probably a strong effect of this EO and fungal incapability for an adaptive response.

Lemongrass EO has been reported for antifungal activity against *A. ochraceus*, *A. niger*, and *A. flavus* (Soliman and Badeaad, 2002; Tzortzakis and Economakis, 2007).

According to Tian et al. (2012), the hydroxylic group present in this compound allows hydrogen bonds to be formed with active enzymes resulting in deactivation and inhibition of fungal growth. In the present study, lemongrass EO presented antifungal activity similar to clove EO, showing also total growth inhibition at 300 μL/L. For all three fungal isolates assayed, lemongrass EO concentration of 200 μL/L was very effective in both MEA and CYA media, since prolonged fungal lag phase duration was observed. This fact indicates that lemongrass EO could be more effective to control fungal growth than toxin production. Indeed, ochratoxigenic effect was not clear since stimulation of OTA occurred in many cases. Low doses of EOs can probably create stress conditions enhancing thus secondary metabolism as a fungal defense mechanism. OTA stimulation by *A. carbonarius* even in the presence of high concentrations of EOs has been previously reported (Passone et al., 2012), whereas Garcia et al. (2011) also reported higher production of OTA by *A. westerdijkiae* in the presence of plant extracts. Regarding citrus EOs there are certain studies demonstrating their inhibitory effects on fungal growth but not on toxin accumulation (Sharma et al., 2006; Singh et al., 2010, Espina et al., 2011). Growth inhibition of *A. niger* by orange, mandarin and lemon EOs has been reported (Viuda-Martos et al., 2008), suggesting mandarin as the best inhibitor for *A. flavus* growth.

Mandarin EO in liquid cultures showed a different effect compared to the other two EOs, since it completely inhibited OTA production. This fact may suggest that a different mode of action was probably responsible for this anti-ochratoxigenic effect. In needs to be noted that all the isolates used in this work were able to produce high amounts of OTA. However, since no OTA was detected in mandarin EO at 1000 μL/L, this could possibly indicate that inhibition was induced by suppression of OTA transcription biosynthetic pathway. The results demonstrated that EOs tested (clove,

mandarin and lemongrass) influenced the relative expression of the selected OTA-related genes, presenting differences between the two OTA producers (*Ac*28 and *Ac*29). These differences underline the complexity of *A. carbonarius* regulation mechanism concerning OTA biosynthetic pathways. Down regulation was observed in many cases that were related to changes in toxin accumulation. Meanwhile, *laeA* expression patterns were similar to all tested samples indicating that this gene was not affected by the specific treatments. In the literature there are limited reports on gene expression studies referring to EOs. Recently, the effect of eugenol on down regulation of aflatoxin biosynthetic genes has been demonstrated for *A. parasiticus* (Jahanshiri et al., 2015) and *A. flavus* (Liang et al., 2015). Down regulation of OTA biosynthetic genes may suggest two possible modes of action. The first refers to the direct inhibition by EOs through mRNA expression. The second refers to the reduction in gene expression that is partially responsible on OTA production, affecting other biosynthetic pathways. A number of post transcriptional mechanisms are tightly connected to gene expression. Results however, could suggest that the specific EOs treatments may present a possible underlying relationship between OTA key genes and OTA production levels that require further elucidation.

Conclusion

Our study demonstrated differences among *A. carbonarius* isolates, concerning the efficacy of the different EOs to inhibit fungal growth and toxin production. Overall, clove, cinnamon, lemongrass and mandarin EOs could become an attractive application for future use against the risk of OTA, since inhibition of fungal growth at certain concentrations of these EOs could result in mycotoxin prevention. Further on, a relationship between transcription levels of the genes *Ac*OTA*nrps*, *Ac*OTA*pks* and OTA production was observed in many cases, since relative expression profiles gave similar

trends with OTA production between treatments and control samples. Investigation of the two structural and one regulatory gene of OTA biosynthetic pathway by qPCR highlighted some variation between the two wild fungal isolates. This finding may result in the elucidation of a possible intra-strain signal pathway involved in the inhibitory effects of these natural compounds in order to develop better strategies to control of OTA contamination.

CHAPTER **6**

Control of *Aspergillus carbonarius* in grape berries by *Lactobacillus plantarum*: A phenotypic and gene transcription study.

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Abstract

The antifungal activity of *Lactobacillus plantarum* against the ochratoxigenic fungus *Aspergillus carbonarius* was investigated in this study. Four different fungal isolates from grape berries were co-cultured with four different strains of *L. plantarum* on agar plates at 30ºC. Bacterial strains inhibited fungal growth up to 88% and significantly reduced toxin production up to 100%. In addition, *L. plantarum* was evaluated as biocontrol agent against *A. carbonarius* growth and OTA production on table grapes. Temporal studies of bacterial antagonism were performed with two different grape cultivars. Artificially wounded and unwounded berries were pre-treated with 10^8 CFU/mL bacteria and inoculated with 10^6 spores/mL of *A. carbonarius* ochratoxigenic isolates. Biocontrol agents displayed high rate of colonization on grapes during 5 days of incubation at 30 ºC. Scanning electron microscopy (SEM) also determined microorganisms' presence in grape surface. Bacterial strains were effective in controlling fungal infection reaching up to 71% inhibition rates. However the presence of wounds on grape skin facilitated infection of berries by *A. carbonarius*, since unwounded berries showed lower levels of infection. Results also revealed significant reduction in mycotoxin production ranging between 32% and 92%. Transcriptome analysis following exposure to co-cultivation, exhibited differential expression for each gene studied (AcOTA*nrps*, AcOT*Apks*, *laeA*) emphasizing the significance of strain variability. The genes AcOTA*nrps* and *laeA* were most influenced by the presence of *L. plantarum*. This work is a contribution for the potential biocontrol of toxigenic fungi in table grapes by lactic acid bacteria (LAB). The above findings underline the significance of bacterial strain variability on the effectiveness of biopreservative features of *L. plantarum* strains.

6.1 Introduction

Table grapes (*Vitis vinifera L.*) are one of the most nutritionally and economically important food products in the world. According to estimates by the US Department of Agriculture (USDA, 2017), Greece is is the second largest producer of table grapes in the EU-28, behind Italy. In recent years, EU production in 2017 is estimated at 1.5 million tons with that of Greece of 315.000 tonnes of table grapes. However, 30–40% of post-harvest table grapes are lost every year owing to inadequate handling and lack of proper methods to prevent decay and senescence (Hashem et al., 2013; Prusky, D. 2011). Fungal decay caused by *Aspergillus spp.* is hazardous to human health for direct consumption because of the production of mycotoxins. *A. carbonarius* is the main cause of Black rot in table grapes (Guzev et al., 2008, Rooney-Latham et al., 2008; Ayoub et al., 2010) and its ability of ochratoxin A (OTA) production is a great food safety issue. In Lasram et al. (2012) analysis of OTA showed the presence of the toxin in both wine and table grapes at a three year survey. Other surveys have been conducted reporting also *A. carbonarius* presence on table grapes demonstrating results shown *A. carbonarius* to be ubiquitous on table grapes (Belli et al., 2007). Melletis et al. (2007) in a survey conducted in Greece observing many varieties to be contaminated with OTA reporting that the incident of the infected berries is low before veraison while at harvest the frequency was twice as high. Exposure of OTA through food consumption poses a health risk. OTA remains a challenge in the face of continuous efforts to produce quality table grapes meeting with food safety standards. Control of fungal decay in table grapes has been achieved by application of sulfur dioxide gas, either by frequent fumigation in storage rooms, or by packing the grapes in polyethylene-lined boxes with SO_2 generator pads (Lichter et al., 2002). SO_2 is usually effective in preventing decay as long as levels are sufficiently high. However, high levels of SO_2 can injure grape

berries, have an unpleasant aftertaste, and could be harmful to humans (Zoffoli et al., 2008).

The use of chemicals is still the main practice to control fungal infection. However, this has fostered fungi to develop resistance to the extensive use of preservatives and antibiotics. Therefore, over the last few decades the interest of scientists has been shifted on natural antimicrobials to control fungal growth on grapes and grape products, employing strategies with less chemical control measures. The approach of biological control agents (BCAs) is increasingly considered by the scientific community as an alternative application either in the field and/or in post-harvest operations and has been already reported on table grapes (Jiang et al., 2014; Sonker et al., 2016). From the available BCAs, lactic acid bacteria (LAB) can inhibit fungal growth and they have also the potential to interact with mycotoxins (Dalié et al., 2010). LAB are generally recognized as safe (GRAS) which usually compete with other microorganisms by secreting antagonistic compounds and modifying the surrounding microenvironment producing several metabolites that are able to inhibit fungal growth (Hassan et al., 2015; Wang et al., 2012).

A number of putative pathways for OTA biosynthesis have been proposed and the role of two genes namely NRPSs and PKSs has been revealed as key enzymes in OTA biosynthetic pathway (Gallo et al., 2009, 2012, 2014). Therefore, they have been employed as targets to detect and quantify OTA producing molds by molecular techniques. Moreover, *laeA* gene has been correlated with the regulation of OTA biosynthesis in *A. carbonarius* as a global transcriptional factor (Crespo-Sempere et al., 2013). In general, there is a limited number of studies elucidating the expression of toxigenic related genes under different environmental and nutritional conditions. Only recently, Al-Saad et al. (2016) used relative gene expression as an indicator of the efficacy of bacterial antagonists against an aflatoxigenic strain of *A. flavus*. Also

Sumsudin et al. (2017) examined two bacterial antagonists for potential contamination of FUM1 in maize through gene expression studies.

The objective of this study was to: (a) investigate the *in vitro* and *in situ* antiochratoxigenic efficacy of different *L. plantarum* strains on *Aspergillus carbonarius* isolated from grape berries, (b) assess of antiochratoxigenic activity on gene transcription level, and (c) confirm bacterial/fungal co-existance on grapes by means of Scanning Electron Microscopy (SEM) images.

6.2 Materials and methods

6.2.1 Microorganisms and inocula preparation

All the microorganisms used in the present study are indicated in Table 6.1. They consisted of four bacterial strains of *L. plantarum* (T571, 345, 195, and 1645), three *A. carbonarius* wild isolates (Ac29, Ac33, and A47) originated from grape berries and one reference strain of *A. carbonarius* (ITEM 5010). All strains were maintained in 20 % (v/v) glycerol at −22 °C.

Lactobacillus plantarum strains were grown in de Man-Rogosa-Sharpe (MRS, Biolife, Italy) broth for 24 h at 30 ºC and sub-cultured in the same medium for 18 h at 30 ºC. Cells were harvested by centrifugation at 5000×g for 10 min at 4 °C. The supernatants were discarded and the cells were washed twice with Ringer solution and resuspended in the same medium to obtain a final concentration of 10^5 CFU/mL (confirmed by plating on MRS agar) for use as inoculum.

Spore suspensions of each *A. carbonarius* isolate were prepared by collecting spores from 7-day old colonies grown on Malt Extract Agar (MEA: malt extract, 20 g; peptone, 1 g; glucose, 20 g; bacteriological agar, 20 g; distilled water, *ca.* 1000 mL) at 25 ºC in the dark to induce sporulation. Conidia were harvested from sub-cultures by adding 10

mL of sterile distilled water containing 0.01 % Tween 80 (Merck, Schuchardt, Germany) and scraping the surface of the mycelium with a sterile glass rod. The suspensions were filtered through a four layer cleaning tissue to remove any mycelia fragments and left still for 5 min to allow spore settling. Supernatants were discarded and the spores were resuspended in Tween 80 to remove any nutrients from the medium. Conidia concentration was adjusted to a final volume of 10^5 spores/mL assessed by a Neubauer counting chamber (Brand, Wertheim, Germany).

Table 6.1 Microorganisms used in the present study.

Microorganism	Strain number	Origin	Country
Aspergillus carbonarius	Ac29[a]	Grape berries	Greece
	Ac33[a]	Grape berries	Greece
	Ac47[a]	Grape berries	Greece
	ITEM 5010[b]	Grape berries	Italy
Lactobacillus plantarum	T571[c]	Feta cheese	Greece
	1645[d]	Fermented cauliflower	Greece
	345[a]	Black olive	Greece
	195[a]	Grape berries	Greece

[a] Food Microbiology Culture Collection (FMCC), Laboratory of Microbiology and Biotechnology of Foods, Agricultural University of Athens.
[b] Provided by Dr. D. Tsitsigiannis, Laboratory of Phytopathology, Agricultural University of Athens.
[c] Provided by the Institute of Technology of Agricultural Products, Hellenic Agricultural Organization "DEMETER".
[d] Provided by Dr. E. Drosinos, Laboratory of Food Quality Control and Hygiene, Agricultural University of Athens.

6.2.2 In vitro experimental settings

The *in vitro* experiments of bacterial/fungal co-cultures were performed in MRS agar for optimal growth of LAB. Specifically, 0.5 mL suspension of each *L. plantarum* isolate was poured into 9 mL of MRS medium (in 6-cm Petri plates) in a final concentration of *ca.* 10^6 CFU/mL. Following media solidification, permeable cellulose membranes were placed onto the agar surface and *A. carbonarius* was centrally spot

inoculated at a concentration of 10^6 spores/mL. Plates not inoculated with bacteria were used as control. Considering the importance of temperature as a modulator factor in fungus-bacteria interaction systems, co-cultures were incubated at 30°C for optimal growth and metabolic activity of fungi and bacteria for three days. The same volume of growth medium (10 mL) was added in the plates to avoid any variation that could contribute to differences in the results among co-cultures. All experiments were undertaken with three replicates per treatment. The percentage of fungal growth inhibition was determined 3 days after *A. carbonarius* inoculation using the formula:

$$Fungal\ inhibition\ (\%) = 100 - \left[\frac{Biomass\ of\ fungal\ colony\ on\ agar\ treated\ with\ LAB}{Biomass\ of\ fungal\ colony\ on\ agar\ without\ LAB} \times 100\right]$$

6.2.3 In situ antagonistic assay

Mature healthy table grapes from two seasonally different and widely consumed grape cultivars, namely Victoria from the area of Tirnavos (white cultivar) and Attika from the area of Korinthos (red cultivar) were purchased from a local retail market. Homogenous bunches were selected according to size, shape, color, weight and absence of mechanical damage and fungal infection. Grape berries were surface disinfected with 70% ethanol for 10 minutes (Tryphinopoulou et al., 2015), rinsed twice with sterile distilled water and allowed to air dry on absorbent paper in a laminar flow bench until inoculation.

Berries were separated into two groups. In the first group, a calibrated wound of about 2 mm in diameter was made on each berry by means of a sterile needle (wounded berries) (Curtis et al., 2012), whereas in the second group no such treatment was given and hence the berries remained intact (unwounded berries). Disinfected unwounded berries were also divided into two groups. The first was used to determine the adherence of

LAB on the surface of berries at the beginning of the experiments and the second to evaluate their ability as biocontrol agents against *A. carbonarius* infection. The artificially wounded berries were also divided into two groups. The first was used to evaluate the efficacy of bacteria to inhibit fungal growth and the second to determine the effectiveness of bacteria against OTA production. Three replicates of 10 equally sized (*ca*. 2 g each) grape berries were used in each treatment.

The potential of bacterial attachment on grape surface was determined by immersing the berries in a bacterial suspension (*ca.* 10^8 CFU/mL) for 2 min and allow to air dry in a laminar flow cabinet for 45 min to facilitate bacterial attachment. Subsequently, the berries were rinsed by pipetting twice with 10 mL of Ringer solution to remove any loosely attached cells. For the assessment of the antagonistic activity, the berries were subsequently submerged in 10^6 spores/mL conidial suspension for another 2 min, allowed to dry in the air, placed in plastic bags and incubated at 30 ^oC. Following the attachment step, the population dynamics of bacteria was evaluated according to Marin et al. (2016) with slight modifications. Each sample was weighted, transferred aseptically to Erlenmayer flasks containing the appropriate volume of Ringer solution and agitated in a rotary shaker at 150 rpm for 10 min to achieve maximum detachment of LAB from grape surface. Detached cells were enumerated in MRS agar plates by 10 fold serial dilutions after incubation at 30 ^oC for 48 h. The typical LAB colonies were counted and results expressed as $\log_{10}$ CFU/g. The level of bacterial colonization on berries was recorded at days 0, 1, 2, 3, 4, and 5. Infection of berries was assessed either directly by visual inspection or with the aid of a stereomicroscope looking at the typical hyphae of *A. carbonarius*. The percentage of fungal infection was determined after 2, 3, 4, and 5 days of incubation according to Nally et al. (2012) by counting the number of berries with typical *A. carbonarius* infection, using the formula:

$$Disease\ incident\ (\%) = \frac{Number\ of\ decayed\ berries}{Number\ of\ total\ grapes} \times 100$$

For the fungal infection assay, *A. carbonarius* Ac29 was indicatively selected on the basis of its high OTA potential as determined in a previous work (Lappa et al., 2015). Positive control samples consisted of grape berries treated only with *A. carbonarius* were also prepared for the infection incidence assay. Finally, negative control samples were prepared by immersing disinfected wounded and unwounded berries in distilled water

6.2.4 Ochratoxin A determination

OTA production of co-cultures was assessed according to Lappa et al. (2017a) with slight modifications. Specifically, mycelium was collected and weighed into a 2 mL eppendorf tube. Mycelium biomass was extracted with 1 mL of 100% methanol. After agitating the tubes for 5 min in a rotary shaker, they were left still for 1 h for toxin extraction. Extracts were filtered through a 0.2 μm syringe-driven filter unit (Millex, Millipore Co., Bedford, Mass) prior to toxin determination by HPLC. Furthermore, toxin extraction from grape berries was similarly assessed based on the toxigenic potential of *A. carbonarius* Ac29 as mentioned above. Specifically, 20 berries were weighted and homogenized using an Ultra Turax homogenizer (Heidolph Instruments, Schwabach, Germany) for 1 min at the highest speed (26 x 10^3 rpm) and 5 mL of each homogenate was mixed with 5 mL of 100% methanol for 30 min. Extracts were filtered through a Whatman No 2 filter paper to remove any suspended solids from grapes and then through a 0.2 mm syringe-driven filter unit (Millex, Millipore Co., Bedford, Mass.). All samples were stored at -20 °C until HPLC analysis. A recovery assay for the

OTA extraction method was performed, spiking known concentrations of OTA (50 and 100 ng/mL) on grape berries leading to satisfactory results with recovery rates of 84 and 95%, respectively.

OTA quantification was undertaken by an HPLC system equipped with a JASCO LC-Net II/ADC system controller, a JASCO AS-2055 Plus auto sampler, with a Model PU-980 Intelligent pump, a Model LG-980-02 ternary gradient unit pump and an FP-2020 Plus fluorescent detector (JASCO Inc., Easton, USA). Injection volume was 10 μL and run time for samples was 20 min with OTA detected at about 11 min. The analysis was performed under isocratic conditions at a flow rate of 1 mL min $^{-1}$ of the mobile phase (water/acetonitrile/acetic acid; 49.5/49.5/1) through a Waters spherisorb C18 analytical column, 5μm ODS2 (4.6 x 250 mm) (Resteck Co., Pinnacle II, Bellefonte, USA). The detection limit of the analysis was 1 ng/g. Percentage of OTA inhibition was calculated as follows:

$$OTA\ inhibition\ (\%) = 100 - \left[\frac{OTA\ production\ after\ LAB\ treatment}{OTA\ production\ without\ LAB\ treatment} \times 100\right]$$

6.2.5 Gene expression studies

Mycelia were collected from Petri dishes, flash freezed in liquid Nitrogen (lN_2) for stabilization of expression and stored at -80 °C until extraction according to Lappa et al. (2017b). Frozen tissues were lyophilized overnight and subjected to nucleic acid extraction. For RNA isolation and extraction, Invitrogen, PureLink RNA mini kit along with Trizol (Ambion, USA) was used according to manufacturer's protocol. Turbo DNase (Ambion, USA) was also used to eliminate possible trace amounts of contaminating genomic DNA according to kit instructions. Quality of RNA samples was checked through a qPCR amplification to ensure absence of genomic DNA.

Nucleic acid quantification was assessed spectophotometrically by measuring the absorbance at 260 nm using a NanoDrop spectrophotometer (IMPLEN, Germany). RNA purity of samples was evaluated through the $A_{260\ nm}/ A_{280\ nm}$ and $A_{260\ nm}/ A_{230\ nm}$ ratio and a 3% ethidium-bromide staining gel verified RNA integrity. First-strand cDNA was synthesized using PrimeScript Reverse Transcriptase (Takara, Japan) according to manufacturer's instructions in a 20 μL final reaction volume. RNA used was 0.5 μg at each sample. For priming Random Hexamers (Invitrogen, USA) was used. Reaction mixture was incubated for 5 min at 65^{o}C and specific set up program was following as 30 ^{o}C for 10 min, 42 ^{o}C for 60 min and 70 ^{o}C for 15 min.

For the relative quantification assay, Real Time PCR was used to amplify *AcOTApks* (polyketide synthase) and *AcOTAnrps* (non ribosomal peptide synthase) genes as target genes, and also *laeA* gene as a global regulator for secondary metabolites in *Aspergillus* spp. Constitute expressed *β-tubulin* gene served as an internal reference for gene expression normalization. Nucleotide sequences of primers used in the qPCR assays are shown in Table 6.2. Real time PCR experiments were conducted according to Lappa et al. (2017). Briefly, for qPCR assays 96-well plates were used in an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, USA). The SYBR Green chemistry was used to monitor cDNA amplification. qPCRBioSyGreen Mix High-Rox (PCR Biosystems, United Kingdom) was used in a 10 μL reaction. Cycling conditions according to specific SYBRGreen protocol were as follows: 95 ^{o}C for 0.5 min, 60 ^{o}C for 0.25 min 72 ^{o}C for 0.25 min [40 cycles]. Melting curve analysis of the PCR products was performed by heating at 95 ^{o}C for 0.15 sec and 60 ^{o}C for 1 min and continuous measurement of the fluorescence to verify the PCR product. Template-free negative controls were used at every run also. Data analysis was undertaken by StepOnePlus RT-PCR System Software v2.1. Expression levels were determined according to Pfaffl

method (Pfaffl, 2002, 2004) by the equation $R = \mathbf{E_{target}}^{\Delta Ct\text{target (control-sample)}} / \mathbf{E_{ref}}^{\Delta Ct\text{ref (control-sample)}}$ and data converted to relative expression and presented in $\log_2$ values as fold changes (Kubista et al., 2007). The above method calculates the expression ratio of a target gene between a test and a control sample ('relative calibrator'). These calibrators corresponded to fungal monocultures.

Table 6.2: Nucleotide sequences of primers for qPCR assay.

Primer pair	Gene	Nucleotide sequences 5'—3'	Reference
F-pks	*AcOTApks*	GTC AAG GTC GGG TGC TAC AA	Lappa at al. (2017a)
R-pks		TCG GAA TGA TAC GCG ACT TT	
F-nrps	*AcOTAnrps*	CTC CAC CCA TCC TCC CGT TC	Crespo Sempere et al. (2013)
R-nrps		AAT CCA TGT CCT CAC CAT CGC	
F-laea	*laeA*	CAC CTA TAC AAC CTC CGA ACC AC	Crespo Sempere et al. (2013)
R-laea		GGT TCG GCC AAC CGA CGA CGC TG	
F-tubβ	*β-tubulin*	CGC ATG AAC GTC TAC TTC AAC GAG	Crespo Sempere et al. (2013)
R-tubβ		AGT TGT TAC CAG CAC CGG ACT	

6.2.6 Scanning electron microscopy (SEM) at in situ co-cultures

Scanning electron microscopy (SEM) was used to evaluate the microstructure of the grape berry samples before and after microorganism colonization. To observe the skin avoiding alteration of the epicuticular waxes and, at the same time, preserving the interaction between berry surface and microorganisms, the samples (*ca*. 5.0 mm × 5.0 mm × 1.5 mm) were fixed with 70% ethanol at 0–4 °C for 36 h and slowly dehydrated through a short alcohol series (80, 95 and 100%), before drying with CO_2 at the critical point in a Critical Point Dryer CPD 030 (Oerlikon Balzers, Balzers, Liechtenstein). The dry tissues were then mounted on aluminium stubs and coated with gold (25 nm thick)

in a Sputter Coater SCD 040 (Oerlikon Balzers, Balzers, Liechtenstein). Representative specimens were examined with a FEI/Philips XL 20 Scanning Electron Microscope (Fei Company, Hillsboro, Oregon, USA) located at the CREA-IT Research Centre for Engineering and Agro-food Processing (Cepagatti (PE), Italy).

6.2.7 Statistical analysis

Measurements were averaged and presented as mean values ± standard error. Statistical analysis was performed using the JMP ver. 10 software (SAS, Institute INV, Cary, NC, USA). Analysis of Variance (ANOVA) was applied to analyze the variation between and within group means at a significant level of $p \leq 0.05$. Least Significant Difference (LSD) with α=5% was applied to evaluate any significant difference of the effect of each bacterial strain in the co-cultures performed.

6.3 RESULTS

6.3.1 In vitro screening of the effect of L. plantarum on A. carbonarius growth and OTA production

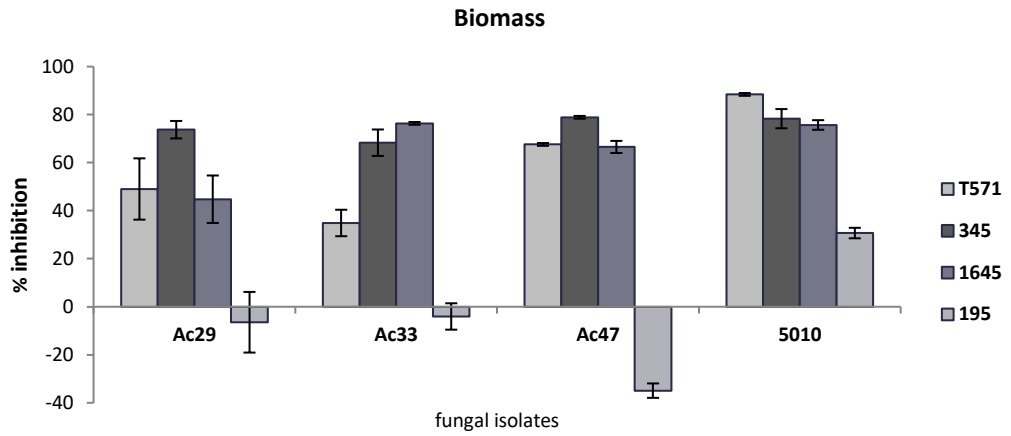
The growth of fungal isolates of *A. carbonarius* in the presence of *L. plantarum* strains is presented in Fig. 6.1A,, whereas an image of growth inhibition of *A. carbonarius* ITEM 5010 by LAB strains is illustrated in Fig. 6E. Antifungal effects in MRS medium were different for each bacterial strain depending on the combination of co-culture among LAB and fungi. All bacterial strains could significantly ($p < 0.0001$) inhibit fungal growth (Table 6.3). More specifically, growth inhibition by *L. plantarum* T571 was estimated from 34% to 88% of fungal biomass. The highest and lowest biomass inhibition rates were noticed in fungal isolates ITEM 5010, and Ac29 respectively. Moreover, *L. plantarum* 345 affected the growth of all fungal isolates with biomass inhibition rates ranging from 68% to 78%. Finally, *L. plantarum* 1645 showed biomass

inhibition rates ranging from 44% for isolate Ac29 to 76% for isolate Ac33. In contrast, in co-cultures between *L. plantarum* 195 and fungal isolates, no biomass inhibition could be observed but rather the presence of the bacterium stimulated fungal growth, with the exception of the reference strain ITEM 5010 where a 30% biomass decrease was observed. Moreover, as shown in Fig. 6.1A, *L. plantarum* 345 displayed the greatest impact on *A. carbonarius* growth while the fungal strain ITEM 5010 was most affected by co-culture with bacterial strains.

Table 6.3: Significance of bacterial impact on *A. carbonarius* growth and OTA production

Fungi	**Bacteria**	**P-value**	
		Growth	OTA
Ac29	T571	<.0001	<.0001
	345	<.0001	<.0001
	1645	<.0001	<.0001
	195	0.4160	<.0001
Ac33	T571	0.0042	<.0001
	345	<.0001	<.0001
	1645	<0.001	<.0001
	195	0.6782	<.0001
Ac47	T571	<.0001	<.0001
	345	<.0001	<.0001
	1645	<.0001	<.0001
	195	0.0053	<.0001
ITEM 5010	T571	<.0001	<.0001
	345	<.0001	<.0001
	1645	<.0001	<.0001
	195	0.0025	<.0001

A.



B.

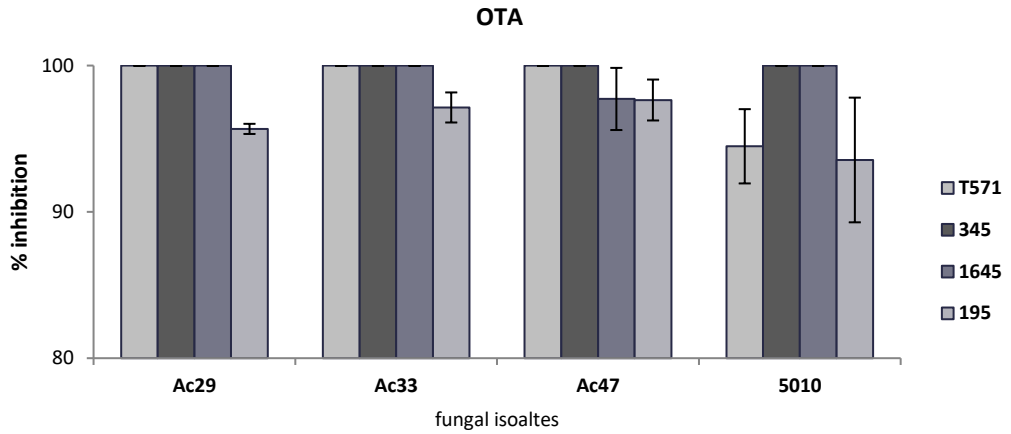


Fig. 6.1 Growth inhibition % (A) and OTA % reduction (B) of *A. carbonarius* in MRS co-cultures with *L. plantarum* strains for 3 days at 30 ºC. Data points are mean values of two replicates ± SE.

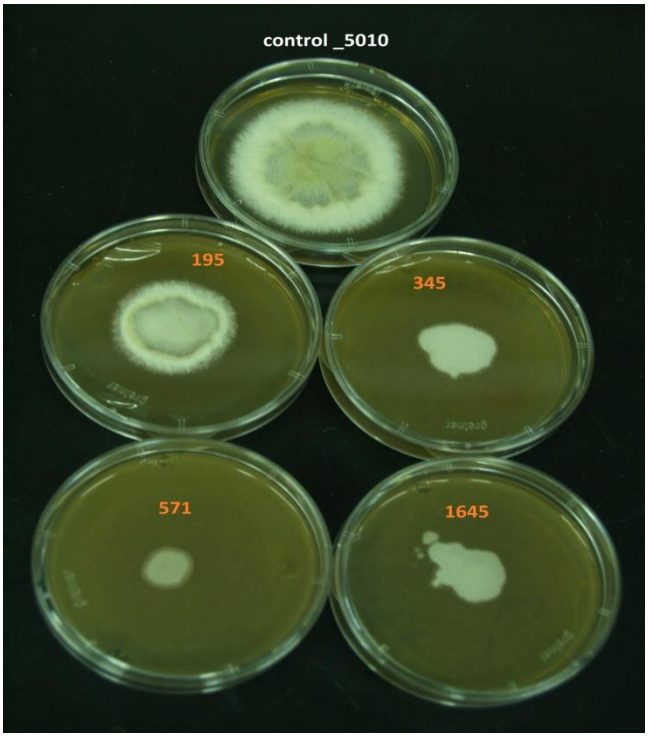


Fig. 6E. Growth inhibition of *A. carbonarius* ITEM 5010 by *L. plantarum* strains.

The effect of different *L. plantarum* strains on OTA production by *A. carbonarius* is presented in Fig. 6.1B. All bacteria had a significant impact regarding OTA accumulation ($p < 0.0001$) presenting a decrease in toxin ranging from 93% to 100%. *L. plantarum* 345 was proved again to have the greatest impact on every fungal isolate, reducing toxin production by 100%. Regarding OTA inhibition, the reference fungal isolate ITEM 5010 was less affected by the presence of LAB compared to wild isolates, although it was the most affected isolate concerning biomass reduction. It needs to be noted that *L. plantarum* 195 had in general a strong effect on OTA inhibition despite the fact that it enhanced fungal growth in the wild isolates of *A. carbonarius*.

6.3.2 Population dynamics of L. plantarum on grapes

The population dynamics of *L. plantarum* on grape berries is shown in Fig. 6.2, where high levels of colonization were observed in both cultivars. Bacterial populations

appeared to have similar trend, starting from almost the same initial counts for all strains and increased rapidly on berries surface. Specifically, the LAB stains presented high attachment on grape surface right after inoculation ranging from 4.6 to 5.1 $\log_{10}$ CFU/g. The highest level of colonization was reached at day 3 (*ca.* 6.5 log units) followed by a slight decrease thereafter. As shown in Fig. 6.2, *L. plantarum* 345 and 571 presented higher populations in the white grape cultivar revealing slight differences among the strains tested.

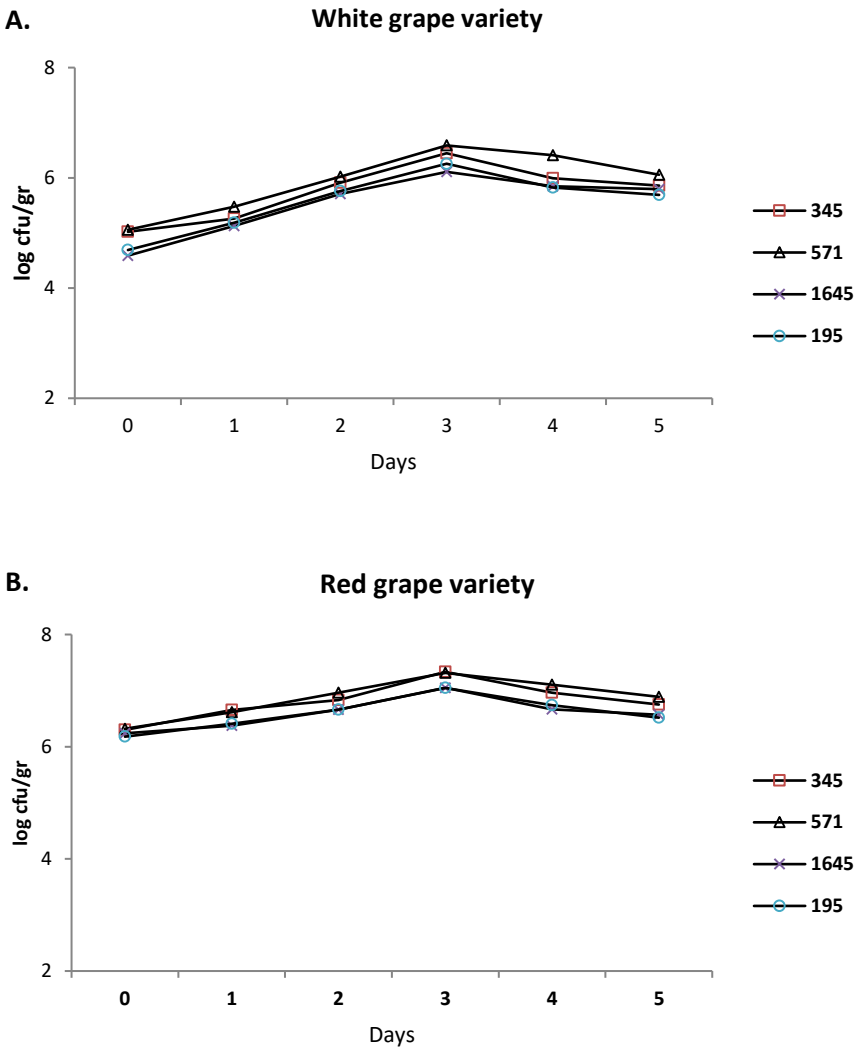


Fig. 6.2 Population dynamics of *L. plantarum* isolates on the surface of grapes of two different table grape varieties stored at 30 °C for 5 days (st. deviation was <5% of the respective value).

6.3.3 Evaluation of antagonistic activity of L. plantarum against A. carbonarius growth in grapes

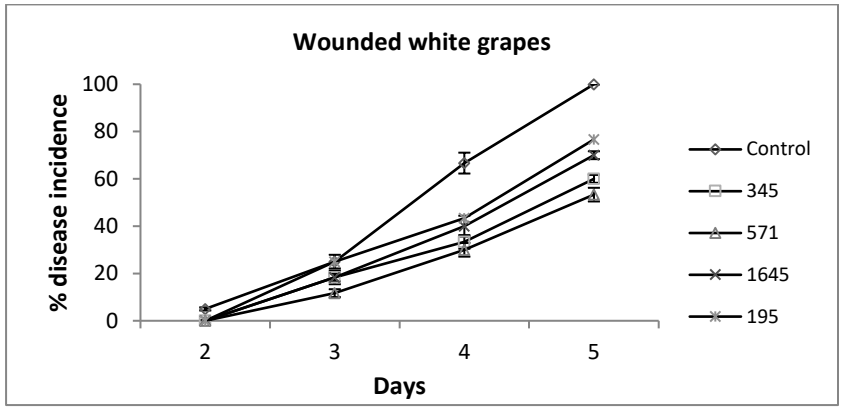
The efficacy of *L. plantarum* to prevent *A. carbonarius* infections on detached grape berries at 30 °C is illustrated in Fig. 6.3. All bacterial strains were able to reduce fungal infection in berries compared to control treatment. Further statistical analyses were conducted to highlight the efficacy of *L. plantarum* to control *A. carbonarius* growth and revealed significant differences ($p<0.0001$) between wounded and unwounded grape berries (Table 6.4). In both cultivars, the percentage of infected berries was lower in the unwounded compared to wounded grape berries group. Fungal infection was delayed for 2 days in wounded berries. With regard to the unwounded group, infection was observed at day 3 including both control samples and samples treated with *L. plantarum* 1645 and 195, whereas *L. plantarum* 345 and 571 delayed fungal infection by one more day.

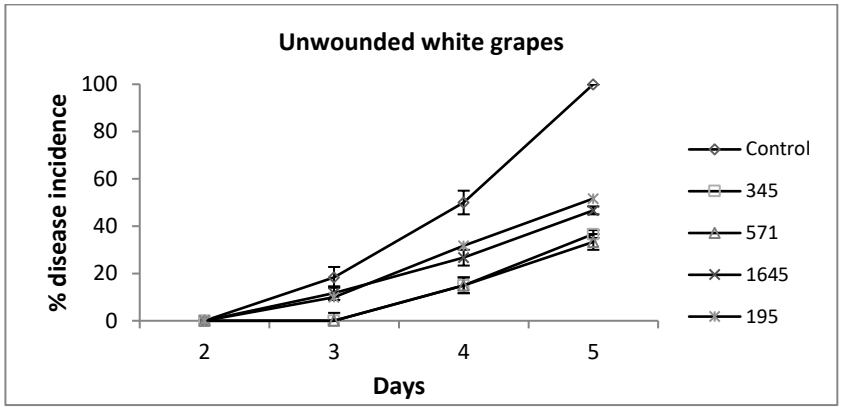
Table 6.4 Analysis of variance (ANOVA) between the two grape cultivars and the parameters involved in the biocontrol experiment for *A. carbonarius* Ac29.

Grapes	Factor	DF	Sum of Squares	F Ratio	Prob > F
WHITE	Treatment (T)	1	163.3333	206.3158	<.0001*
	Bacterial strain (Bs)	4	469.9500	148.4053	<.0001*
	Day (D)	3	2644.0000	1113.263	<.0001*
	T ×Bs	4	6.5833	2.0789	0.0913
	T ×D	3	46.6667	19.6491	<.0001*
	Bs ×D	12	249.2500	26.2368	<.0001*
	T ×Bs × D	12	30.7500	3.2368	0.0008*
RED	Treatment (T)	1	147.4083	194.3846	<.0001*
	Bacterial strain (Bs)	4	481.8833	158.8626	<.0001*
	Day (D)	3	2269.0917	997.4029	<.0001*
	T ×Bs	4	12.0500	3.9725	0.0054*
	T ×D	3	62.6250	27.5275	<.0001*
	Bs ×D	12	308.1167	33.8590	<.0001*
	T ×Bs × D	12	18.7500	2.0604	0.0291*

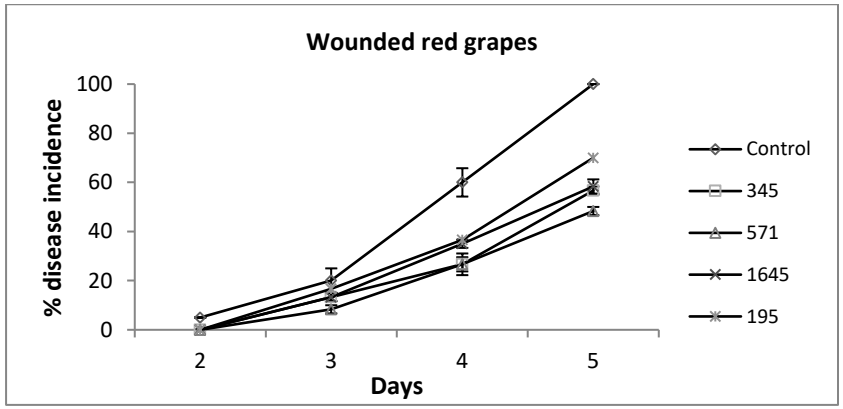
Higher differences in infection rates were observed at day 4 in both groups. Specifically, unwounded grapes pre-treated with bacterial strains were able to control fungal infection *ca.* 25% more effectively than in wounded ones (Fig. 6.3). Even thought on day 5, 100% infection was observed in both groups (wounded and unwounded) for control samples, all bacterial strains displayed good biocontrol activity with considerable reduction in infection rates. *Lactobacillus plantarum* 571 and 345 have been proved as the most effective agents on fungal control. Specifically, the former strain reduced infection on the white grape cultivar to 53.3% and 33.3% for wounded and unwounded berries, respectively. Higher reduction was observed for the same strain in the red grape cultivar since the infection rate was reduced to 48.3% and 30.0% (Fig.s 6.3 A,B) for wounded and unwounded berries, respectively. Similarly, *L. plantarum* 345 reduced infection on the white grape cultivar to 60.0% and 36.6%, respectively, for wounded and unwounded berries, whereas the respective rates in the red grape cultivar were 56.6% and 28.3% (Fig.s 6.3A,B). Moreover, the cross effects between the two cultivars and the experimental parameters are presented in Table 6.4, highlighting the significance of interactions between strain, treatment and time on antifungal activity.

A.





B.



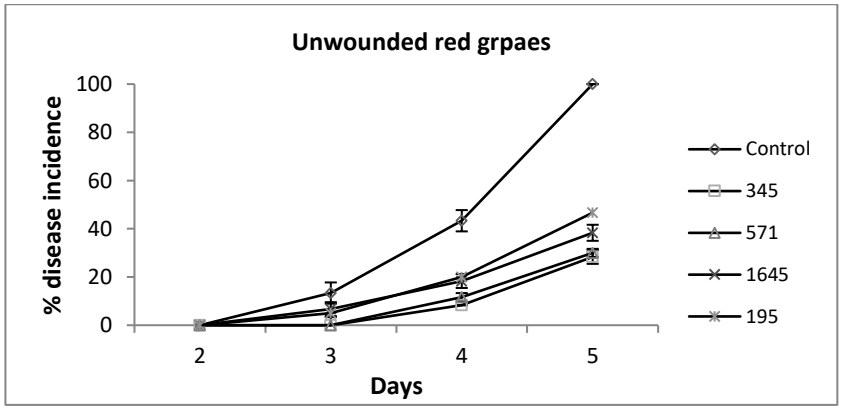


Fig. 6.3 Percentage of infection (%) of *A. carbonarius* on white grape cultivar (A) and red grape cultivar (B) berries during co-culture with *L. plantarum* strains for 5 days at 30 ºC. Data points represent mean values of three replicates ± SE.

6.3.4 In situ assessment of L. plantarum potential to control OTA production by A. carbonarius

On the basis of the results of antagonistic activity, the antiochratoxigenic potential of *L. plantarum* strains was evaluated against *A. carbonarius* Ac29 in grapes. This particular wild fungal isolate was selected as a high OTA producer isolated from grapes in Greek vineyards according to a previous study (Lappa et al., 2015). Infected grapes from wounded berries were also investigated for the presence of OTA. The average OTA accumulation under the interaction effects of *L. plantarum* after 5 days at 30 °C is presented in Fig. 6.4.

OTA content of grape berries was significantly reduced by the biocontrol agents ($p<0.0001$). Interestingly, *L. plantarum* 345 and 571 were proved more effective to reduce toxin production in the red grape cultivar by *ca.* 84.5% and 92.5% respectively, compared to *ca.* 78.7% and 81.4% reduction in the white grape cultivar. On the other hand, *L. plantarum* 1645 and 195 showed also antiochratoxigenic activity reducing OTA by 56.7% and 40.0% as well as by 44.5% and 32.3% for the white and red grape cultivars, respectively.

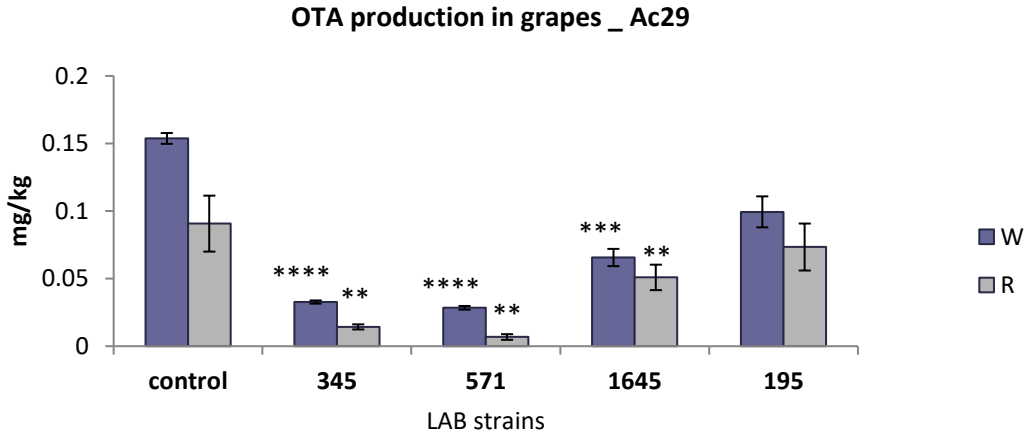


Fig. 6.4 Impact of *L. plantarum* strains on OTA production in white grape cultivar (W) and red grape cultivar (R) after 5 days at 30 ºC. Bars represent mean values of two replicates ± SE. Significant differences are indicated by * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), and **** ($p<0.0001$).

6.3.5 Transcription responses of A. carbonarius isolates under co-cultivation with L. plantarum

The influence of bacterial strains in the gene expression assay of *A. carbonarius* was evaluated and compared with the control samples (fungal growth without bacterial co-culture). All fungal wild isolates used in this study along with the reference strain ITEM 5010, showed expression of the biosynthetic cluster genes AcOTA*nrps* and AcOTA*pks*, whereas *laeA* exhibited also regulation in all samples. The interactions between microorganisms were apparent in fungal isolates affecting expression levels of these specific genes (Fig.s 6.5). Regarding isolate Ac29, transcriptional profile of AcOTA*nrps* and AcOTA*pks* was down regulated up to 8 folds in the majority of treatments. However, *laeA* did not show any difference in transcription between co-cultures and control samples (Fig. 6.5A). In contrast, in Ac33 the expression of *laeA* was highly affected by bacterial antagonists presenting up to 12 folds up regulation (Fig. 6.5B). However, the genes AcOTA*nrps* and AcOTA*pks* exhibited differential expression in Ac33, whereas isolate Ac47 did not present any significant changes in transcription profile. More specifically, fungal co-culture with *L. plantarum* T571 and 1645 showed down regulation of both structural genes while *L. plantarum* 345 caused down regulation only to AcOTA*nrps.* On the other hand, up regulation of AcOTA*pks* was observed for *L. plantarum* 195. Gene expression for the reference strain ITEM 5010 revealed also a different profile. Specifically, *laeA* was most affected by all bacterial strains, since it was up regulated up to 9.3 folds. AcOTA*pks* seemed to be influenced only by *L. plantarum* T571 where down regulation was observed. In the case

of AcOTA*nrps*, up regulation was observed by treatments with *L. plantarum* T571, 345 and 1645.

6.3.6 Microstructural analysis of grape surface by SEM

The ultrastructure of grape berries untreated and treated with microorganisms as revealed by SEM images is presented in Fig. 6.6. Specifically, Fig. 6.6A illustrates fresh healthy berries before treatments where the surface shows uniformLy distributed epicuticular wax deposits. Fig. 6.6B shows the grape skin surface of berries inoculated with LAB where high levels of colonization by *L. plantarum* were observed. The structure of epicuticular waxes could be of paramount importance in the skin-adhesion by microrganisms, as in the case of table olives fermentation by *L. plantarum* (Lanza and Di Serio, 2015). The grape skin of berries inoculated with *A. carbonarius* is shown in Fig. 6.6C. The surface of injured berries shows spherical structures formed by a mass of vegetative hyphae that could represent the initial phase of infection starting from the inoculation point. Finally, the co-culture between *L. plantarum* and the fungus is illustrated in Fig. 6.6D, where the proliferation of lactobacilli seems to reduce the formation of the fungal plaques.

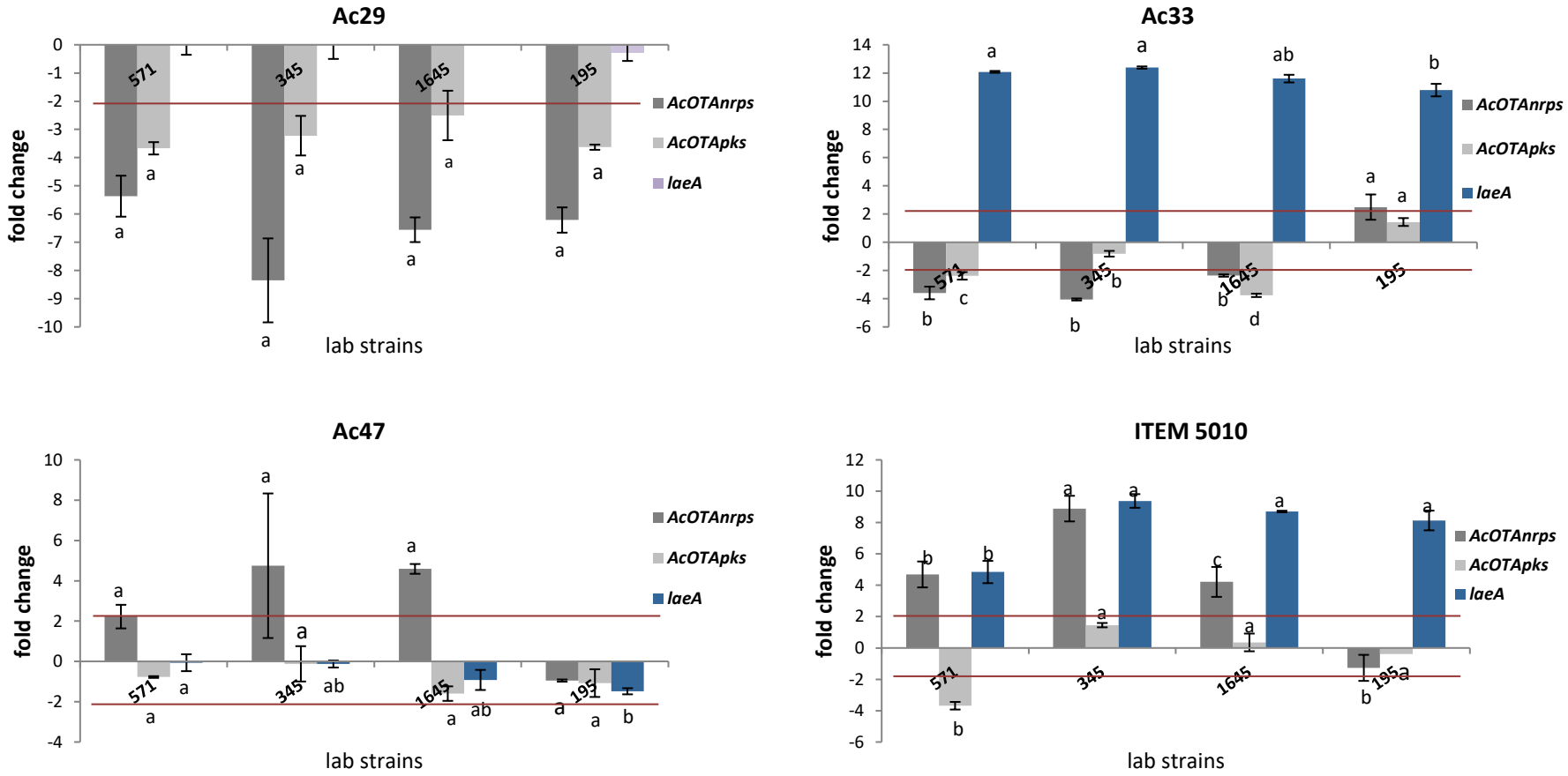
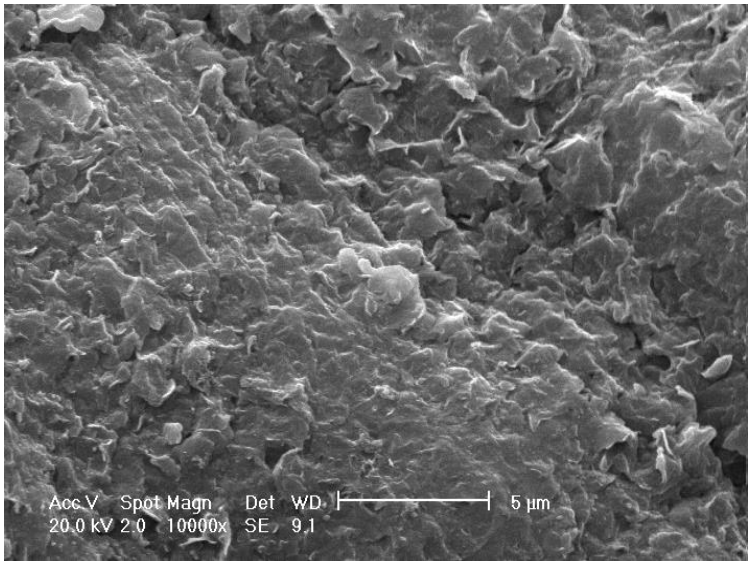
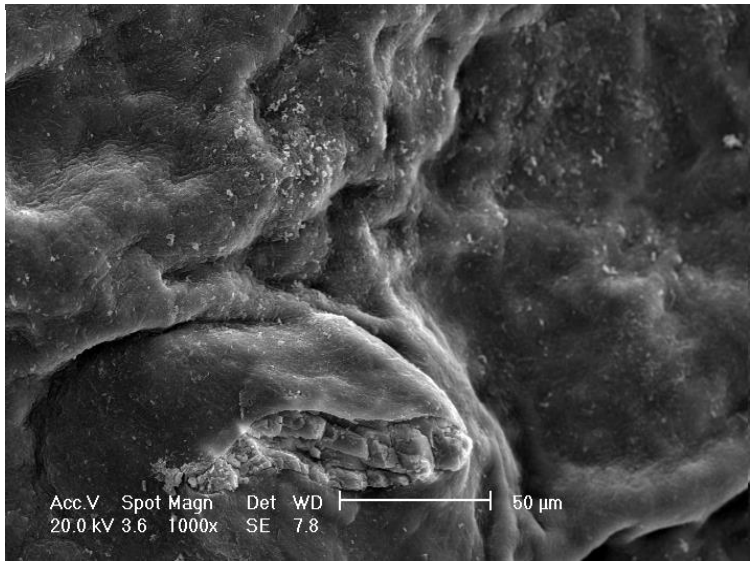


Fig. 6.5 Relative gene expression of *A. carbonarius* in MRS co-cultures with *L. plantarum* strains for 3 days at 30 ºC. Bars represent mean values of two replicates ± SE. Significant differences between relative gene expression for each OTA related gene and bacterial/fungal co-culture are indicated by different letters.

A.


Acc.V Spot Magn Det WD 5 µm
20.0 kV 2.0 10000x SE 9.1

B.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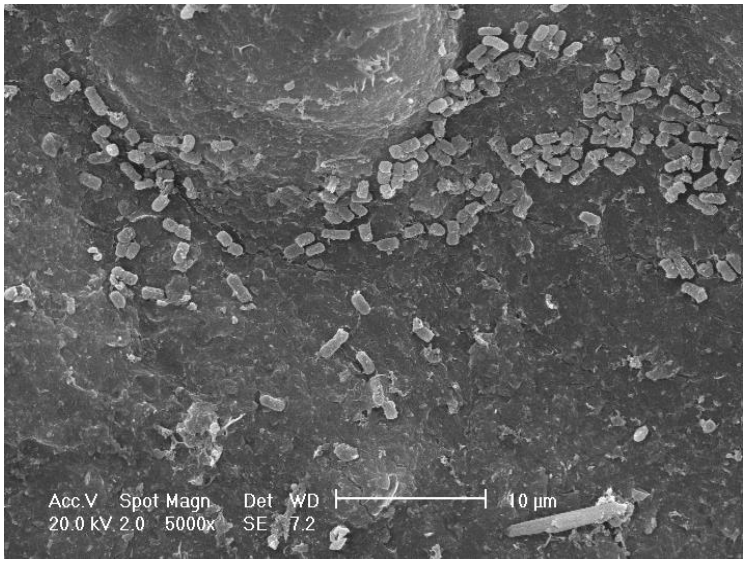
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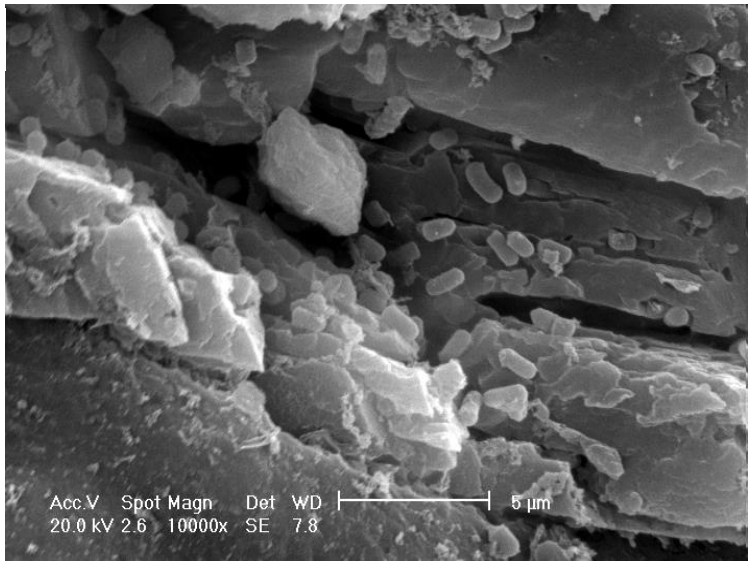
B.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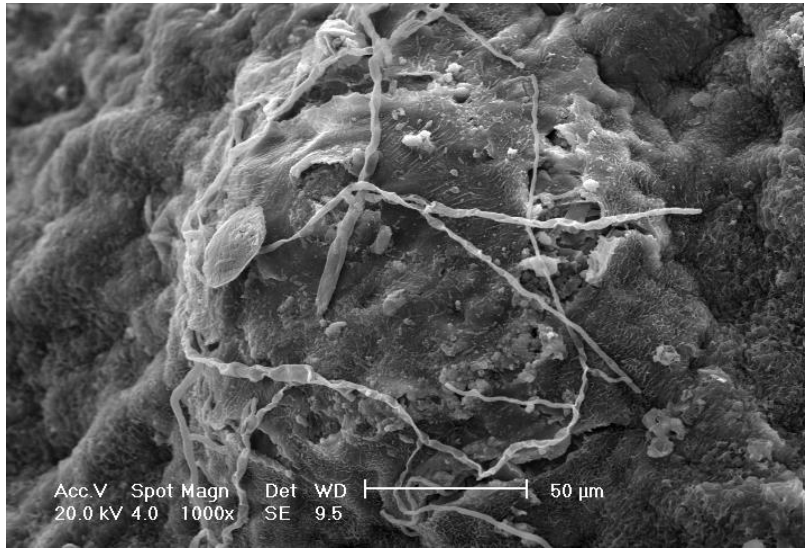
20.0 kV 2.6 10000x SE 7.8

C.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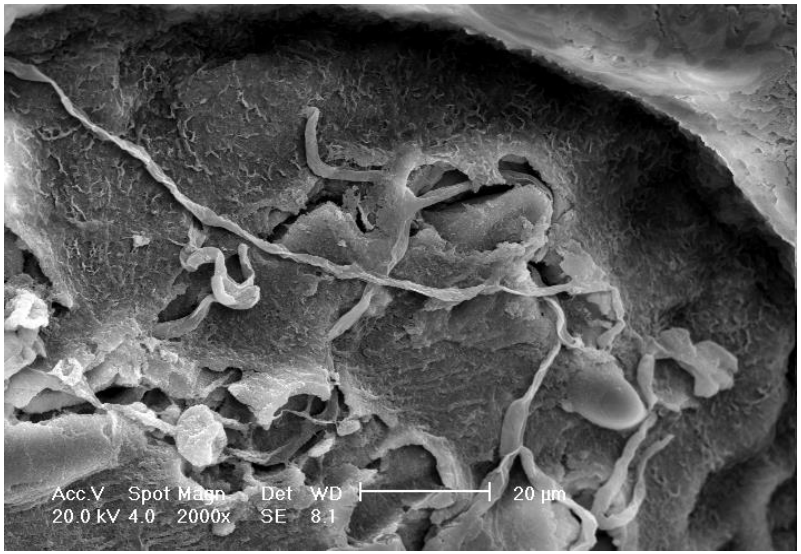
C.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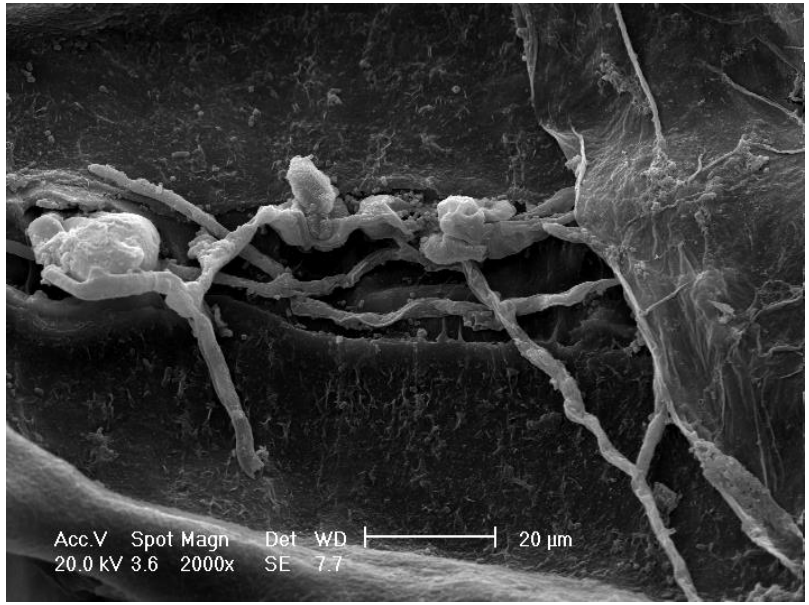
D.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D.

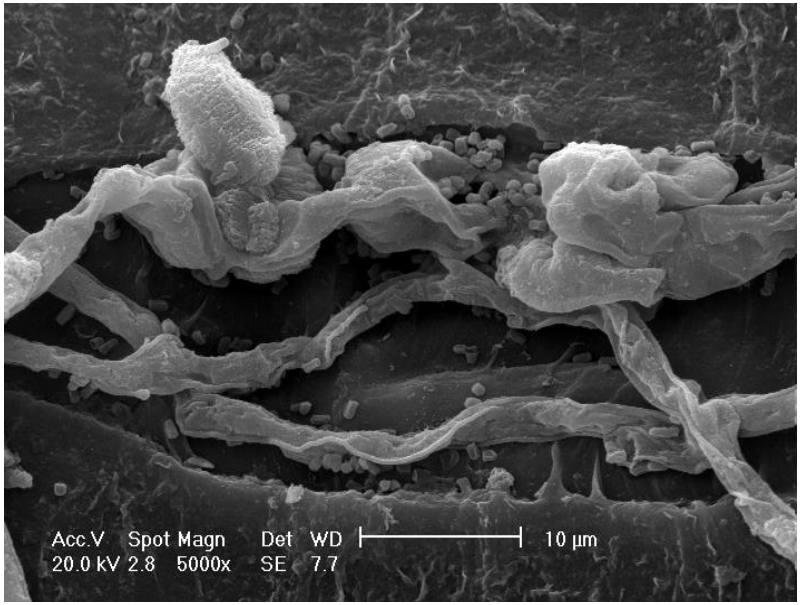
Acc.V Spot Magn Det WD 10 µm
20.0 kV 2.8 5000x SE 7.7

Fig. 6.6 Ultrastructure of grape berry skin before and after colonization of microorganisms. (A) untreated grape berry; (B) grape berry inoculated with *L. plantarum*; (C) grape berry inoculated with *A. carbonarius*; (D) grape berry inoculated with both microorganisms.

6.4 Discussion

Filamentous fungi are widely distributed food spoilage microorganisms responsible not only for significant losses in the field but also for safety concerns due to their potential to produce mycotoxins. LAB have already been highlighted as promising bioprotective agents, among which *L. plantarum* is considered as a prominent microorganism in the field of antifungal research (Djossou et al., 2011; Lavermicocca et al., 2000; Luz et al., 2017). In this work, the focus was given on screening different strains of *L. plantarum* isolated from heterogenous food matrices against *A. carbonarius* grape isolates. An *in vitro* co-culture assay was undertaken to determine the effects of *L. plantarum* strains on fungal growth and OTA production. Results revealed that *L. plantarum* presented significant effects on the growth of *A. carbonarius* and fungal inhibition was dependent on the bacterial strain assayed. Significant reduction in fungal biomass was observed for the majority of co-cultures with the exception of *L. plantarum* 195 that stimulated fungal growth in wild grape isolates. This could be indicative of bacterial strain dependent stress or some specific fungal/bacterial interaction. Therefore, the imposed additional stress by fungicidal agents could possibly stimulate fungal growth as a defense response of the fungus in sub-lethal levels of the agent employed. It is also likely that the specific bacterial strain is adapted to co-existence with fungi due to the fact that it is also isolated from grapes and it is less resistant against fungal competition. Another possible hypothesis of strain differences could be attributed to metabolic compounds presenting lower antifungal potential. Recently, Dong et al. (2017) elucidated the genetic diversity among *L. plantarum* strains associated with mechanisms of antifungal action and reported that the antimicrobial activity of LAB is expressed

either directly through competition of live cells for growth substrates or indirectly as a result of the synthesis of a wide variety of active antagonistic metabolites. Our results are also consistent with previous studies which have reported great inter-specific variability among the antifungal activity spectrum of *L. plantarum* (Russo et al., 2017).

It is known that certain LAB are able to pose anti-mycotoxins such as patulin (Fuchs et al., 2008), aflatoxins (Sangwanee et al., 2014) and fumonsin B1 (Pizzolitto et al., 2012). In a recent work, (Saladino et al., 2016), the effect of *L. plantarum* to reduce growth of *Aspergillus parasiticus* and *Penicillium expansum* was reported together with a reduction in AFs biosynthesis. Even though there is little information about the anti-ochratoxigenic effect of LAB against *A. carbonarius,* the results obtained in this study are in agreement with previous reports that evaluated *L. plantarum* mycotoxigenic control. Specifically, Belkacem-Hanfi et al. (2014) reported high effects against an *A. carbonarius* strain concerning OTA reduction in liquid medium. Concerning the mechanisms of action involved in the inhibition of OTA accumulation by *L. plantarum*, it is hypothesized that the acid compounds produced by bacteria could also be partly responsible for OTA reduction apart from their direct interaction effect on growth. Nevertheless, binding toxin in LAB cell wall is another well supported hypothesis by previous researchers (Haskard et al., 2001; Piotrowska and Żakowska, 2000). *Lactobacillus plantarum* 195 was proved less efficient to inhibit OTA production from all fungal isolates suggesting inter-strain differences in terms of OTA elimination. In contrast, *L. plantarum* 571 and 1645 were able to inhibit totally OTA production only in some fungal isolates. In this regard, intra-strain differences indicate also differences in resistance mechanisms of fungal isolates upon bacterial antagonism. Interestingly, the strain ITEM 5010 was proved to be the least resistant isolate to bacterial antagonism in terms of growth but more prone to reduced toxin accumulation. This fact underlines the

different response of the reference strain compared to the wild grape fungal isolates at genetic level and defense action mode.

In order to assess the potential application of our findings, the reduction of *A. carbonarius* colonization in grape berries was temporally monitored and the positive effect of biocontrol treatment in terms of OTA inhibition was determined at the same time. *Lactobacillus plantarum* strains presented high colonization and biocontrol activity resulting in reduced *A. carbonarius* incidence on grape berries. Despite the fact that total fungal inhibition was not observed, it is promising that considerable elimination of infection occurred using high initial fungal populations. Colonization, nutrient competition and inhibition of fungal metabolites may be a mechanism of action of this antagonistic outcome. Ability of *A. carbonarius* to colonize and penetrate intact berries has been previously shown (Battilani et al., 2001). It is also well known that table grapes have much more soft skin that wine grape berries. The antagonistic activity of biological agents against *A. carbonarius* in grapes has been demonstrated mainly for yeasts confirming their positive role in decreasing fungal infection and OTA accumulation in wine grapes (Bleve et al., 2006; Ponsone et al., 2011; De Curtis et al., 2012; Nally et al., 2013). Nevertheless, the results of the present work are in accordance with Ponsone et al. (2016), presenting that biological agent was not the same efficient to control *A. carbonarius* on damaged berries. Furthermore, the authors also reported that the inhibitory effects were dependent on the ochratoxigenic species and yeast strains.

This study has also attempted to investigate the efficacy of LAB against *A. carbonarius* by examining the effect on genes involved in OTA biosynthetic pathway. The focus was given on two structural genes localized in the ochratoxin cluster and one global regulator. In general, with regard to the expression of the two biosynthetic genes AcOTA*nrps* and AcOTA*pks* and the global regulator *laeA*, all isolates showed

regulation in all cases studied and different expression patterns were observed. These results indicate that OTA production was not consistent with the effect of gene expression which is in agreement with Al-Saad et al. (2016) who observed different responses expressed after the influence of four bacterial antagonists against *Aspergillus flavus* on aflatoxin related genes. The data obtained in our work demonstrated that *L. plantarum* influenced relative expression of the selected OTA related genes, presenting differences between OTA producers. Down regulation was observed in many cases that were related to changes in toxin accumulation supporting the hypothesis of toxin inhibition induced by the suppression of the OTA transcription biosynthetic pathway. Gene expression in co-culture experiments has been characterized by transcriptional responses of *A. carbonarius* with high variations among fungal isolates. Moreover as mentioned above, results among bacterial strains were also inconsistent. Transcripts were differentially expressed after *L. plantarum* influence even in cases where no toxin was detected. Hencefore, this fact may suggest that a different mode of action was probably responsible for this anti-ochratoxigenic effect. Antifungal activity could induce a metabolic alteration, which is likely to affect expression of other genes leading to final protein expression. The complete understanding of this complexity requires comprehensively cataloging the gene expression changes that accompany infection. While *A. carbonarius* responded to *L. plantarum* by down regulation of genes, the most interesting result is the trigger of expression of the global regulator, enforcing thus the findings of a previous study (Crowley et al., 2013) in which *L. plantarum* increased transcprition of global regulator *laeA.* This result indicates a cellular stress response and exerts an attempt of *A. carbonarius* isolates to be protected against antifungal activities. Transcriptome experiments were performed in order to assess findings as opposed to secondary and thus indirect response. Direct antifungal effect was not observed except for particular cases. Significant reductions have been observed in the fold change of

mRNA in the presence of bacterial antagonists. The decreased expression of mRNA therefore provides additional evidence for changes in *A. carbonarius* metabolism in response to *L. plantarum* influence. This suggests that some bacteria may indeed control toxin production through transcription. Instead, our data point to a far underscribed alteration of *A. carbonarius* metabolism in response to *L. plantarum.* Results suggest that the protective effect of L. plantarum is mediated by a synergy of actions. It is likely however that the reduced level of expression is a consequence rather than a cause of OTA production. When compared to the gene expression for the control of A. carbonarius, although these effects were significant, they may not have sufficiently clarified and other key regulatory genes may be needed to explore. The question is whether the differential results are because of inter-species variability or because of post transcriptional even post translational mechanisms.

Conclusions

In conclusion, this work is a contribution for the application of microbial antagonists against OTA producing fungi in table grapes through the implementation of a gene transcription approach. *Lactobacillus plantarum strains* proved effective as potential biocontrol agents against *A. carbonarius* isolates since they greatly reduced fungal growth *in vitro*. In addition OTA production was minimized by bacterial strains presence at inoculation level of 10^6 cfu/mL for all fungal isolates. Moreover, the *in situ* results showed that biocontrol treatment with *L. plantarum* decreased infection by *A. carbonarius* on table grapes. All bacterial strains successfully colonized and influenced fungal adhesion on grape berries as confirmed also by SEM images. Furthermore, greater control efficacy revealed especially for unwounded berries. A positive effect of *L. plantarum* strains was also observed on the level of OTA accumulation, even when

protection from fungal infection was not complete. These positive effects were proved to be strain dependent for both microorganisms. Finally, OTA related genes, mainly OTA*nrps* and *laeA*, were significantly affected by the bacterial co-culture, emphasizing also strong strain dependence for *A. carbonarius* isolates in terms of gene expression.

CHAPTER 7

Concluding remarks and Future perspectives

Aspergillus carbonarius is a mycotoxigenic fungus of high concern for grapes and grape derivatives and is considered one of the most potent sources of OTA contamination. Although half a century has elapsed since OTA was isolated for the first time, its study remains a major challenge in science until today. Contamination of different food and feed with OTA remains an important health issue that still requires much attention. Climate, agronomic and global market changes are importanct factors affecting mycotoxigenic hazards. Therefore, the knowledge of fungal behavior, biosynthetic mechanisms and interactions along with toxin production is still essential to face effectively the exposure to this toxin.

In this thesis, OTA kinetics was determined in an attempt to correlate primary (growth) and secondary metabolism (toxin production) at optimum conditions, developing also a model for OTA prediction. A variety of growth parameters (diameter, biomass, dry weight, colony area, colony density) were studied in an inter-specific assay. The observations reported in Chapter 3 indicated that the dependence of the results from each growth determination method assayed is due not only to inter- but also to intra-species level. Even though highly correlated results were obtained between fungal growth and toxin production for the majority of grape isolates, variability in toxin potential among grape isolates could not be efficiently explained, indicating that phenotypic OTA production should somehow be connected to the activity of OTA related genes. In these terms, further investigation was undertaken at molecular level.

Despite the great progress in genome sequencing of *A. carbonarius* which has allowed the elucidation of a putative gene cluster involved in OTA biosynthesis, the biosynthetic mechanism of this mycotoxin has been partially revealed until now. In general, gene transcription coincides with OTA production, demonstrating that its induction can be used as a molecular signal to monitor toxin production. Transcriptional regulation is the

first step used by fungi to modulate mycotoxin production. The knowledge of the genes involved in OTA biosynthesis and understanding of the mechanisms involved in their regulation are essential to develop new effective control methods. Therefore, studies on the regulation of expression of OTA biosynthetic genes are important to elucidate how and why fungi produce the toxin and thus help to establish efficient control methods.

In these terms, in Chapter 4 gene regulation was investigated with regard to environmental factors effects. However, gene transcription could not be correlated with OTA production for all genes studied. These findings provided insight on the complexity of OTA regulation. As stated before, OTA biosynthetic key genes have been proved to be regulated by environmental stimuli, suggesting that the relative expression profiles of both AcOTA*pks* and AcOTA*nrps* could provide critical information on the ecological conditions that influence OTA production. So far, however, there is no positive or negative established trend between gene expression and temperature or water activity. Lack of consistent correlations could be due to the fact that the measured OTA concentration is an accumulated value, whereas gene expression data are actual values at the time point at which the samples were taken. In general, OTA is very stable and the amount produced during growth accumulates in the mycelium and the medium. Additionally, the amount of mRNA may change rapidly since it is also not stable over time and may vary at a certain time point depending on culture age and maturity. These fluctuations in the level of mRNA over time have also been taken into account in this work. This type of information has been obtained for the first time and demonstrates the dynamics of the regulation of expression of OTA key genes, especially in response to different environmental conditions, emphasizing thus the need to understand these mechanisms.

The economic loss and health hazards caused by toxigenic fungi are of paramount importance and as highlighted above the most efficient way to prevent food contamination with mycotoxins is to avoid their growth. Alternative natural strategies for the management of mycotoxins in food and feed, which have received much attention lately, have been proved promising for mycotoxin biosynthesis inhibition. On the other hand, focus should be given to control both fungal growth and mycotoxin production, as opposed to controlling fungal growth only, since partial control of growth might lead to higher mycotoxin production and thus reduce the quality of the harvested commodity. In addition, a better understanding of the impact of sublethal doses of the control agent should be evaluated along with the environmental conditions that could affect its capacity to reduce toxin production.

The ability of natural compounds, such as essential oils, to suppress mycotoxigenesis could become an alternative strategy to limit food and feed contamination as demonstrated in Chapter 5. The use of different assays indicated that the physicochemical characteristics and microstructure of different substrates have a strong impact on fungal growth that may shape both the transcriptional responses and phenotypes related to growth or gene expression. Not surprisingly, inter and intra strain differences were a major issue observed in the obtained results.

Finally, in Chapter 6 the efficacy of LAB strains as anti-ochratoxigenic agents was studied in terms of fungal growth and assosiated toxin reduction. Moreover, the determination of transcription levels under co-culture conditions was conducted by targeting the biosynthetic genes involved in toxin biosynthesis. It needs to be noted that in the case of food (grape berries) contamination, bacterial antifungal efficacy was proved moderately lower compared to the *in vitro* study. Given that the generated data

should be as relevant as possible to real conditions in foods and food-related environments, it is really important to undertake additional *in situ* and *in vivo* studies.

Chapters 5 and 6 could contribute to the development of improved control strategies to reduce the risk of OTA contamination in grapes and related products. However, the importance of isolate variability was stressed throughout this thesis as a critical factor. The inherent differences among identically treated strains of the same microbial species constitute an important source of variability in microbiological studies. Taking into account that it is often difficult to compare findings from different studies, assessing isolate variability is of paramount importance and multiple strains should be tested, under well described conditions, with appropriate replicate experiments as well as reference strains. As previously described, bacterial strains of different origin employed in this work resulted in strain-dependent responses that corresponded to different antifungal activity. Moreover, different fungal wild isolates from Greek vineyards resented biological variability due to differences in phenotypic and transcriptomic toxigenic behavior.

The difficulty to predict and interpret the intricate relationships and interspecies differences within these microbial communities was a major challenge in this thesis. Tracking and defining molecular interactions among microorganisms is critical for understanding how single species and complex communities impact their associated host on the natural environment, providing an in-depth insight into ecological interactions of microbial consortia.

Secondary metabolite production is also controlled at an upper hierarchical level by global transcription factors encoded by genes related to the biosynthetic gene clusters. Such genes regulate multiple physiological processes and generally respond to environmental cues such as pH, temperature, and nutrition. To understand the

biosynthesis of mycotoxins as secondary metabolites, the important task is to target transcription factors that catalyze steps within the biosynthetic pathway. Nevertheless, due to different regulatory mechanisms and pathways, prediction of mycotoxin biosynthesis in a natural environment is important.

Generally, transcription and phenotypic biosynthesis of mycotoxins are coupled and gene induction occurs usually some time before the phenotypic biosynthesis of higher amounts of the respective mycotoxin. However it has to be underlined that measuring transcription generates only relative data. Transcription is not always ultimately coupled with the phenotypic production of mycotoxins, since post-transcriptional regulatory mechanisms may uncouple both processes. Furthermore, the amount of mRNA is much more variable over time than the amount of mycotoxin produced. Thus, monitoring transcription of mycotoxin biosynthesis genes provides an in-depth picture of the molecular events leading to mycotoxin biosynthesis activation in foods. The signal from certain environmental conditions at transcriptional level is mediated via transduction pathways. This situation demonstrates the complexity of biosynthesis regulation of fungal secondary metabolites during growth in their natural environment.

Among molecular tools, the reverse transcription qPCR (RT-qPCR) technique could be employed to analyze the temporal expression of genes involved in the mycotoxin biosynthetic pathway. RT-qPCR is highly sensitive and allows quantification of small changes in gene expression. In this perspective, highly efficient quantitative RT-qPCR protocols have been designed and the new protocols developed in this thesis will favor studies on the expression of OTA biosynthetic genes in response to different ecophysiological and environmental factors and, therefore, improve the knowledge on the regulation of OTA production.

Within a planet experiencing ecological shifts due to climate change, differences in phenotypic responses among strains of the same microbial species constitute an important source of variability in microbiological studies, and as such they need to be assessed, characterized and taken into account. There is extensive variability among fungal species with respect to their behavior concerning toxin production. Differences in phenotypic responses such as growth, virulence, even sporulation activity among strains of the same species can be significant and should not be overlooked. In order to underline the above regulatory mechanisms, it is critical to explore and understand strain variability of toxigenic fungi. Phenotypic variability data should be assesed in conjunction with corresponding findings of molecular studies on the basis of mycotoxin biosynthesis. Intra-species variability may have an important impact on the accuracy of microbial risk assessment outcomes, and therefore, should be systematically assessed and accounted for in the framework of such molecular approaches.

Elucidation of molecular principles behind these complex interaction regulatory networks is a big challenge and will provide not only a deeper insight into how fungi translate differential signals into mycotoxin biosynthesis but will also result in a deeper understanding of their ecological role in the future. Moreover, genome modifications and activation of silent genes can be investigated through the use of complex media and co-culture conditions. Hence, further studies are needed to clarify the regulatory mechanisms underlying the activation of OTA production. In addition, the availability of additional data from different -omics approaches will allow to fill the gap of knowledge in the near future.

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List of publications

1. **Lappa, I.K**, Mparampouti, S., Lanza, B., Panagou, E.Z., **2018**. Control of *Aspergillus carbonarius* in grape berries by *Lactobacillus plantarum*: A phenotypic and gene transcription study. *International Journal of Food Microbiology* 275, 56-65.

2. **Lappa, I.K**., Kizis, D., Panagou, E.Z., **2017**. Monitoring the temporal expression of genes involved in ochratoxin A production of *Aspergillus carbonarius* under the influence of temperature and water activity. *Toxins* 9, 10.

3. **Lappa**, **I.K.,** Simini, E., Panagou, E.Z., **2017**. *In vitro* evaluation of essential oils against *Aspergillus carbonarius* isolates and their effects on ochratoxin A related gene expression in synthetic grape medium. *Food Control* 73, 71-80.

4. **Lappa, I.K.**, Kizis, D, Natskoulis, P.I., Panagou, E.Z., **2015**. Comparative study of growth responses and screening of intra-specific OTA production kinetics by *A. carbonarius* isolated from grapes. *Frontiers in Microbiology* 6, 502.

Conferences:

5. **Lappa, I.K.**, Mparampouti, S., Panagou, E.Z., 2017. The effect of *Lactobacillus plantarum* on the ochratoxigenic potential of *Aspergillus carbonarius* at gene expression level. IAFP European Symposium on Food Safety, 29-31 March, Brussels, Belgium.

6. Gkana, E., **Lappa, I.K.**, Nychas, G.-J.E., Panagou, E.Z., 2016. Evaluation of forming mixed biofilms of *Aspergillus carbonarius* with *Lactobacillus plantarum* and *Lactobacillus pentosus*. 25th International ICFMH Conference-FoodMicro 2016, 19-22 June, Dublin, Ireland.

7. **Lappa, I.K.**, Simini, E., Panagou, E.Z., 2016. *In vivo* screening of 3 essential oils against ochratoxin A by *Aspergillus carbonarius* isolates in grapes. IAFP European Symposium on Food Safety, 11-13 May, Athens, Greece.

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