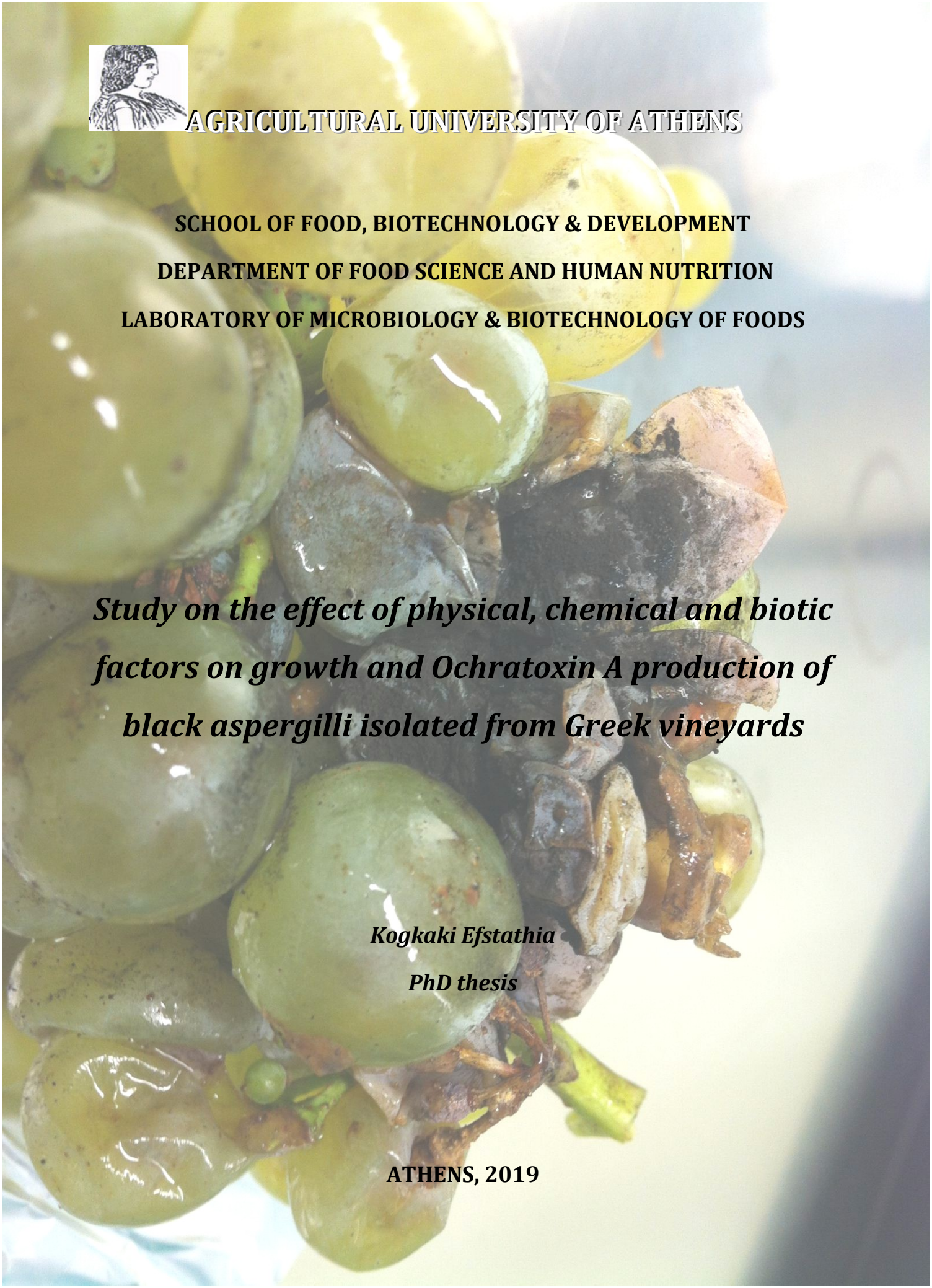

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LABORATORY OF MICROBIOLOGY & BIOTECHNOLOGY OF FOODS

Study on the effect of physical, chemical and biotic factors on growth and Ochratoxin A production of black aspergilli isolated from Greek vineyards

Kogkaki Efstathia

PhD thesis

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SUMMARY

Contamination of grapes and their products may be caused by several fungal species at preharvest, postharvest or grape processing period. Fungal isolates belonging to the *Aspergillus* section *Nigri* group have been reported as the main responsible species for Ochratoxin A (OTA) contamination. OTA is a fungal secondary metabolite with nephrotoxic, immunosuppressive, teratogenic, and carcinogenic properties with adverse effects in human and animal health. More specific, *Aspergillus carbonarius* has been referred as the major OTA producer of this group. Fungal spoilage control in grapes must be seriously considered in order to prevent growth of mycotoxin-producing species and ensure the lowest possible consumer exposure in such natural occurring toxins.

This thesis has initially focused on the development of a modelling approach in order to quantify the effect of temperature (15-38 °C), water activity (0.88-0.98) and sodium metabisulphite (NaMBS) concentration (0-200 mg L^{-1}) on the *Aspergillus carbonarius* growth and OTA production of on a Grape Juice based Medium (GJM). According to the results, the effect of NaMBS on the growth and lag phase of *A. carbonarius* was elucidated. Particularly when higher concentrations of the antifungal compound applied the inhibitory effect on the growth parameters was increased. Contrary, when studying the OTA production, at the temperature conditions of 38 & 20 °C and optimum a_w levels of 0.985 a_w, treatments with high NaMBS concentrations stimulated the toxin production. OTA content was enhanced even in the cases where fungal growth was delayed by the presence of the antifungal agent.

Further on, experiments based on *A. carbonarius* interspecific interactions with other black *Aspergillus* species on OTA production were undertaken. Fungal mixed spores were prepared by using three *A. carbonarius* isolates and two non-OTA producers of *A.* section *Nigri* species. These mixtures were grown on a Synthetic Grape Juice Medium (SGM) at different ratios (0:100, 25:75, 50:50, 75:25 and 100:0) and environmental conditions, such as temperature (15, 20 and 25 °C), water activity ranges (0.95 and 0.98), and incubation time (7, 14, 21 and 28 days). A great variation in OTA production of the three ochratoxigenic isolates of *A. carbonarius* after the co-inoculation with the competing non-toxigenic *Aspergillus* section *Nigri* species was noticed. The non-toxigenic species of *Aspergillus* section *Nigri* group either inhibited or stimulated the toxin amount released by *A. carbonarius*.

Additionally, experiments in fungal intermingling, as an approach to find new bio-control agents, were conducted. Although several studies have examined the ecophysiology of *A. carbonarius* at different environmental conditions, only few investigated the interactions between *A. carbonarius* and other fungal species**.** This experiment examined the effect of different environmental factors (a_w and temperature) and fungal interactions on the growth rates and OTA production, between three *A. carbonarius* isolates and eleven fungal species commonly isolated by grapes. Results showed that OTA production by *A. carbonarius* was mainly decreased after co-inoculation with the antagonist species. Few exceptions were observed with some of the competing species, resulting in OTA stimulation by the target fungal species. Nevertheless, the most important observation was that *Botrytis cinerea*, a common fungal species existed in the mycobiota of grapes, notably reduce growth and OTA production by *A. carbonarius*.

Besides the experiments with biological control agents, the effect of two antimicrobial compounds (natamycin and pine-resin) on the growth rate, lag phase duration and OTA accumulation of three *A. carbonarius* isolates was also studied. The effect of natamycin (0-1000 ng mL^{-1}) and pine-resin (0-2.61%, *w/v*) in combination with temperature (16.6-33.4 °C) and water activity (0.90-0.97) was investigated by means of a Response Surface Methodology (RSM). Results showed that natamycin was able to prolong the lag phase duration and decrease OTA production at intermediate temperature conditions (20 and 25 °C) whereas pine-resin had no effect on the growth rate and increased OTA levels at the same conditions assayed. Overall, OTA production was detected at lower levels after treatment with both antifungals at higher temperature conditions (30 °C).

Finally, in this study a first effort to distinguish black aspergilli species isolated from grapes of Greek vineyards with the use of FT-IR spectroscopy was attempted. Since characterization of these fungal members is considered as one of the most confusing and difficult tasks, alternative methods such as Diffuse Reflectance Infrared Fourier Transform (DRIFT) Spectroscopy have gained interest lately. *A. carbonarius* and species of *Aspergillus niger* aggregate (*A. niger* and *A. tubingensis*) showed classification accuracy of 100% in both model calibration and validation. Classification rate for calibration reached 95.3% for *A. carbonarius* species, whereas for validation the accuracy was 100%. Overall, correct classification for both model calibration and validation, based on molecularly identified black aspergilli species, was 98.3%.

In summary, the findings of this thesis may contribute to a better understanding of the effect of ecophysiological factors on OTA production and contamination, as well as on the improvement of strategies for OTA minimization in grapes and their derivatives. Additionally, this study may be considered as a first approach and guide for fungal discrimination and classification of closely-related species belonging to *Aspergillus* section *Nigri* group by using the FT-IR spectroscopy.

Scientific field: Food Mycology and Safety

Keywords: *Aspergillus carbonarius*, black aspergilli, Ochratoxin A, fungal growth inhibition, toxin control, biological control, fungal taxonomy

ΠΕΡΙΛΗΨΗ

Η μόλυνση των σταφυλιών και των προϊόντων τους μπορεί να προκληθεί από διάφορα είδη μυκήτων πριν το στάδιο της συγκομιδής (προσυλλεκτικά), μετά την συγκομιδή (μετασυλλεκτικά) ή στο στάδιο της μεταποίησης των σταφυλιών. Οι μύκητες που ανήκουν στην ομάδα *Aspergillus* section *Nigri* (μαύροι ασπέργιλλοι) έχουν αναφερθεί ως οι κυριότεροι επιμολυντές των σταφυλιών και των προϊόντων τους με Ωχρατοξίνη Α (ΩΤΑ). Η ΩΤΑ είναι ένας δευτερογενής μεταβολίτης των μυκήτων με νεφροτοξικές, ανοσοκατασταλτικές, τερατογόνες και καρκινογόνες ιδιότητες οι οποίες έχουν αρνητικές επιπτώσεις στην υγεία του ανθρώπου και των ζώων. Πιο συγκεκριμένα, ο μύκητας *Aspergillus carbonarius* έχει αναφερθεί ως ο ισχυρότερος παραγωγός ΩΤΑ μεταξύ των μαύρων ασπέργιλλων. Για το λόγο αυτό, θα πρέπει να λαμβάνεται πολύ σοβαρά υπόψη ο έλεγχος της αλλοίωσης των σταφυλιών από μύκητες, προκειμένου να ελαχιστοποιηθεί η ανάπτυξη ειδών που παράγουν μυκοτοξίνες, ώστε να εξασφαλιστεί η χαμηλότερη δυνατή έκθεση των καταναλωτών σε τέτοιες φυσικές τοξίνες.

Αυτή η εργασία επικεντρώθηκε αρχικά στην ανάπτυξη μαθηματικών μοντέλων για την ποσοτικοποίηση της επίδρασης της θερμοκρασίας (15-38 °C), της ενεργότητας ύδατος (0,88-0,98 a_w) και της συγκέντρωσης μεταδιθειώδους νατρίου (NaMBS) (0-200 mg L^{-1}) στην ανάπτυξη του μύκητα *Aspergillus carbonarius* και στην παραγωγή ΩΤΑ σε μικροβιολογικό θρεπτικό μέσο ανάπτυξης με βάση το χυμό σταφυλιού. Η αύξηση του μύκητα καταγράφηκε σύμφωνα με τη μεταβολή της διαμέτρου του μυκηλίου σε σχέση με το χρόνο. Στη συνέχεια τα δεδομένα προσαρμόστηκαν στο πρωτογενές μοντέλο Baranyi και Roberts για την εκτίμηση των κινητικών παραμέτρων της διάρκειας φάσης προσαρμογής (lag phase) και του ρυθμού αύξησης (growth rate) του μύκητα. Τέλος, οι κινητικές παράμετροι συσχετίστηκαν με τη θερμοκρασία, την ενεργότητα ύδατος και τη συγκέντρωση του μεταδιθειώδους νατρίου μέσω του δευτερογενούς μοντέλου Rosso. Παράλληλα με την αύξηση του μύκητα, μετρήθηκε η παραγόμενη ΩΤΑ, η οποία συσχετίστηκε με τις ανωτέρω παραμέτρους μέσω πολυωνυμικού μοντέλου δευτέρου βαθμού. Σύμφωνα με τα αποτελέσματα, παρατηρήθηκε ότι όσο υψηλότερη ήταν η συγκέντρωση του αντιμυκητιακού παράγοντα NaMBS τόσο μεγαλύτερη ήταν η ανασταλτική επίδρασή του στο ρυθμό αύξησης του μύκητα *A. carbonarius*. Αντιθέτως, για την παραγωγή ΩΤΑ, στις συνθήκες θερμοκρασίας 20 & 38 °C και βέλτιστης ενεργότητας νερού 0,985 a_w παρατηρήθηκε ότι οι υψηλές συγκεντρώσεις

NaMBS διέγειραν την παραγωγή τοξίνης. Η συγκέντρωση της ΩΤΑ αυξήθηκε ακόμη και στις περιπτώσεις όπου η ανάπτυξη του μύκητα μειώθηκε με την προσθήκη NaMBS.

Στη συνέχεια πραγματοποιήθηκαν πειράματα προκειμένου να προσδιοριστεί η επίδραση διαφορετικών ειδών μυκήτων στην παραγωγή ΩΤΑ από τον μύκητα *Aspergillus carbonarius*. Για τον σκοπό αυτό, σπόρια τριών στελεχών του μύκητα *A. carbonarius* που χαρακτηρίστηκαν *in vitro* από υψηλή παραγωγή τοξίνης και δύο μη ωχρατοξινογόνων μυκήτων της ομάδας *Aspergillus* section *Nigri* (*Aspergillus tubingensis* και *Aspergillus japonicus*) αναμίχθηκαν σε διαφορετικές αναλογίες (0:100, 25:75, 50:50, 75:25 και 100:0) σε τρυβλία που περιείχαν υπόστρωμα συνθετικού χυμού σταφυλιών. Τα τρυβλία επωάστηκαν σε διαφορετικές θερμοκρασίες (15, 20 και 25 °C), τιμές ενεργότητας ύδατος (0,95 και 0,98 a_w) και χρόνο επώασης (7, 14, 21 και 28 ημέρες). Τα αποτελέσματα έδειξαν μεγάλη διακύμανση ως προς την παραγωγή ΩΤΑ από τα τρία ωχρατοξικογόνα στελέχη του μύκητα *A. carbonarius* μετά από την συν-καλλιέργεια με τα ανταγωνιστικά μη τοξικογόνα είδη της ομάδας *A.* section *Nigri*, η παρουσία των οποίων είτε ανέστειλε είτε διέγειρε την παραγωγή τοξίνης στα είδη του *A. carbonarius* που μελετήθηκαν.

Επιπρόσθετα, μελετήθηκε *in vitro* η επίδραση της ενεργότητας ύδατος (0,90, 0,94 και 0,98 a_w) και της θερμοκρασίας (15, 20 και 25 °C) στην αύξηση και παραγωγή ΩΤΑ τριών στελεχών του μύκητα *A. carbonarius* με υψηλό δυναμικό παραγωγής τοξίνης, κατά την συγκαλλιέργεια με 11 στελέχη μη ωχρατοξινογόνων μυκήτων που σχετίζονται με τη μυκητοχλωρίδα των σταφυλιών. Παρόλο που αρκετές μελέτες έχουν εξετάσει την οικοφυσιολογία του μύκητα *A. carbonarius* σε διαφορετικές περιβαλλοντικές συνθήκες, λίγες μόνο μελέτες έχουν διερευνήσει τις αλληλεπιδράσεις μεταξύ του εν λόγω μήκητα και άλλων γενών μυκήτων. Η συγκαλλιέργεια των μυκήτων πραγματοποιήθηκε σε υπόστρωμα συνθετικού χυμού σταφυλιού για χρονικό διάστημα 15 ημερών. Τα αποτελέσματα έδειξαν ότι στις περισσότερες περιπτώσεις τα στελέχη του μύκητα *A. carbonarius* επικράτησαν έναντι των υπολοίπων μυκήτων, με εξαίρεση τους μύκητες *Penicillium spinulosum* και *Cladosporium* spp. σε θερμοκρασία 15 °C και *Botrytis cinerea* σε θερμοκρασία 20 °C που παρουσίασαν υψηλότερο ανταγωνισμό κατά τη συγκαλλιέργεια με τα στελέχη του μύκητα *A. carbonarius*. Αναφορικά με την παραγωγή ΩΤΑ, η συγκαλλιέργεια με τα ανταγωνιστικά είδη μυκήτων οδήγησε κυρίως σε μείωση ή σπανιότερα σε ελαφρά αύξηση της συγκέντρωσης της τοξίνης, ανάλογα με τη θερμοκρασία και την ενεργότητα ύδατος. Η σημαντικότερη παρατήρηση ήταν ότι το είδος *Botrytis cinerea* που συμπεριλαμβάνεται στη φυσική

μυκητοχλωρίδα των σταφυλιών, μείωσε σημαντικά την ανάπτυξη και την παραγωγή ΩΤΑ από τα τοξικογόνα στελέχη του μύκητα *A. carbonarius*.

Εκτός από τα παραπάνω πειράματα, μελετήθηκε επίσης η επίδραση δύο αντιμικροβιακών ενώσεων (ναταμυκίνη και ρητίνη πεύκου) στον ρυθμό αύξησης, τη διάρκεια της φάσης προσαρμογής και την παραγωγή ΩΤΑ από τρία τοξικογόνα στελέχη του μύκητα *A. carbonarius*. Συγκεκριμένα, μελετήθηκε η επίδραση της ναταμυκίνης (0-1000 ng mL^{-1}) και της ρητίνης πεύκου (0-2,61%, *w/v*) σε συνδυασμό με την θερμοκρασία (16,6-33,4 °C) και την ενεργότητα ύδατος (0,90-0,97 a_w) με την εφαρμογή πειραματικού σχεδιασμού που βασίστηκε στη μεθοδολογία της επιφανειακής απόκρισης (Response Surface Methodology, RSM) και κεντρικού σύνθετου σχεδιασμού (Central Composite Design, CCD). Τα αποτελέσματα έδειξαν ότι η ναταμυκίνη ήταν ικανή να επιμηκύνει τη διάρκεια της φάσης προσαρμογής του μύκητα και να μειώσει την παραγωγή ΩΤΑ στις ενδιάμεσες συνθήκες θερμοκρασίας (20 και 25 °C) ενώ η ρητίνη του πεύκου δεν έδειξε να έχει επίδραση στον ρυθμό αύξησης και στην παραγωγή ΩΤΑ του μύκητα στις ίδιες συνθήκες του πειράματος. Τέλος, η ΩΤΑ ανιχνεύθηκε σε χαμηλότερη συγκέντρωση μετά την προσθήκη και των δύο αντιμικροβιακών ενώσεων στις υψηλές θερμοκρασίες (30 °C).

Επιπρόσθετα, σε αυτή τη διατριβή μελετήθηκε ο διαχωρισμός και η ταξινόμηση μαύρων ασπέργιλλων, απομονωμένων από σταφύλια ελληνικών αμπελώνων, με τη χρήση φασματοσκοπίας υπερύθρου με μετασχηματισμό Fourier (FT-IR). Ιδιαίτερη έμφαση δόθηκε στο διαχωρισμό του ωχρατοξικογόνου μύκητα *A. carbonarius* από τα άλλα είδη μυκήτων της ομάδας *A. niger* aggregate (*A. niger* και *A. tubingensis*). Για το σκοπό αυτό, 182 συνολικά απομονώσεις μυκήτων των ειδών *A. carbonarius, A. niger* και *A. tubingensis,* οι οποίοι προηγουμένως είχαν ταυτοποιηθεί με μοριακές τεχνικές, αναλύθηκαν με φασματοσκοπία FT-IR. Η πρώτη παράγωγος συγκεκριμένων περιοχών κυματαριθμών του φάσματος (3002-2801 cm^{-1}, 1773-1550 cm^{-1} και 1286-952 cm^{-1}) επιλέχθηκε και υποβλήθηκε σε πολυμεταβλητή στατιστική ανάλυση (Διακριτική Ανάλυση). Τα φάσματα 130 απομονώσεων μυκήτων χρησιμοποιήθηκαν για την ανάπτυξη του μοντέλου και τα υπόλοιπα 52 φάσματα χρησιμοποιήθηκαν για την εξωτερική επικύρωση του μοντέλου. Το ποσοστό των ορθών προβλέψεων κατά την ανάπτυξη του μοντέλου ανήλθε σε 95,3% για τις απομονώσεις του μύκητα *A. carbonarius*, ενώ κατά την επικύρωση του μοντέλου το ποσοστό ανήλθε σε 100%. Το συνολικό ποσοστό ορθής ταξινόμησης για τις δύο κλάσεις των μυκήτων (*A. carbonarius* και *A. niger aggregate*) ανήλθε σε 97.6% και 100% κατά την ανάπτυξη και επικύρωση του μοντέλου αντίστοιχα.

Εν κατακλείδι, τα ευρήματα αυτής της διατριβής συμβάλουν στην καλύτερη κατανόηση της επίδρασης των οικοφυσιολογικών παραγόντων στην παραγωγή ΩΤΑ, καθώς επίσης και στη βελτίωση των στρατηγικών για την ελαχιστοποίηση της εν λόγω μυκοτοξίνης στα σταφύλια και τα παράγωγά τους. Επιπλέον, αυτή η μελέτη μπορεί να θεωρηθεί ως μια πρώτη προσέγγιση για τη διαφοροποίηση και ταξινόμηση διαφορετικών ειδών μυκήτων που ανήκουν στην ομάδα *A.* section *Nigri* χρησιμοποιώντας τη φασματοσκοπία FT-IR.

CHAPTER 1

Literature Review

1.1 *Aspergillus* spp. section *Nigri*: morphology and taxonomy

Aspergillus is one of the most economically important genera of fungi. It is classified to the *Aspergillaceae* family and belongs to the order *Eurotiales* (class *Eurotiomycetes*, phylum *Ascomycota*) as shown in the diagram below (Varga et al., 2011).

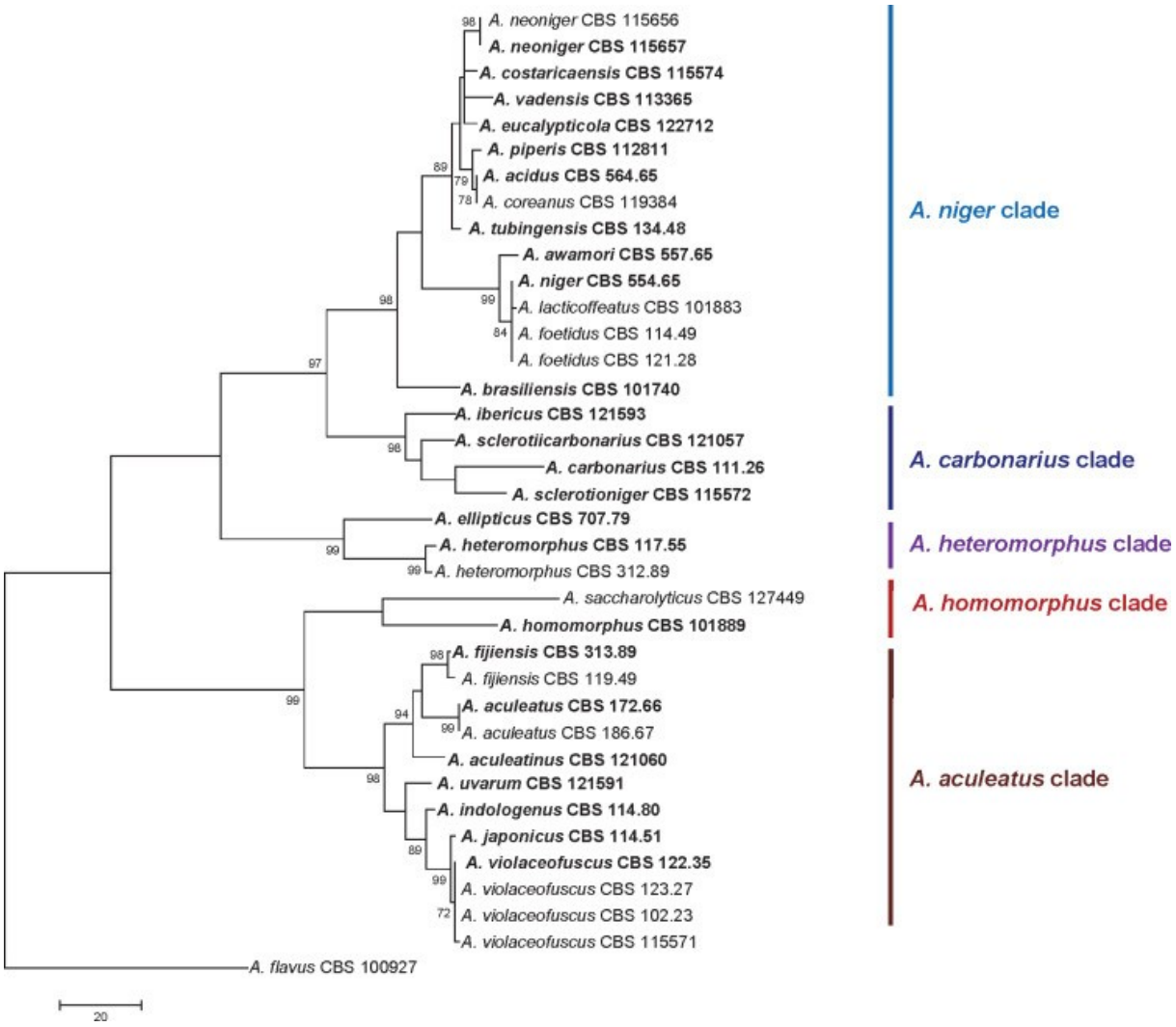

Diagram by Varga et al. (2011)

One of the MP trees obtained based on phylogenetic analysis of calmodulin sequence data of *Aspergillus* section *Nigri*. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

Fungal species belonging to the *Aspergillus* genus can reproduce only by asexual means. It is known that the so called black aspergilli species can develop black colonies and spores and thus they classified in the *Aspergillus* section *Nigri* group. Fungal members of this group are classified to the *Hyphomycetes* class and referred as anamorphic fungi. Conidiophore, the structure that bears asexual spores, is considered as the most important taxonomic characteristic in *Aspergillus* taxonomy. Specifically, *Aspergillus* species are characterized by the formation of conidiophores with smooth walled, elongated stipes which are ending up to roughly spherical or ellipsoidal vesicles-heads. These vesicles may bear either phialides, or metulae along with phialides, which are all borne simultaneously and give rise to spherical conidia (Fig.1.1). When vesicles produce only phialides the species are called uniseriate, whereas when vesicles produce metalue along with phialides, two palisades of the conidiophore's structure, the species are referred to as biseriate. Several species produce large conidia up to 7–9 μm including *A. carbonarius*, *A. ibericus*, *A. homomorphus*, *A. sclerotiicarbonarius* and *A. sclerotioniger*. Most other species produce conidia in the size range of 2.5–5 μm such as *A. aculeatus*, *A. japonicus*, *A. tubingensis* and *A. niger* (Samson et al., 2007). Finally, the color of the colony, the conidial heads, the shape and ornamentation of the conidia are those characteristics that unambiguously distinguish *Aspergilli* by any other genera and subgenera (Abarca et al., 2004; Pitt and Hocking, 2009; Bennett, 2010).

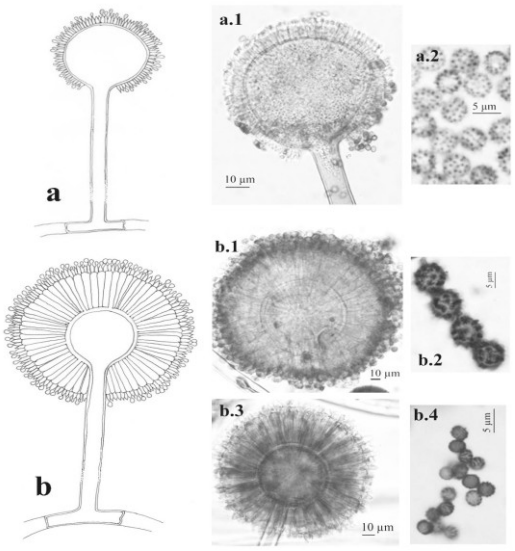

Figure 1.1: Uniseriate *Aspergillus* conidiophore. Note the single palisade of cells (phialides) which give rise to conidia. Conidial head (a.1) and conidia (a.2) of *A. japonicus* var. *aculeatus* (A-969, UAB Mycology group). b: Biseriate *Aspergillus* conidiophore. Note the two palisades of cells (metulae and phialides). *A. carbonarius* (A-1157, UAB Mycology group), conidial head (b.1) and conidia (b.2); *Aspergillus niger* aggregate (A-219, UAB Mycology group), conidial head (b.3) and conidia (b.4) (picture from Abarca et al., 2004).

1.2 *Aspergillus* mycotoxins

Aspergillus species are filamentous fungi that are commonly found in soil, decaying vegetation, seeds and grains, where they thrive as saprotrophs and generally considered ubiquitous in nature. They are frequently colonizing plant debris and causing infection in several agricultural products (Perrone et al., 2007; Palumbo et al., 2010; Copetti et al., 2012; Hayrettin et al., 2012; Perrone et al., 2013). During their life cycle, several *Aspergillus* species are capable of producing a wide range of hazardous mycotoxins with negative impact in human and animal health (Scott, 1994, 2004). In specific, mycotoxin production by *Aspergillus* species is mainly a matter of fruit and vegetable decaying and spoilage as well as environmental conditions, species and other parameters may play an important role. Namely, mycotoxins in fruits and vegetables are mostly derived by these species: i) Aflatoxins, which are mainly produced by the aflatoxigenic species *A. flavus* and *A. parasiticus*, in figs, dates, citrus and other fruits grown in tropical and subtropical climates (Doster et al., 1996; Shenasi et al., 2002) and ii) Ochratoxin A (OTA), produced mainly by the ochratoxigenic species *Aspergillus carbonarius*, *Aspergillus niger* aggregate and other *Aspergillus* species in grape derived products, as well as in various food and feed commodities (grains, legumes, coffee, dried fruits, beer and meat) (Abrunhosa et al., 2001; Cabañes et al., 2002; Perrone et al., 2007; Kizis et al., 2014). The main naturally produced aflatoxins are B_1, B_2, G_1 and G_2, with B_1 aflatoxin being the most commonly found mycotoxin of this group producing the highest concentration in contaminated products (Sweeney and Dobson, 1998; Perrone et al., 2007). Even though, OTA and aflatoxins are the most important toxins released by *Aspergillus* species, other mycotoxins can be also synthesized by these species. Some other mycotoxins/secondary metabolites often released by *Aspergillus* spp. are mainly cyclopiazonic acid, sterigmatocystin, citrinin, penicillic acid, secalonic acid B, D and F, aculeasin, eumodin, malformins, naphtopyrones, fumonisins and pyranonigrin which may also cause toxicological effects on humans and animals (Samson et al., 2007; Barkai-Golan, 2008).

1.2.1 OTA – chemical structure and parameters

Ochratoxin A (OTA) is a secondary metabolite produced by many filamentous species belonging to the genera *Aspergillus* and *Penicillium* (Sweeney and Dobson, 1998; Moss,

2002; Cabañes et al., 2010; Labrinea et al., 2011). It can be found in several food products such as cereals, coffee, beans, cocoa beans, nuts, beer, wine, spices, vine fruits (raisins, currants and sultanas) (Aish et al., 2004; Varga and Kozakiewicz, 2006). OTA has carcinogenic, nephrotoxic, immunotoxic, tetratogenic and genotoxic properties. It has been also implicated as the agent responsible for Balkan Endemic Nephropathy, a fatal kidney disease (Vukelic et al., 1991; JECFA, 2001). The International Agency for Research on Cancer (IARC) has given OTA a Group 2B classification, a possible human carcinogen (IARC, 1993).

Analyzing OTA structure, the isocoumarin ring of OTA is a pentaketide derived from the dihydrocoumarins family coupled to β-phenylalanine (Khoury and Atoui, 2010; Wang et al., 2016). Its chemical name is: L-phenylalanine-*N*-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyrane-7 yl)carbonyl]-(*R*)-isocoumarin and its chemical structure is presented in Figure 1.2.

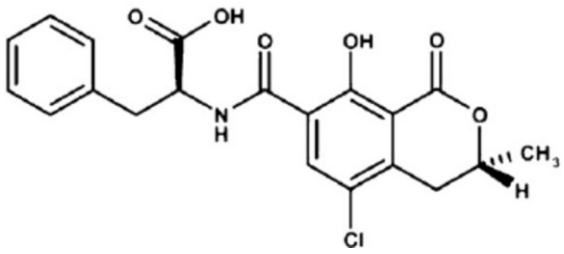

Figure 1.2: Chemical structure of OTA.

There are many analogues to OTA, particularly, ochratoxin B (OTB) which is the dechloro analog of OTA, ochratoxin C (OTC) its ethyl ester, the isocoumaric derivative of OTA, ochratoxin α (OTα), and its dechloro analog, ochratoxin β (OTβ), all of them characterized as fungal metabolites (Khoury and Atoui, 2010).

Production of OTA released by *Aspergillus* spp. is associated with fungal growth rate combined with several intrinsic and extrinsic parameters. These parameters affecting fungal growth on grapes and their derivatives are mainly water activity (a_w), environmental and temperature conditions, fungal strain, vineyard position, soil and culture type, harvest as well as storage conditions (Clarke et al., 2004; Battilani et al., 2006a). Battilani et al. (2004b) reported that susceptibility to *A. carbonarius* colonization and OTA production varied to different grape cultivars. Other studies have demonstrated that OTA amount depended on the latitude of the region, and reported that mycotoxin contamination in lower latitudes is higher and thus OTA accumulation is more likely to occur (Zimmerli and Dick, 1996; Stander and

Steyn, 2002; Bellí et al., 2005a). Additionally, other factors like wounded berries, by abiotic and/or biotic causes, are also a favorable substrate for fungal colonization and OTA increasement (Battilani et al., 2004b; Cozzi et al., 2006).

1.2.2 OTA producers of *Aspergillus* spp. section *Nigri*

1.2.2.1 *Aspergillus carbonarius*

More than 20 species in the *Aspergillus* genus have been cited as ochratoxigenic fungi, but only a few of them are known as regularly OTA sources (Frisvad et al., 2004; Samson et al., 2004). Several studies have demonstrated that the main ochratoxigenic black aspergilli species are the so-called *Aspergillus niger* aggregate species and the biseriate species *Aspergillus carbonarius*, although the highest percentage of OTA-producing strains has been detected in the latter species (Serra et al., 2006). The ecology and significance of these ochratoxigenic species has been studied by researchers across the Mediterranean, South American countries and Australia (Cabañes et al., 2002; Da Rocha Rosa et al., 2002; Battilani and Pietri, 2002; Magnoli et al., 2003; Serra et al., 2003; Leong et al., 2007), with *A. carbonarius*, as the most prevalent OTA producer species which have been investigated the most. Reported sources of infection with *Aspergillus carbonarius* are grapes, wine, dried vine fruits, figs, peanuts, maize, bee pollen, paprika, red bay berries and fermenting cocoa beans (Pitt and Hocking, 2009). Despite of the higher incidence of species belonging to the *A. niger* aggregate found in vineyards, only 5-10% of *A. niger* OTA-producing strains were detected, whereas more than 50% and in some cases up to 100%, is *A. carbonarius* species (Heenan et al., 1998; Serra et al., 2005; Battilani et al., 2006a). There is an argument as regards the percentage of OTA producing strains of *A. carbonarius* isolated from grapes. Nevertheless, Somma et al. (2012) reached to the conclusion that almost 100% of isolates were OTA producers, based on literature published before 2006. Other studies based on *A. carbonarius* identification with molecular techniques, showed that less than 50% of the identified species were OTA producers (Martínez-Culebras and Ramón, 2007; Spadaro et al., 2012). Recently, another study based on morphological and genotypic methods for fungal identification, has shown the existence of non-ochratoxigenic *A. carbonarius* (Cabañes et al., 2013). In the previous study, three non-ochratoxigenic wild strains belonging to the consistent OTA producing species of *A. carbonarius*, have been isolated and clearly identified. However, a general overview suggested that *A. carbonarius* is the main species responsible for the

presence of OTA in wine since OTA mean production by this species is always higher compared to the toxin released by other producing species (Accensi et al., 2001; Bau et al., 2006; Perrone et al., 2006a; Spadaro et al., 2012).

The optimum conditions for OTA production by this species have been reported at cooler temperatures 15 °C and 0.95-0.97 a_w or at 20 °C and 0.98-0.99 a_w. At temperatures above 35 °C, only low levels of OTA or no toxin at all has been detected, whereas the lowest a_w level for OTA production has been reported near 0.92 (Tassou et al., 2007a; Natskoulis et al., 2009; Pitt and Hocking, 2009). Moreover, researchers have found that suitable temperature conditions for OTA production by *A. carbonarius* on *in vitro* conditions were between 15-20 °C (Esteban et al., 2004; Bellí et al., 2005b; Tassou et al., 2007b). Regarding pH, OTA can be produced over a wide pH range between 2–10 in combination with various temperature conditions (Esteban et al., 2005).

1.2.2.2 *Aspergillus niger* aggregate

Black aspergilli species with high ochratoxigenic potential, are mainly those belonging to the so-called *Aspergillus niger* aggregate (Perrone et al., 2007). Even though OTA can be primarily produced when *Aspergillus carbonarius* infects berries before harvest, other relative toxigenic species of this group, may also contribute to OTA contamination, with *A. niger* being by far the most common species of *Aspergillus* present on grapes (Chulze et al., 2006; Leong et al., 2006a; Leong et al., 2006c; Varga and Kozakiewicz, 2006; Leong et al., 2007). It has been found that *A. niger* can produce OTA under various conditions (Bellí et al., 2004c; Esteban et al., 2004, 2006a; Leong et al., 2006b). OTA production by *A. niger* aggregate normally occurs at 20-25 °C and 0.95-0.98 a_w but usually only a low percentage of *A. niger* isolates can produce this toxin (Esteban et al., 2004). *A. niger* has been isolated from vine fruits, dried, smoked and cured fish, biltong, cocoa beans and spices (Pitt and Hocking, 2009).

Studies have been reported that the percentage of ochratoxigenic isolates in *A. carbonarius* ranges from 25 to 100 %, whereas in the *A. niger* aggregate group the toxigenic potential is lower, ranging from 0.6 to 50 %. Therefore, *A. carbonarius* is considered as the main OTA producer within the *Aspergillus* section *Nigri*, yet it is very difficult to know the extent of its natural occurrence in products because all black aspergilli are commonly referred to as '*A. niger*' (Abarca et al., 2004). Molecular studies support the division of the *A. niger* aggregate into two morphologically indistinguishable species, *A. niger* and *A. tubingensis*

(Accensi et al., 1999; Perrone et al., 2006b; Martínez-Culebras and Ramón, 2007). Perrone (2006a) detected a low percentage of OTA producing strains among the *A. niger* aggregate species. Although the ability of *A. niger* to produce OTA has been previously described, species of *A. tubingensis* have been subsequently reported to have also ochratoxigenic potential. Medina et al. (2005) were the first to report that *A. tubingensis* species are able to produce OTA and concluded that the difficulty to discriminate *A. niger* and *A. tubingensis* based on morphological characteristics may have provided misidentifications. Therefore isolates of black aspergilli that were identified and studied for their toxigenic profile were classified as *A. niger* instead of *A. tubingensis*.

1.3 Incidence of OTA in grapes and grape products

The presence of OTA in grapes, musts, wines and dried vine fruits has been investigated around the Mediterranean countries (Greece, France, Portugal, Italy and Spain), South American countries and Australia (Cabañes et al., 2002; Da Rocha Rosa et al., 2002; Battilani and Pietri, 2002; Magnoli et al., 2003; Serra et al., 2003; Chulze et al., 2006; Leong et al., 2007). The aforementioned studies have demonstrated that fungal species belonging to *Aspergillus* section *Nigri*, and particularly *A. carbonarius*, are the major responsible species for OTA contamination in grapes and their products (Perrone et al., 2007). Wine is considered as the second most important source of OTA (*ca.* 10% to 15% of the total daily intake), after cereals in human consumption (*ca.* 50% of the total daily intake) (Otteneder and Majerus, 2000; Cabañes et al., 2002; Stefanaki et al., 2003; Khoury and Atoui, 2010).

The presence of OTA in musts and wine is due to fungal contamination of the grapes at pre- and/or post-harvest operations, or during the wine-making process. Therefore, OTA occurrence is considered as a consequence of grape contamination in the vineyard from véraison onward and sometimes even as early as setting, by various ochratoxigenic species and especially by species of the *Aspergillus* section *Nigri* group (*A. carbonarius* and *A. niger* aggregate). OTA production increases rapidly at the maturation stage and for this reason the date of grape harvest has an important effect on OTA content in these products (Leong et al., 2006c). Red wine has been found to have higher amounts of OTA compared to white or rosé wines, and so processing has been considered as an important stage in diminishing the potential risk of OTA contamination in wines (Grazioli at el., 2006).

1.4 Commission Regulation- Limits of legislation

As a potential health hazard, OTA ingestion has been limited by the Commission of the European Communities (EC) to protect consumer's health from the harmful effects of this mycotoxin. European countries with frequent OTA incidences have inducted regulations for the maximum OTA levels in some food commodities. In the European Union (EU), the maximum OTA level allowed in wine, grape must and grapes is 2 μg kg^{-1} and in dried vine fruits is 10 μg kg^{-1} (Regulation No. 1881/2006). Moreover, maximum levels of daily OTA intake have been set up for various food products, such as roasted coffee (5 μg kg^{-1}), instant coffee (10 μg kg^{-1}), raw cereal grains (5 μg kg^{-1}), processed cereal products (3 μg kg^{-1}), baby foods and processed cereal products for infants and young children (0.5 μg kg^{-1}), grape-based wines (2.0 μg kg^{-1}), grape juices (2.0 μg kg^{-1}) and dried vine fruits (10 μg kg^{-1}). These are the most important products with permissible dosages of OTA intake per day as established by the European Community (European Commission, 2005a).

1.5 OTA control

1.5.1 Physical control

A basic approach to suppress *Aspergillus* growth and control mycotoxin contamination in fruits and vegetables intended for consumption is the minimization of postharvest fungal pathogens. Initially, effective control of *Aspergillus* mycotoxins is based on good agricultural practices in the field before harvest. This may limit the growth of mycotoxigenic fungi, whereas postharvest contamination can be minimized by proper practices such as curing, sorting, drying, and heat treatments (Tamm, 2001). On the other hand, mycotoxins are very stable to heat with high inactivation temperature conditions (Betina, 1989). Notwithstanding, destroying the spores and mycelia of the mycotoxigenic fungi by heat treatments, in combination with other means (e.g., microwave irradiation, high ultrasound amplitudes, gaseous ozonation), might reduce or eliminate fungal growth and toxin production.

Researchers have studied the kinetics of the conidia survival of pathogenic *Aspergillus* species, like *A. fumigatus*, *A. flavus* and *A. niger*, by exposing them to heat treatment at 60 °C and microwave irradiation (Araujo et al., 2006). Heating the conidia of *A. flavus* and *A. niger*

at 60 °C for 45 min was found to be fungicidal (reduction > 10^4 conidia mL^{-1}), but was not effective against the conidia of *A. fumigatus*. Additionally, short periods of microwave irradiation (40 s) resulted in a significant reduction of conidia viability for both fungal species. Other treatments corresponding to physical control such as hot-water dipping (HWD), were undertaken in chilli pepper fruits at 52 °C for 15 min resulting in 100% inhibition of *Aspergillus* spp. growth (Ajithkumar and Naik, 2006).

1.5.2 Chemical control

The use of chemical compounds is a very attractive strategy to prevent mycotoxin production in order to minimize exposure of human and animal health in such natural occurring mycotoxins. However, controlling fungal diseases and toxin production on grapes by using several fungicides, is quite complex, since recent evidence indicates that the application of fungicides to grapes, under certain conditions, may stimulate toxin production (Bellí at el., 2006b; Medina et al., 2007a). Nonetheless, other studies have previously examined the effectiveness of various commercial fungicides, such as Carbendazim and Switch, against fungal growth and OTA production by *A. carbonarius* (Tjamos et al., 2004; Bellí et al., 2006b; García-Cela at el., 2011). Carbendazim, which is widely used against fungal infections in grapes, showed a positive influence on OTA production by *A. carbonarius* in the field, increasing OTA content in grape juices and wines, whereas Switch was effective in reducing grape infection and OTA production (Bellí et al., 2006b; Medina et al., 2007b). Chemical treatments with mepanipyrim, pyrimethanil, fluazinam, iprodione and a mixture of cyprodinil and fludioxonil have been found effective in reducing to a different extent both fungal growth and OTA production in grape bunches. The latter mixture of fungicides was confirmed as the most effective in field trials carried out in several Mediterranean countries including France, Spain, Greece and Italy (Tjamos et al., 2004; Kappes et al., 2005; Bellí et al., 2007).

Bellí et al. (2007) investigated the antifungal effect of Chorus and Switch *in vitro* against *Aspergillus* section *Nigri* and found that the fungicide Switch was the most effective in reducing the infection of grapes by *A. carbonarius* and subsequently OTA production. Valero et al. (2007b) confirmed that these two fungicides remained active on grapes during dehydration and were effective in OTA reduction. Lo Curto et al. (2004) observed that application of some other fungicides commonly used in grapes, such as Azoxystrobin (a

strobilurin derivative) or Dinocap (a dinitrophenyl derivative) in combination with sulfur were able to decrease OTA content in wines.

Moreover, *in vitro* studies undertaken by Bellí et al. (2006b) screened 26 fungicides and resulted that the fungicides that were able to inhibit *A. carbonarius* growth were also effective in reducing OTA production. In general, fungicides that contained copper or strobilurins reduced both, growth and OTA production, contrary to sulfur fungicides. Among the fungicides that inhibited *A. carbonarius* growth in synthetic medium, cyprodinil seemed to be the most effective active ingredient to stop fungal growth when reduced doses were tested. It is important to underline that fungicides must be applied with care since some of them, such as carbendazim, have been found to reduce fungal flora but stimulate OTA production (Bellí et al., 2006b; Medina et al., 2007b).

Another strategy to reduce fungal growth and mycotoxin production is the use of antioxidants and essential oils extracted from plants. For example, vanillic acid or 4-hydroxybenzoic acid and the essential oils from *Thymus vulgaris* or *Aframomum danielli*, have affected both, fungal growth and OTA biosynthesis (Aroyeun et al., 2007; Palumbo et al., 2007; Kabak et al., 2009). From a human health perspective, the use of antioxidants as antimicrobial agents is allowed by the US Food and Drug Administration (FDA) and regarded as safe (GRAS). Even though these antioxidants have not been tested on dried vine fruits, some studies have shown that these compounds have a protective action in food since they could maintain their organoleptic properties (Nguefack et al., 2007; Barberis et al., 2009). Lappa et al. (2017) studied four essential oils (clove, mandarin, cinnamon and lemongrass) for their efficacy against *Aspergillus carbonarius* growth and OTA production. The aforementioned researchers concluded that all the essential oils examined could become an attractive application for future use against the risk of OTA, since inhibition of fungal growth in certain concentrations could result in mycotoxin prevention.

Overall, a strict legislation concerning the use of chemicals and maximum residue levels of pesticides (MRLs) on the field has been established in the European Union for many products, including grapes, in order to ensure the lowest possible consumer exposure (European Commission, 2005b). For this reason, there is a growing interest in examining alternative antifungal compounds, against the presence of *A. carbonarius* on grapes and their by-products, based on a biological approach (Medina et al., 2007a).

1.5.3 Biological control

The need to develop natural alternatives to chemical control strategies has led to the application of various yeast strains or other microorganisms as biological control agents against ochratoxigenic fungi. Biological control, as a part of Integrated Pest Management (IPM) against OTA–producing fungi causing rot in grapes, has been investigated pre-harvest in vineyards and post-harvest (Bleve et al., 2006; De Felice et al., 2007; Valero et al., 2007b; Dimakopoulou et al., 2008). This treatment is an alternative to chemical strategy and it has been proposed as an environmental approach to prevent fungal spoilage and therefore OTA contamination.

Strains of *A. carbonarius* can produce OTA from grape harvesting to the beginning of the wine-making process, which usually does not exceed 24 to 36 hours in total. Control of *A. carbonarius* in the field and in the short time between harvest and wine-making is an important step to decrease OTA accumulation in the final product. Studies have reported that OTA levels have decreased during wine fermentations, due to the action of lactic acid bacteria and yeasts participating in this procedure (Bejaoui et al., 2004; Shetty and Jespersen, 2006; Angioni et al., 2007; Patharajan et al., 2010). The ability of microbial antagonists to compete with the resident microbiota and their interactions has been evaluated. More specific, researchers have studied the ability of some *Aureobasidium pullulans* strains to suppress rot caused by *A. carbonarius* on detached and wounded wine grape berries (De Felice et al., 2007). Interestingly, this study demonstrated that certain strains of this yeast were able to decrease OTA to the less toxic compound, ochratoxin a, on *in vitro* experiments and finally OTA content was less compared to infected and non-treated with the yeast berries. In another study, a strain of *Aureobasidium pullulans* was proved as an effective yeast species in reducing sour rot infection by *A. carbonarius* on berries at harvest and subsequent OTA accumulation in must (Dimakopoulou et al., 2008). Cubaiu et al. (2012) studied the ability of *Saccharomyces cerevisiae* wine strains in growth inhibition and OTA accumulation by *A. carbonarius* and *A. ochraceus*. The results of this work showed that some antagonist yeasts into the microbial community among grape berries was able to control growth and toxin production of black aspergilli species.

Apparently, the microorganisms with potential utility in biological control-based strategies appeared to be yeasts, due to their ability to colonize plant surfaces or wounds for longer periods under dry conditions in combination with their mechanisms of antagonism

during the wine making process. However, during the post-harvest period, species of *A.* section *Nigri* can grow together with other fungal species that may play an important role in the final OTA content through their interaction with fungal OTA producers. For this reason, many studies have employed dual fungal cultures on *in vitro* assays to investigate the interactions among fungal species, in order to undertake a screening for potential biocontrol agents that may contribute to OTA reduction by *A. carbonarius* or other ochratoxigenic species (Valero et al., 2006a, 2007c; Kogkaki et al., 2015). Valero et al. (2007a) studied the effect of intra and interspecific interaction on OTA accumulation of different populations of *A.* section *Nigri* in grapes, as affected by other fungi, during a simulated *in vitro* sun-drying process. The aforementioned researchers concluded that OTA content by *A. carbonarius* was reduced when competing fungi were co-inoculated in both healthy and injured grapes. *Eurotium amstelodami* was the only competing fungus which increased OTA accumulation in healthy grapes. The complex fungal interactions which may take place during sun-drying process could act as an additional control factor in OTA accumulation by mycotoxigenic fungal species. Another study by Kogkaki et al. (2015) examined the efficacy of some non-ochratoxigenic grape-associated fungal isolates on growth inhibition of *A. carbonartius* and OTA reduction. It was reported that some fungal species were capable to reduce toxin production and especially *Botrytis cinerea*, a highly grape-associated fungus in the wine making process, was proved to be a strong inhibitor of OTA accumulation in combination with different environmental factors.

Biological control and microbial competition has been proposed so far as an effective option to control plant pathogens. An advantage of this strategy is that it could be used in combination with reduced dosages of fungicides in order to decrease fungal growth and toxin production. Competition between microorganisms may have a catalytic effect on the secondary metabolism of filamentous fungi. Among these microorganisms, yeasts could become a promising bio-control agent towards ochratoxigenic *Aspergillus* because of their biology and non-toxic properties.

1.6 Predictive mycology

The need to ensure the microbiological quality and safety of food products has gained interest lately and the use of mathematical models for quantifying and predicting microbial behaviour has been applied (Lahlali et al., 2005). Quantitative predictions of the responses of

several microorganisms to various environmental conditions, have been implemented and numerous studies are available in the literature dealing with the predictive modelling approach of fungi (Tassou et al., 2007b; Gougouli and Koutsoumanis, 2010; Garcia et al., 2011; Kapetanakou et al., 2011).

In predictive mycology, mathematical models are used to predict growth and toxin production by fungal species, and also to study their response to diverse environmental factors. Microbial kinetic models can be classified as primary, secondary or tertiary (Whiting, 1995). Primary models demonstrate how microorganism populations change against time in certain conditions (Marks, 2007). Secondary models refer to primary models in relation with the influence of environmental factors, such as water activity, pH, temperature etc., on fungal germination and growth. Tertiary models combine primary and secondary models with a computer interface, providing a complete prediction tool (Marks, 2007). Initially, there was a tendency to employ models that have been developed for bacteria due to the lack of specific models for fungi.

Starting with the primary models, Baranyi et al. (1993) developed a growth model to predict fungal development (expressed as increase in colony diameter) against time. Later on, these primary models were adapted by other researchers to fit colony diameter growth curves of *A. flavus* (Gibson et al., 1994), *Penicillium roqueforti* (Valík et al., 1999), *P. brevicompactum* (Membré and Kubaczka, 2000), *Fusarium verticillioides* and *F. proliferatum* (Samapundo et al., 2005).

Fungal colonization and/or mycotoxin production are generally influenced by a variety of factors such as temperature (T), substrate, pH or water activity (a_w), with the latter being as the most important environmental factor affecting fungal germination and growth (Aldars-García et al., 2015). Most studies in predictive mycology were mainly focused on the effect of environmental factors, on fungal growth and mycotoxin production under static conditions, whereas in realistic situations the environmental conditions can fluctuate. However, reports on fungal behavior under environmental fluctuating conditions are missing and limited information is available (Gougouli and Koutsoumanis, 2010, 2012; Peleg and Normand, 2013; Kalai et al., 2014).

It is important to take into account these fluctuations in model development and validation, otherwise their applicability is compromised. Extrapolation of data to predictions in food products is not straightforward due to the fact that predictive models are often built under laboratory conditions with synthetic media and take a limited number of factors into account compared to the numerous factors influencing fungal growth in food products (Garcia

et al., 2009). Therefore, a good way to validate a model is to compare its prediction to data obtained from food products. Moreover, OTA prediction in foods is also a challenging task due to the variety of factors influencing its substance. Finally, the existence of robust predictive models for quantifying the extent of fungal growth and the rate of OTA production in food commodities would be useful in order to control the presence of such a hazard and prevent risks for human and animal health.

1.7 FT-IR spectroscopy and fungal classification

Lately, new techniques, such as Fourier transform infrared spectroscopy (FT-IR), have gained interest in fungal identification and classification. Many authors have reported the use of mid-infrared spectral data and chemometrics as promising discrimination tools for microorganisms (Fischer et al., 2006; Tralamazza et al., 2013; Lecellier et al., 2014, 2015). Fourier transform infrared spectroscopy (FT-IR) is a powerful technique for characterizing microorganisms where a sample is irradiated by IR light, resulting in absorption of energy by molecular vibrations. The absorption bands of the FT-IR spectra can be assigned to specific vibrations of molecular compounds such as lipids, proteins, phosphate-containing compounds such as RNA and DNA, phospholipids, and carbohydrates, providing a spectral fingerprint of the sample studied that may aid microorganism classification (Tralamazza et al., 2013).

FT-IR spectroscopy was successfully used by Garon et al. (2010), for *Aspergillus* differentiation at species level (*A. flavus*, *A. fumigatus* and *A. parasiticus*) and allowed discrimination rates of 75 and 100% in non-toxigenic and toxigenic *A. flavus* and *A. parasiticus* isolates, respectively. Tralamazza et al. (2013) also studied the potential of FT-IR spectroscopy to discriminate *Aspergillus* species (*A. niger*, *A. ochraceus* and *A. westerdijkiae*). They reported that differentiation of similar species, namely *A. ochraceus* and *A. westerdijkiae* during internal model validation presented robust results, with overall correct classification between both species of 83.3%. Moreover, they noticed that *A. ochraceus* and *A. westerdijkiae* spectra were much closer to each other compared to *A. niger* and they were also consistent in morphology and molecular phylogeny, yet full-spectrum multivariate analysis techniques were employed to distinguish features with discriminating capacity for fungal classification.

To our knowledge, even though several studies have highlighted the potential of FT-IR to discriminate fungal species, no research has been undertaken so far to investigate its effectiveness as a rapid tool in the differentiation of black aspergilli.

1.8 Aim and objectives of this study

The aim of this thesis was focused on *A. carbonarius* fungal control and therefore OTA minimization in grapes and their derivatives using chemical, biotic or abiotic factors. Moreover, discrimination of black aspergilli species isolated from grapes of Greek vineyards was attempted using FT-IR spectroscopy as a rapid alternative technique to classical phenotypic and molecular identification.

Specifically, the objectives of this study were: i) to develop and validate kinetic models for *A. carbonarius* growth on both Synthetic and Natural Grape Juice Media as a function of temperature, water activity and antifungal compounds (metabisulphite, pine-resin and natamycin), ii) to evaluate alternative treatments for fungal and OTA minimization, such as the use of antagonistic fungal strains in mixed cultures or dual culture treatments, and iii) to evaluate the use of FT-IR spectroscopy as a new method for fungal discrimination among black aspergilli species.

Findings of this study may contribute to a better understanding of OTA production and contamination, as well as to improve strategies for OTA minimization in grapes and their by-products. Additionally, this study may be considered as a first approach for fungal discrimination and identification of fungal members belonging to *Aspergillus* section *Nigri* group using FT-IR spectroscopy as a new, rapid and innovative technique in food mycology.

CHAPTER 2

Modelling the influence of temperature, water activity and sodium metabisulphite on the growth and OTA production of Aspergillus carbonarius isolated from Greek wine grapes

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Abstract

The purpose of the present study was to develop a modelling approach to quantify the effect of temperature (15-38 °C), a_w (0.88-0.98) and sodium metabisulphite (NaMBS) concentration (0-200 mg L^{-1}) on the growth and OTA production of *Aspergillus carbonarius* on a Grape Juice based Medium (GJM). Growth responses of the fungus were recorded over time in terms of colony diameter changes, and fitted to the primary model of Baranyi and the estimated maximum growth rates (μ_{max}) and lag phases (λ) were subsequently modelled as a function of temperature, a_w and NaMBS concentration using the cardinal values model with inflection (CMI). Moreover, OTA production was measured during fungal growth and modelled as a function of the same parameters through a quadratic polynomial model. Results showed that NaMBS increased the lag phase of *A. carbonarius,* particularly at 38 °C/0.98 a_w and 38 °C/0.96 a_w, as well as at lower a_w levels regardless of temperature. In the lowest NaMBS concentration (50 mg L^{-1}) there was no inhibitory effect, while at higher concentrations (100 and 150 mg L^{-1}) fungal growth was delayed. No growth was observed at 200 mg L^{-1} of NaMBS irrespective of temperature and a_w levels. The optimum values for growth were found in the range 30-35 °C and 0.96 a_w, while for OTA production at 20 °C and 0.98 a_w. The developed models were subjected to internal and external validation and presented satisfactory performance as inferred by graphical plots and statistical indices (bias and accuracy factors). The present study will complement the findings on the ecophysiology of *A. carbonarius* using NaMBS as an inhibitory agent.

Keywords: Aspergillus carbonarius, Ochratoxin A, Sodium metabisulphite, predictive mycology

2.1 Introduction

A number of certain filamentous fungi are able to cause food spoilage with considerable economic losses in the food chain. In the last years, the focus on these fungi has been shifted on their ability to produce secondary metabolites, such as mycotoxins, which can cause deleterious effects on animals and humans. Ochratoxin A (OTA) is a mycotoxin of a major concern which can be produced by several species of *Aspergillus* section *Nigri* (black aspergilli), *Circumdati* and *Penicillium* genera (Pitt and Hocking, 2009). Black aspergilli can be isolated from a wide range of products, including cereals, cocoa, coffee, dried fruits, dried grapes, must, wine etc. (Bucheli and Taniwaki, 2002; Abarca et al., 2003; Leong et al., 2004; Magnoli et al., 2004; Varga et al., 2004; Valero et al., 2005; Bellí et al., 2006a). In particular, *A. carbonarius* is the most distinct member of this section and it has been reported as the main source of OTA contamination in grapes and related products (Cabãnes et al., 2002; Counil et al., 2005; Anli and Bayram, 2009). OTA has been accused for teratogenic, mutagenic, carcinogenic, and immunosuppressive effects to humans and animals (IARC, 1993; Castegnaro et al., 1998), while it is also associated with the Balkan Endemic Nephropathy (Abouzied et al., 2002). Concerning the European Union legislation for OTA, authorities have set up a limit of 2.0 μg kg^{-1} for wine, must or grape juice and 10 μg kg^{-1} for dried vine products (European Commission, 2006).

The most powerful strategy to control OTA contamination in grapes and their products is the prevention of mycotoxigenic fungal growth before crop harvest (Magan and Aldred, 2007; Ponsone et al., 2012). Specifically, the incidence of *A. carbonarius* ochratoxigenic strains reduction in vineyards could be successfully achieved by chemical control measures which focus on the use of appropriate fungicide applications in the field (Bellí et al., 2006b). Fungicide formulations such as Azoxystrobin, Dinocap, Chorus and Switch have been tested for their efficacy to prevent fungal growth of black aspergilli in the field and consequently suppress OTA accumulation in grapes and wine (Lo Curto et al., 2004; Tjamos et al., 2004; Bellí et al., 2006b). Results obtained by the aforementioned researchers showed that the use of these fungicides could influence either positively or negatively the accumulation of OTA. In addition, toxigenic fungi can produce toxins not only in the field, but also post-harvest, during processing and storage of grape products. Post-harvest control of toxigenic fungi could be achieved with the application of NaMBS, a salt of sulphurous acid with known antimicrobial action, extensively used in foods and beverages for many decades (Jay, 2000).

NaMBS is often used as a postharvest antifungal agent for table grapes' storage, during processing of raisins and grape juice, and during grape crushing for wine production (Prabhakar and Reddy, 2000; Magan and Aldred, 2007). Moreover, Pateraki et al. (2007) have examined the antifungal efficacy of NaMBS and reported that an effective control against fungal growth and OTA production of *A. carbonarius* could be achieved at high NaMBS concentrations. Interestingly, Jiang et al. (2014) demonstrated that the residual OTA in the musts treated with SO_2 increased with the addition of *A. carbonarius* spores in the must. Moreover, aforementioned researchers presented that absence of SO_2 resulted to lower residual OTA in the must compared with the control, and thus application of NaMBS may have a key role in OTA accumulation during winemaking.

An innovative and promising approach to assess fungal responses and therefore mycotoxin contamination in food products, in relation to key controlling parameters in the food environment, is based on predictive mycology (Dantigny et al., 2005; Garcia et al., 2009; Dagnas and Membré, 2013). Until now, many studies have been focused in modelling fungal growth under diverse environmental conditions (Dantigny et al., 2005; Samapundo et al., 2005; Tassou et al., 2007b; Marín et al., 2008; Gougouli and Koutsoumanis, 2010; Panagou et al., 2010), but few have taken into account the possibility to model toxin production (Medina et al., 2007; Giorni et al., 2011; Garcia et al., 2013). Due to the high variability in mycotoxin potential by different fungal strains, modelling of toxin formation could be difficult to deal with (Marín et al., 2006; Kapetanakou et al., 2011) and hence the use of mathematical models for mycotoxin quantification should receive more attention to enhance food safety.

The purpose of the present work was to quantify the effect of temperature, a_w and NaMBS on growth and OTA production of an *A. carbonarius* isolate from Greek vineyards on a grape juice based medium, and develop a modelling approach that would allow prediction of growth and OTA production under the studied factors. The developed models were validated with independent data from other similar studies on *A. carbonarius* as well as with data from additional experiments undertaken in our laboratory.

2.2 Materials and methods

2.2.1 Fungal isolates

Two isolates of *A. carbonarius* (Ac-57 and ATHUM 5659) with high ochratoxigenic potential, 745 and 4.900 ng OTA g^{-1} CYA (Czapek Yeast Agar medium; 7 days at 25 °C), respectively, isolated from Greek vineyards were selected in this study. The former was isolated from table grapes of *cv.* Calmerian from Corinth region of the Peloponnese and was used in model development, while the latter from wine grapes of *cv.* Rhoditis of Achaia region of the Peloponnese and was used in model validation. The isolates were stored at 4 °C in the fungal collection of the Laboratory of Microbiology and Biotechnology of Foods (LMBF) of the Agricultural University of Athens.

2.2.2 Media preparation and sodium metabisulphite formulation

A Grape Juice based Medium (GJM) was used as the basal medium in this study by mixing 50% (*v/v*) organic pasteurized white grape juice (composition per 100 mL: fat, 0.02 g; proteins, 0.2 g; carbohydrates, 15.8 g; energy, 64 Kcal; Vitamin C, 0.2 mg; data provided on the nutritional label of the product) and 1.5% agar (LabM, Agar No. 1 Bacteriological, UK) in distilled water. The a_w of this medium was 0.985, measured by an AquaLab LITE (Degacon, USA) water activity meter at 25 °C. Different a_w levels were achieved by adding the required amounts of glycerol (99% purity, Lach-Ner, Czech Republic) to the basic medium and adjust to 0.88, 0.90, 0.93 and 0.96. Low levels of a_w were selected in order to take into account the possibility of fungal growth during sun-drying dehydration process taking place for production of raisins and sweet wines (Valero et al., 2007a). The pH of the medium was adjusted to 3.5 using 2 M KOH. A stock solution of 20% (*w/v*) sodium metabisulphite ($Na_2O_5S_2$; Sigma-Aldrich, Germany) was prepared from which appropriate volumes were added to the substrate to obtain 50, 100, 150 and 200 mg L^{-1}. Petri plates with no NaMBS were also prepared and served as control treatment. The adjustment of pH and incorporation of NaMBS to the medium occurred after autoclaving at 121 °C for 15 min and cooling to approximately 50 °C, in order to assure medium solidification at low pH and antifungal activity of NaMBS, respectively.

2.2.3 Inoculation and incubation conditions

Inocula were prepared from sub-cultures grown on malt extract agar (MEA; Biolab, Hungary) for 7 days at 25 °C to obtain sporulating cultures. Spore suspension of each isolate was prepared by flooding 10 mL of sterile distilled water containing 0.05% Tween 80 (Merck, Schuchardt, Germany) and gently scratching the colony surface of the media using a sterile spatula. The final concentration of the spore suspension was adjusted to 10^5 spores mL^{-1} using a haemocytometer slide (Brand, Wertheim, Germany). Petri dishes (9 cm diameter) containing *ca.* 20 mL of the solidified growth medium were centrally inoculated with 5 mL of the spore suspension and incubated at 15, 20, 25, 30, 35 and 38 °C in high precision (± 0.2 °C) incubators (MIR-153, Sanyo Electric Co., Osaka, Japan). Plates of the same water activity were enclosed in polyethylene bags to minimize desiccation. The effect of temperature, a_w and NaMBS on fungal growth and OTA production was examined by means of a full factorial design. Each treatment was carried out in triplicate and the whole experiment was repeated twice ($n = 6$).

2.2.4 Model development

2.2.4.1 Modelling of growth responses

A two-step modelling approach was followed including both primary and secondary model development to determine the effect of temperature, a_w and NaMBS concentration on fungal growth responses. Specifically, fungal growth was quantified by daily diametric measurements of the mycelium of growing colonies at right angles. Measurements were preceded for a period of 40 days or until Petri dishes were fully colonized by fungal mycelium depending on the combination of the environmental conditions assayed. Changes in colony diameter were plotted against time and the maximum growth rate (μ_{max}) and lag phase duration (λ) were obtained by fitting the data to the primary model of Baranyi and Roberts (1995) modified accordingly to adapt to fungal growth (Marín et al., 2008):

$$D(t) = \mu_{max} A - log\left\{1 + \frac{[exp(\mu_{max} A) - 1]}{exp(D_{max})}\right\} \quad (1)$$

and

$$A = t + \frac{1}{\mu_{max}} log\left[e^{(-\mu_{max}\, t)} + e^{(-\mu_{max}\, \lambda)} - e^{(-\mu_{max}\, t - \mu_{max}\, \lambda)}\right] \quad (2)$$

where D(t) is the changes in colony diameter versus time (mm), μ_{max} is the maximum growth rate (mm h^{-1}), and λ is the lag phase duration (h). Estimates of μ_{max} and λ were further fitted to the secondary cardinal model with inflection (CMI) (Rosso and Robinson, 2001) to describe the effect of temperature, a_w and NaMBS concentration on fungal growth. The model is described by the general equation:

$$\mu_{max}(T, a_w, NaMBS) = \mathrm{CTPM}_3(T, a_w, NaMBS) = \mu_{opt} \cdot \tau(T) \cdot \sigma(a_w) \cdot \rho(NaMBS) \quad (3)$$

where

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

and

$$\sigma(a_w) = \left\{ \frac{(a_w - a_{w_{min}})^2 \cdot (a_w - a_{w_{max}})}{(a_{w_{opt}} - a_{w_{min}}) \cdot [(a_{w_{opt}} - a_{w_{min}}) \cdot (a_w - a_{w_{opt}}) - (a_{w_{opt}} - a_{w_{max}}) \cdot (a_{w_{opt}} + a_{w_{min}} - 2a_w)]} \right\}$$

and

$$\rho(NaMBS) = \left(\frac{1 - NaMBS}{MIC_{NaMBS}}\right)$$

The terms T_{min}, T_{max}, $a_{w,min}$, $a_{w,max}$ correspond to the theoretical minimum and maximum values of temperature and a_w, respectively, below and above which no growth occurs, and MIC_{NaMBs} is the minimum inhibitory concentration of NaMBS. Moreover, T_{opt} and $a_{w,opt}$ are the values of temperature and a_w at which μ_{max} is to its optimal value (μ_{opt}). The programme DMFit (available at http://www.combase.cc/) was fitted to the experimental data to estimate the kinetic parameters. Additionally, the parameters of the CMI secondary model were estimated using the non linear regression module of Statistica version 8.0 software (Statsoft Inc., Tulsa, OK). Only the combinations of ecological factors (i.e., temperature, a_w, NaMBS concentration) where fungal growth was observed (growth cases) were taken into account in model development.

2.2.4.2 OTA analysis and modelling

OTA was measured at 3, 7, 10, 14 and 17 days of incubation depending on the environmental conditions assayed for growth and OTA production, according to the protocol of Bragulat et al. (2001) with slight modification in the OTA extraction step. Specifically, the whole content of the Petri dish was used for OTA extraction to achieve a more accurate determination of OTA in the case where infusion of toxin to the substrate was taking place during incubation (Valero et al., 2006a). The content of each Petri dish (substrate and mycelium) was weighted and extraction was performed with 100 mL of an 80/20 methanol/water solution (*v/v*) of HPLC grade purity (Kapetanakou et al., 2009) by blending for 2 min. Extracts were left still for 30 min and filtered, first through a Whatman No1 filter paper, and subsequently through Millex® nylon membrane syringe-driven filter of 0.2 mm pore size (EMD Millipore Corp. Billerica, USA), and finally kept at 4 °C until analysis.

HPLC analysis was performed with an 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). Chromatographic separations were performed with a Waters spherisorb C18 analytical column, 5 mm ODS2 (4.6 × 250 mm) (Resteck Co., Pinnacle II, Bellefonte, USA), at a flow rate of 1 mL min^{-1} of the mobile phase consisted of water/acetonitrile/acetic acid: 49.5:49.5:1 (*v/v/v*). Run time of each sample was 20 min and OTA was detected at about 11 min. Additionally, known concentrations of OTA (50, 100 and 500 ng g^{-1}) were spiked on the substrate and recovery rates for the method were estimated, resulting in satisfactory recovery percentages of 96-99%. OTA determinations were carried out in triplicate for each sampling time.

OTA concentrations were plotted against sampling times (3, 7, 10, 14 and 17 days) and the OTA production rate (OTA_r, μg g^{-1} d^{-1}) was estimated by the slope of the straight line by means of linear regression. In some cases, where a decrease in OTA was present, usually in the last two OTA sampling times, these points were excluded from the regression and only the linear segment of the curve was taken into account. Satisfactory fitting was obtained in all cases with regression coefficients (R_2) ranging between 0.85 and 0.99. During an experiment with Petri-dishes *in vitro*, a decrease of OTA production takes place when the mycelium has grown enough to deplete nutrients of the medium, and thus decompose the produced toxin as carbon and nitrogen sources for metabolic purposes (Leong et al., 2006b; Valero et al.,

2006b). Further on, multiple (non linear) regression analysis was used to describe the effect of temperature, a_w and NaMBS concentration on the OTA production rate (OTA_r) using a quadratic polynomial model:

$$Log(OTA_r) = b_0 + b_1 \cdot T + b_2 \cdot a_w + b_3 \cdot NaMBS + b_4 \cdot T \cdot a_w + b_5 \cdot T \cdot NaMBS + b_6 \cdot a_w \cdot NaMBS + b_7 \cdot T^2 + b_8 \cdot a_w^2 + b_9 \cdot NaMBS^2 \quad (4)$$

where *b0, b1, … b9,* are constant parameters to be estimated and T, a_w, NaMBS are the exploratory (independent) variables. Data were analysed with the non-linear regression module of JMP version 8.0 software (SAS Institute Inc., Cary NC, USA). Finally, analysis of variance (ANOVA) was performed on maximum growth rate (μ_{max}), lag phase (λ) and OTA production rate (OTA*r*) considering all the factors involved at a significant level of $p < 0.05$.

2.2.5 Model validation

In the case of fungal growth, the performance of the developed model was compared with data from independent experiments using another isolate of *A. carbonarius* (ATHUM 5659) from the fungal collection of the LFMB, grown on the same grape juice medium and under the same conditions (T, a_w, NaMBS) with Ac-57 isolate. Additional validation was undertaken with literature data (Pateraki et al., 2007) referring to four isolates of *A. carbonarius* grown on a similar grape juice medium within a range of 0.930-0.985 a_w at 25 °C and for several NaMBS concentrations (0, 100, 250, 500, 750 and 1000 mg L^{-1}). This dataset was considered most appropriate for validation as it contained a wide range of a_w and NaMBS levels although the fungi were grown only at one temperature. For OTA validation, as there was a lack of comparable published data in the literature from similar studies with the same medium and the presence of NaMBS, assessment of model performance was based only on internal validation. In all cases, model validation was performed by graphical comparison between observed and predicted values and also by estimating the statistical indices of root mean squared error (RMSE), as well as the bias (B_f) and accuracy (A_f) factors (Ross, 1996).

2.3 Results and discussion

2.3.1 Fungal growth

Analysis of variance revealed that all factors examined and their interactions had a significant effect on lag phase duration (λ) and maximum growth rate (μ_{max}) of *A. carbonarius* at a level of $p < 0.0001$ (data not shown). The effect of temperature, a_w and NaMBS on the lag phase duration of Ac-57 is illustrated in Fig. 2.1. No fungal growth was observed at 200 mg L^{-1} regardless of environmental conditions studied in the time frame of the experiment. At 0 and 50 mg L^{-1} of NaMBS the highest lag phase duration was observed at 15 °C and gradually decreased with increasing temperature until 35 °C, whereas at 38 °C, lag phase duration presented an increase again. At all temperatures and for a_w levels between 0.96 and 0.98, lag phase duration did not varied regardless of NaMBS concentration, with the exception of 150 mg L^{-1}/38 °C and 100 & 150 mg L^{-1}/15 °C where a significant increase in lag phase duration occurred. No fungal growth was observed (absence of bars in Fig. 2.2) for the combination of low a_w (0.88-0.93) and the highest NaMBS concentrations (100 and 150 mg L^{-1}), irrespective of temperature.

Concerning colony growth rate (μ_{max}) of Ac-57, the highest reduction on fungal growth was observed after the treatment with NaMBS at 100 and 150 mg L^{-1}, as well as at low a_w levels for all the temperatures considered. Specifically, fungal growth was not recorded at 0.88-0.93a_w/150 mg L^{-1} NaMBS, and 0.88 and 0.90 a_w/100 mg L^{-1} NaMBS (absence of bars in Fig. 2.2). Regarding the lower concentration of NaMBS (50 mg L^{-1}), no growth was observed at 0.88 a_w/15 °C and 0.88-0.90 a_w/38 °C.

Our results are in good agreement with previous works reporting 30 °C and 0.960-0.985 a_w as optimum conditions of temperature and water activity, respectively, in the absence of antifungal agents (Mitchell et al., 2004; Tassou et al., 2007b; Patharajan et al., 2011). Moreover, our findings are in line with Lasram et al. (2010) who reported a significant decrease of *A. carbonarius* growth when temperature increased over 37 °C.

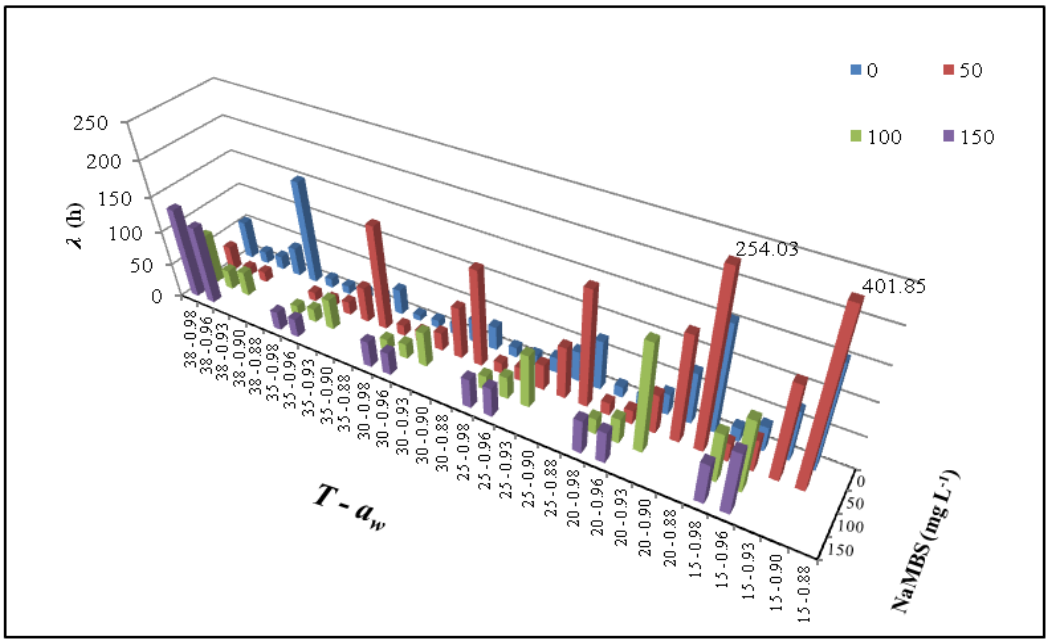


Figure 2.1: Effect of sodium metabisulphite (NaMBS) concentration (mg L^{-1}), temperature (T) and water activity (a_w) on the lag phase duration (λ, hours) of *A. carbonarius* (Ac-57) isolate grown on a grape juice based medium.

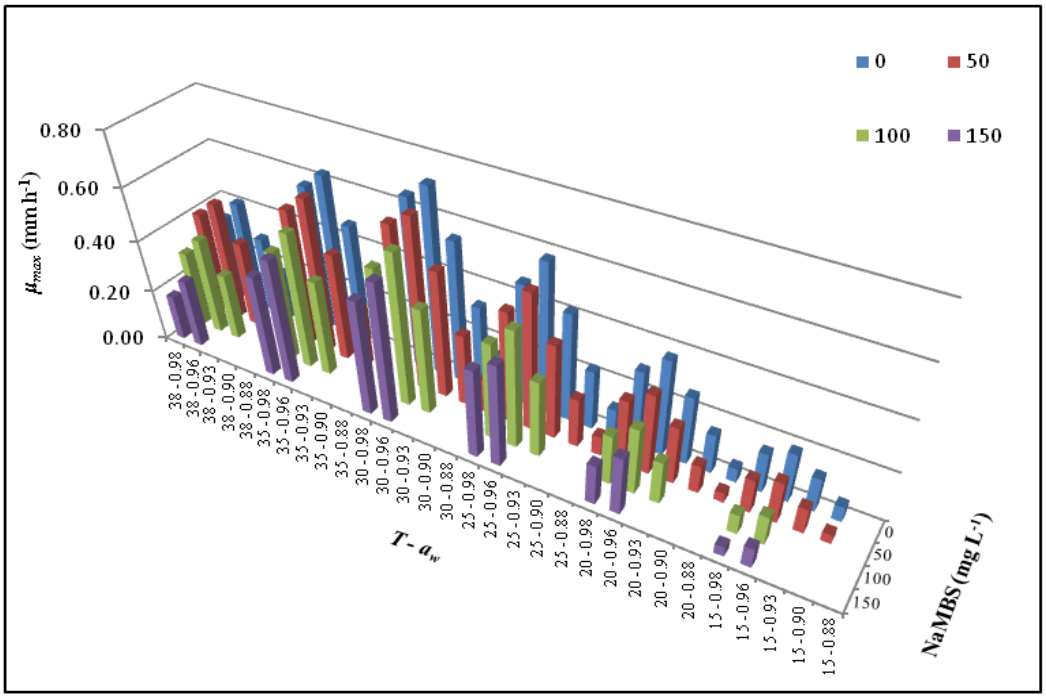


Figure 2.2: Effect of sodium metabisulphite (NaMBS) concentration (mg L^{-1}), temperature (T) and water activity (a_w) on the maximum growth rate (μ_{max}, mm h^{-1}) of *A. carbonarius* (Ac-57) isolate on a grape juice based medium.

Pateraki et al. (2007) after assessing the germination of five *A. carbonarius* strains under different a_w levels and NaMBS concentrations at 25 °C on a similar GJM, reported no significant differences between 0.985 and 0.965 a_w in the range of 0-250 mg L^{-1} NaMBS. Medina et al. (2007b) also reported that the presence of carbendazim as an antifungal agent in GJM could not effectively change the lag phase duration and growth rates at intermediate temperatures (25 and 28 °C) and high water activity levels (0.96 and 0.98 a_w). Similarly, our results showed that significant differences between the lag phase duration were observed only at 15 and 38 °C for the same a_w levels. Moreover, the aforementioned researchers reported that as a_w levels decreased, lower concentrations of the antifungal agent were required for the inhibition of growth, which is also in accordance with the findings of the present study. Pateraki et al. (2007) reported 750-1000 mg L^{-1} of NaMBS for complete inhibition of fungal growth at 0.985 a_w, while at 0.965 a_w lower concentrations (500 mg L^{-1}) were required. Nevertheless, effective concentrations for growth inhibition were reported to be greater than those claimed from our study. Thus, according to our results, growth inhibition occurred at 200 mg L^{-1} regardless of temperature and a_w, with the exception of 0.88-0.90 a_w and 0.93 a_w where the required NaMBS concentrations were 150 mg L^{-1} and 100 mg L^{-1}, respectively.

Table 2.1: Parameter estimation and statistics of the coefficients of the CMI model for *A. carbonarius* (Ac-57) at different conditions of temperature, a_w and NaMBS concentration.

Parameter	Estimated value[a]	Parameter	Estimated value[c]
μ_{opt}	0.768 ± 0.007	$1/\lambda_{opt}$	0.104 ± 0.008
T_{max}	40.69 ± 0.11	T_{max}	39.69 ± 0.33
T_{min}	7.36 ± 0.31	T_{min}	-6.85 ± 2.88
T_{opt}	31.69 ± 0.89	T_{opt}	33.21 ± 0.49
a_{wmax}	0.990 ± 0.001	a_{wmax}	0.986 ± 0.012
a_{wmin}	0.831 ± 0.002	a_{wmin}	0.821 ± 0.011
a_{wopt}	0.965 ± 0.001	a_{wopt}	0.974 ± 0.002
MIC	458.77 ± 11.66	*MIC*	223.62 ± 7.60
RMSE[b]	0.0266	*RMSE*[b]	0.0120

All parameters were significant at $p < 0.05$.

[a] μ_{opt} = the optimal value of maximum growth rate; $1/\lambda_{opt}$ = the optimal value of lag phase; T_{max}, T_{min}, a_{wmax}, a_{wmin} = the theoretical minimum and maximum values of temperature and a_w below and above which no growth occurs; T_{opt}, a_{wopt} = the temperature and a_w values at which maximum growth rate or lag phase equals to its optimal value; [b] RMSE = Root mean squared error; [c]Estimated value ± standard error.

These differences could be attributed to the adjustment of a_w with glycerol in our experiments, whereas in Pateraki et al. (2007) the a_w of the medium was adjusted with additional incorporation of glucose. It is known that a percentage of free SO_2 can be absorbed and bound by glucose resulting in reduced antifungal activity (Pateraki et al., 2007). Furthermore, in the present study the incorporation of the antifungal agent in the substrate took place after sterilization of the growth medium, whereas Pateraki et al. (2007) added NaMBS before the thermal treatment and this may have led to greater reduction of the fungicidal activity.

The cardinal values of environmental factors of the CMI model of Rosso for maximum growth rate and lag phase duration are shown in Table 2.1, whereas the fitted model is graphically illustrated in Figs. 2.3 & 2.4. The model exhibited good fit to experimental data in terms of calculated RMSE. Optimum conditions for growth were in the range 31.7-33.2 °C (T_{opt}) and 0.96-0.97 ($a_{w,\ opt}$), which is in accordance with the raw data for fungal growth presented in Fig. 2.2. The differences in parameter estimations between the two models, especially for the MIC term, could be attributed to the fact that for lag phase modelling the MIC represents the concentration of NaMBS to inhibit fungal growth, since this model was developed on lag phase data, whereas for growth modelling the MIC represents the concentration of NaMBS to suppress fungal growth once growth has been initiated, as this model was developed on maximum growth rate data. Moreover, the minimum and maximum conditions for growth were at 7.3 °C (T_{min}) and 40.7 °C (T_{max}) and 0.83 ($a_{w,\ min}$) and 0.99 ($a_{w,\ max}$), respectively. These results are in line with Tassou et al. (2007b), who after applying the same model to growth data of *A. carbonarius* on SGM medium as a function of a_w and temperature, reported similar cardinal values of 0.82 ($a_{w,\ min}$), 0.99 ($a_{w,\ max}$), 0.96 ($a_{w,\ opt}$), 40.5 °C (T_{max}), and 34.2 °C (T_{opt}), while for T_{min} an underestimated value of -0.08 °C was obtained. Nevertheless, the developed model for lag phase duration failed to give a reliable prediction for T_{min} and underestimation was evident for this cardinal value (*ca.* -7 °C, Table 2.1). Moreover, the estimated MIC value of NaMBS needed to prolong the lag phase of the fungus until the end of the experiment (40 days) was 223.6 mg L^{-1} (Table 2.1) which is in agreement with the commercially employed maximum level of NaMBS in white and rosé wines (European Commission, 2009). When the same model was fitted to the growth data, the estimated MIC value to suppress fungal growth was 458.7 mg L^{-1}. In a previous work (Ioannidis et al., 2013) the effect of a_w, temperature and NaMBS on *A. carbonarius* growth was investigated using a rapid screening method based on turbidimetric measurements. The MIC value of NaMBS was estimated using the model of Lambert and Pearson (2000) and was

found between 420 and 440 mg L^{-1} depending on temperature and a_w level which is in good agreement with the MIC value obtained in this work.

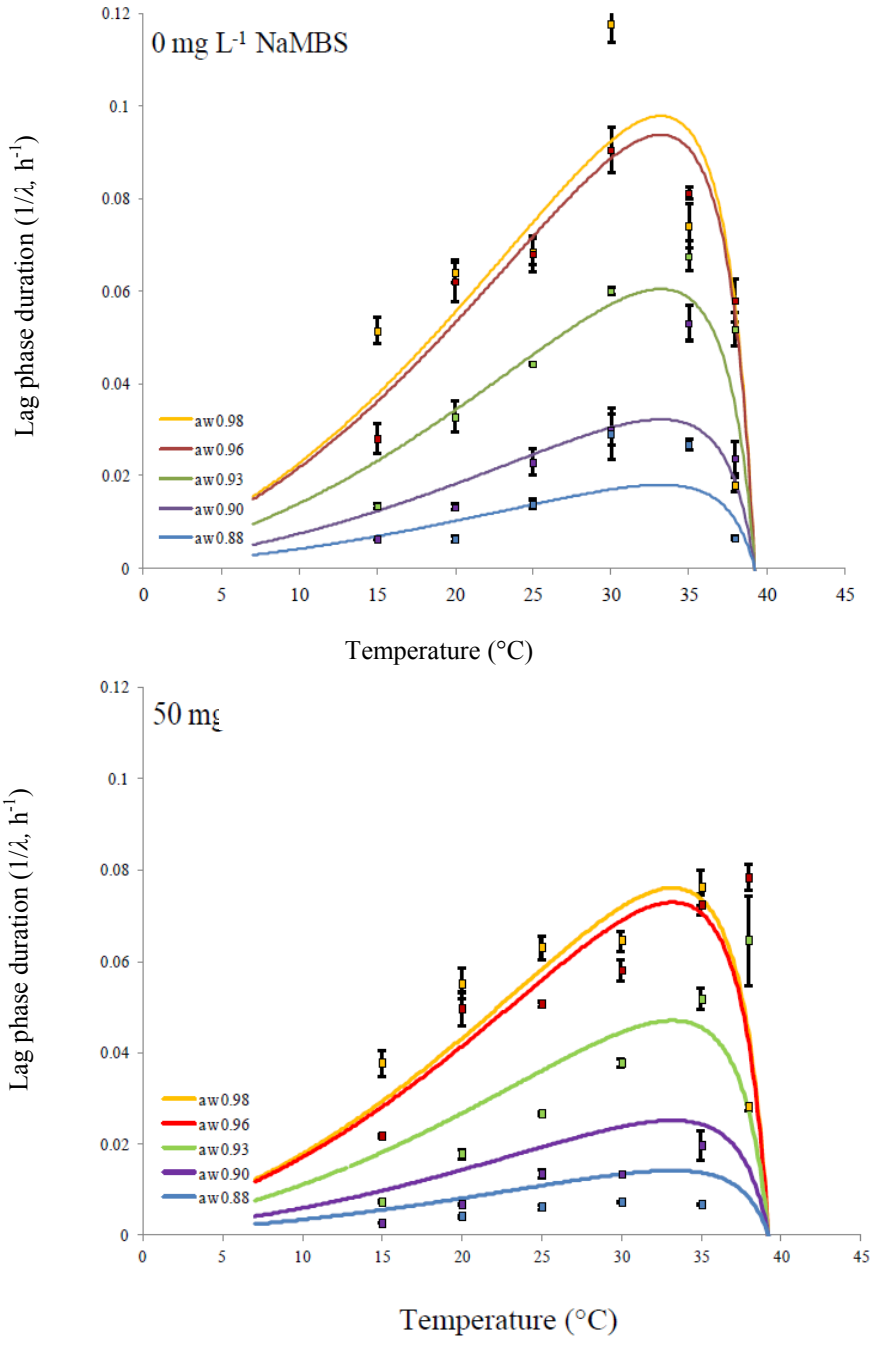


Figure 2.3: Fitted curves of the CMI model describing the effect of temperature (T), water activity (a_w) and sodium metabisulphite (NaMBS) concentration on the lag phase duration ($1/\lambda$, h^{-1}) of *A. carbonarius* (Ac-57) on a grape juice based medium.

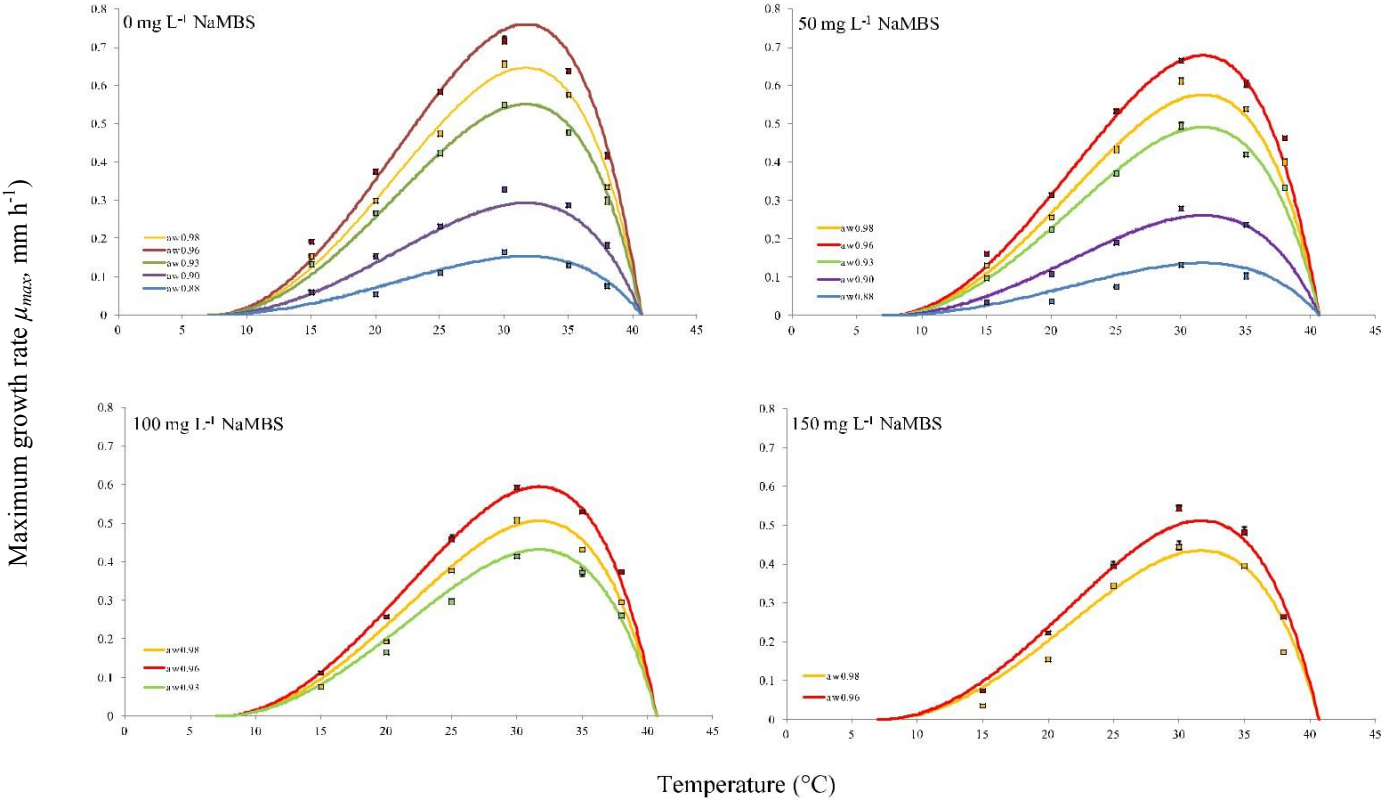


Figure 2.4: Fitted curves of the CMI model describing the effect of temperature (T), water activity (a_w) and sodium metabisulphite (NaMBS) concentration on the maximum growth rate (μ_{max}, mm h^{-1}) of *A. carbonarius* (Ac-57) on a grape juice based medium.

2.3.2 OTA accumulation

The effect of NaMBS concentration on OTA production rate (OTA*r*) for the different environmental factors is shown in Fig. 2.5. Maximum rates for OTA production were mainly detected at 20 °C/0.98 a_w followed by 38 °C/0.98 a_w, regardless of NaMBS concentration. The statistical analysis showed that all single factors and the interaction of NaMBS with temperature had significant effects on OTA accumulation ($p < 0.05$) (Table 2). Patharajan et al. (2011) also reported conditions of 30 °C and 0.98 a_w as optimum for OTA production, followed by 25 °C and 35 °C, but not within the range of 15-20 °C that is usually reported by other researchers (Mitchell et al., 2004; Bellí et al., 2005b; Leong et al., 2006b; Lasram et al., 2010). Esteban et al. (2006b) also reported high OTA amounts at temperatures of 30 °C and 0.98 a_w, although the optimum was detected at lower temperature (15 °C) for the same a_w level. Moreover, Astoreca et al. (2007) reported that the highest OTA amounts were measured

at 30 °C/0.99 a_w when assessing two *A. carbonarius* strains isolated from dried grapes. The highest levels of OTA were found at 0.98 a_w and decreased when a_w decreased as reported by other researchers as well (Mitchell et al., 2004; Bellí et al., 2005b; Medina et al., 2007a; Lasram et al., 2010; Patharajan et al., 2011).

After treatment with NaMBS, results showed that OTA content was enhanced even in the cases where fungal growth was delayed by the presence of the antifungal agent. Specifically, OTA was markedly increased at 38 °C/0.98 a_w and 20 °C/0.98 a_w at 150 mg L^{-1} concentration of NaMBS. Similar observations were made at lower levels of the antifungal agent (50 and 100 mg L^{-1}), where an increase in OTA content was measured at 15 °C/0.98 a_w for 50 mg L^{-1} and 25 °C/0.98 a_w for 100 mg L^{-1} NaMBS. Medina et al. (2007b) also reported that treatment with fungicide, namely carbendazim, in the concentrations of 0-450 ng mL^{-1} can increase OTA production and thus an application with this fungicide is not suggested for OTA control in grapes. Bellí et al. (2006a, b) reported an increase in OTA accumulation at 20 °C and 30 °C for three *A. carbonarius* strains after application with sulphur and copper hydroxide on SGM medium. Pateraki et al. (2007) reported that concentrations of 100-250 mg L^{-1} NaMBS increased OTA production when compared with the control. Nevertheless, they observed inhibition of OTA production at high a_w (0.985) and 25 °C after treatment with concentrations of at least 650-700 mg L^{-1} of NaMBS. Overall, application of some fungicides to grapes can reduce fungal growth but simultaneously stimulate OTA production by *A. carbonarius*, and thus a more thorough approach related to OTA accumulation and the applied fungicides in the field should be considered.

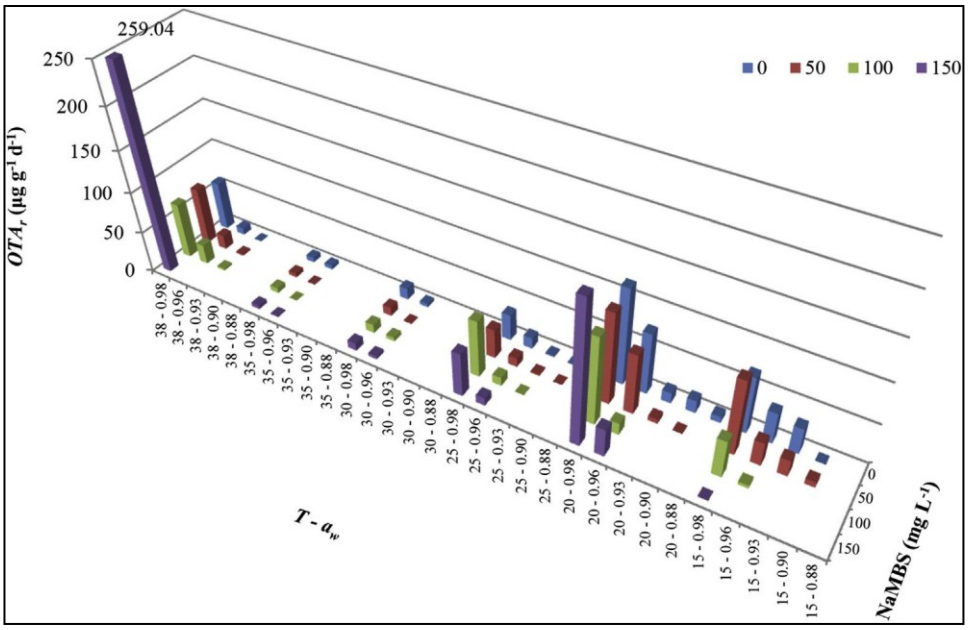


Figure 2.5: Effect of sodium metabisulphite (NaMBS) concentration, temperature (T), and water activity (a_w) on OTA production rate (OTA*r*) of *A. carbonarius* (Ac-57) on a grape juice based medium.

2.3.3 Validation

The graphical illustration of the observed vs. predicted values for $1/\lambda$, μ_{max} and OTA production rate (OTA*r*) are presented in Fig. 2.6. Internal validation of the developed models, as well as external validation with independent data obtained from another *A. carbonarius* isolate (ATHUM 5659) showed satisfactory performance. Regarding internal validation, it is inferred by graphical illustration that the CMI model was an acceptable secondary modelling approach for the investigation of the effects of environmental and abiotic factors on growth of *A. carbonarius*. This secondary model has received the attention of researchers in predictive mycology as it provides parameters with biological meaning and facilitates future use in potential mechanistic models describing cell biological mechanisms (Deschuyffeleer et al., 2013). Observed vs, predicted values were equally distributed above and below the line of equity (y = x) without presenting any systematic over- or under-prediction (Fig. 2.6a and b). The performance of the CMI model in terms of coefficient of determination (R_2) and RMSE were 0.978 and 0.057 for maximum growth rate (μ_{max}), and 0.769 and 0.011 for lag phase duration ($1/\lambda$), respectively. Regarding the OTA production rate (OTA*r*) a higher deviation from the line of equity was evident (Fig. 2.6c) that was clearly depicted in the calculated values for R_2 (0.548) and RMSE (0.624). Marín et al. (2006) also calculated the rate of OTA

accumulation on CYA medium at different temperatures using the Gompertz equation and reported that the maximum rates occurred at 20 °C. In another study, Garcia et al. (2013) used a primary model for aflatoxin (AFB1) accumulation by *Aspergillus flavus* applying the Luedeking-Piret equation and reported that toxin formation did not present a clear correlation with growth at certain conditions. Finally, to our knowledge only Medina et al. (2007b) applied a second order polynomial model on OTA accumulation as a function of a_w, temperature, time and carbendazim concentration and reported an acceptable level of correlation between observed and predicted values of the model with the exception of low and high OTA values where predictability was more difficult. Concerning external validation, the CMI model appeared satisfactory in predicting the growth rate and the lag phase of the isolate ATHUM 5659, as indicated by graphical comparison of observed vs. predicted values (Fig. 2.7a and b) and statistical indices (Table 2.3). The value of the bias factor (B_f) for the maximum growth rate (μ_{max}) was <1 indicating a slight underestimation of growth rates, whereas the average deviation between predictions and observations as indicated by the accuracy factor (A_f) was 22%. For the lag phase duration, the model presented underestimation ($B_f > 1$) with higher average deviation between predictions and observations (42%). This variation in external validation could be attributed to intraspecific variability of fungal strains, since different strains of *A. carbonarius* present diverse mycotoxigenic potential (Kizis et al., 2014) and hence some authors have proposed the use of mixed inocula of different isolates to obtain an average representation within a fungal species (Garcia et al., 2014), probably resulting in better model building. In a previous work, Tassou et al. (2007b) working on SGM with *A. carbonarius* reported values of 1.31 and 1.59 for B_f and A_f, respectively, after performing external validation for the Rosso model, with independently derived data from the literature which is in line with the respective values reported in this work.

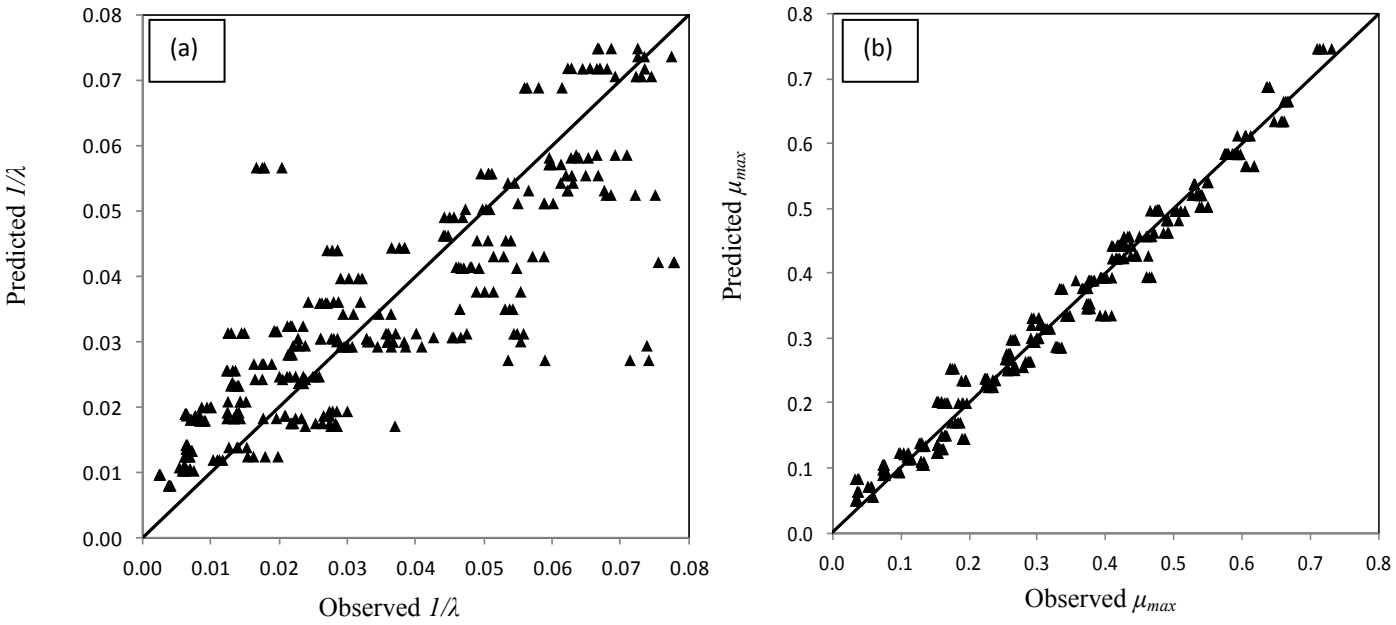
Table 2.2: Parameter estimation and statistics of the quadratic polynomial model for the OTA production rate of *A. carbonarius* (Ac-57) on grape juice based medium.

Parameter	Estimated value[a]	RMSE[b]
Intercept	0.6166±0.1593*	0.6046
T	0.0371±0.0094*	
a_w	-26.6976±4.0052*	
NaMBS	0.0040±0.0016*	
NaMBS *NaMBS	0.0001±0.0001[n.s.]	
a_w*a_w	60.1294±109.0358[n.s.]	
T*T	0.0025±0.0013[n.s.]	
a_w*T	0.5610±0.4386[n.s.]	
NaMBS*T	0.0004±0.0001*	
NaMBS*a_w	0.1191±0.0850[n.s.]	

*Statistically significant at $p < 0.05$; n.s.: not significant.

[a] Value ± standard error.

[b] Root mean squared error.



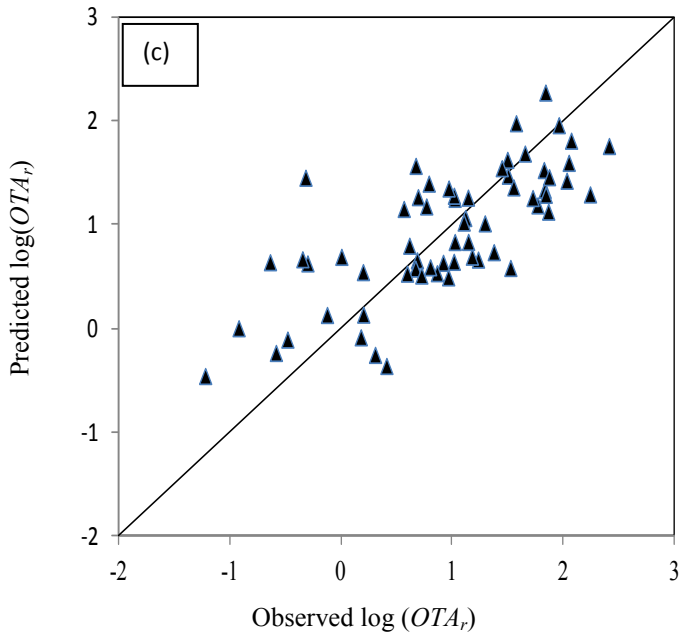


Figure 2.6: Plot comparing the observed against the predicted values obtained by CMI model for the effect of temperature (T), water activity (a_w) and sodium metabisulphite (NaMBS) concentration on the lag phase duration (a), maximum growth rate (b) and OTA production rate (c) of *A. carbonarius* (Ac-57) on grape juice based medium.

2.4 Conclusion

To our knowledge only few attempts have been made for secondary modelling on OTA accumulation. Besides there is need for a common protocol on modelling experiments in order to develop secondary modelling of OTA as a function of abiotic/processing factors in food substrates. According to our results, the effect of NaMBS on the growth and lag phase duration of *A. carbonarius* was expected, with higher concentrations of the antifungal compound presenting higher inhibitory effect. In contrast, when studying the OTA production, apart from the expected optimum temperature and a_w range, high NaMBS concentrations seemed to stimulate toxin production. It is clear that NaMBS can play a key role in grape processing and preservation, especially for OTA production, but further work is necessary to clarify its effect under several environmental factors. Finally, the Rosso CMI model could be a very good approach to study and predict fungal behaviour, although additional research is needed regarding OTA modelling in order to build more reliable models.

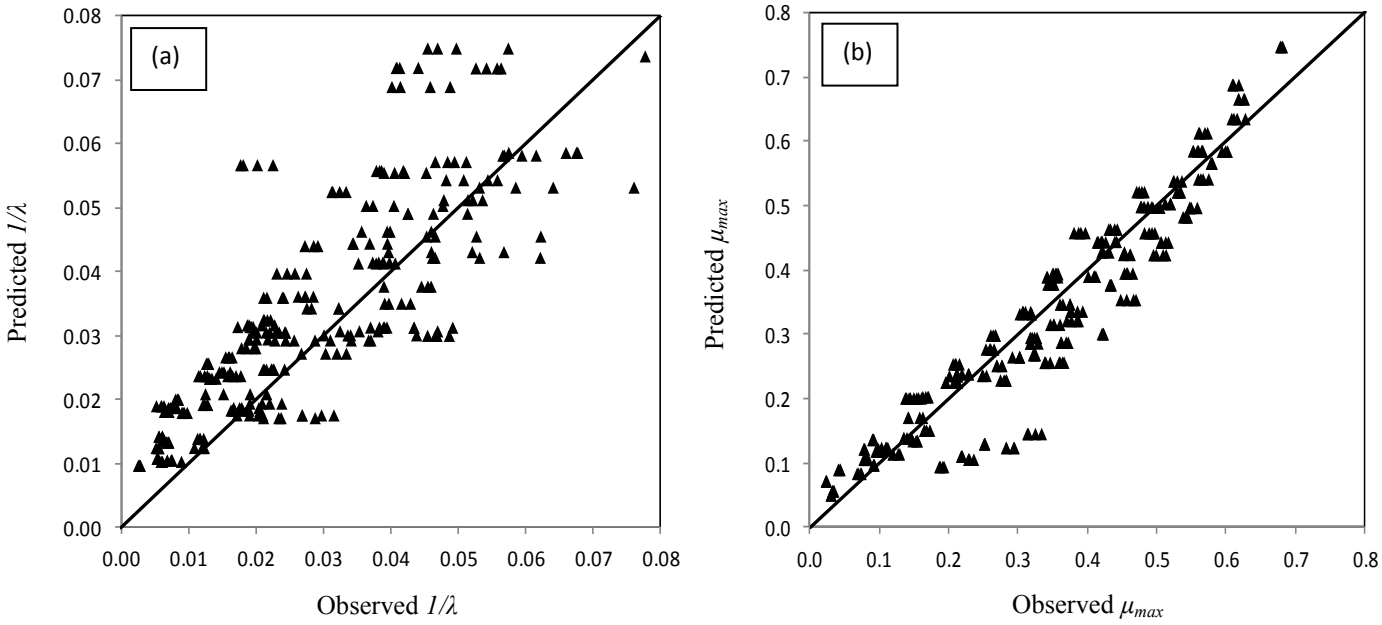


Figure 2.7: Plot comparing the observed against the predicted values obtained by CMI model for the effect of temperature (T), water activity (a_w) and sodium metabisulphite (NaMBS) concentration on the lag phase duration (a) and maximum growth rate (b) of *A. carbonarius* (ATHUM 5659) on grape juice based medium from independently derived data.

Table 2.3: Validation indices for the performance of the CMI model on independently derived data from the growth of *A. carbonarius* (ATHUM 5659) on grape juice based medium.

Parameters	RMSE	Bias factor (B_f)	Accuracy factor (A_f)
Maximum growth rate (μ_{max})	0.0572	0.98	1.22
Lag phase duration *(1/λ)*	0.0118	1.28	1.42

CHAPTER 3

Effect of water activity, temperature and mixed fungal spore interactions on Ochratoxin A production by Aspergillus carbonarius

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Abstract

The purpose of this work was to investigate the potential of two non-toxigenic *Aspergillus* section *Nigri* species (*Aspergillus tubingensis* and *Aspergillus japonicus*) to influence the *in vitro* Ochratoxin A (OTA) production of three toxigenic *Aspergillus carbonarius* isolates (Ac-28, Ac-29 and Ac-33) from Greek vineyards of different geographical areas. OTA accumulation was evaluated by inoculation of 0:100, 25:75, 50:50, 75:25 and 100:0 ratios of mixed spore suspensions on a Synthetic Grape-juice Medium for up to 28 days at different temperatures (15, 20 and 25 °C), water activity (a_w) levels (0.95 and 0.98 a_w) and incubation time (7, 14, 21 and 28 days). Results confirmed that environmental factors and fungal species had a significant effect on OTA production. Specifically, maximum OTA concentration for Ac-28 (3.21 μg g^{-1}) and Ac-29 (7.69 μg g^{-1}) was observed at 20 °C/0.98 a_w and for Ac-33 (9.13 μg g^{-1}) at 15 °C/0.95 a_w, regardless of incubation time. Moreover, *A. tubingensis* had no significant influence on OTA concentration of all toxigenic isolates assayed, regardless of temperature, a_w, and incubation time. On the other hand, the presence of *A. japonicus* slightly inhibited OTA production of Ac-29 and Ac-33, while for Ac-28 stimulation of OTA was observed in some cases. Overall, lower a_w levels reduced OTA accumulation for Ac-28 and Ac-29, regardless of temperature, inoculum ratio, and time. On the contrary, for Ac-33 low a_w increased OTA production, regardless of the investigated parameters. The importance of this study concerns the understanding of interspecific interactions on OTA diffusion by *A. carbonarius* in an attempt to find ways to prevent the presence of toxins in grapes and their derivatives.

3.1 Introduction

A number of ubiquitous fungi belonging to *Aspergillus* and *Penicillium* genera are able to colonize wine and table grapes during the preharvest and harvest period, as well as during the drying process or storage (Bejaoui et al., 2006; Bellí et al., 2006a). Species of these molds, namely, members of *Aspergillus* section *Nigri* and *Penicillium verrucosum* have been found to be responsible for the presence of ochratoxin A (OTA) in various foods, such as cocoa, coffee, grapes, and vine products (Cabañes et al., 2002; Abarca et al., 2004; Leong et al., 2004; Suárez-Quiroz et al., 2004; Battilani et al., 2006c; Bellí et al., 2006a; Copetti et al., 2010). OTA is a secondary metabolite with nephrotoxic, immunosuppressive, teratogenic and carcinogenic properties that has been classified as a group 2B carcinogen by the International Agency for Research on Cancer (IARC, 1993). Hence, the European Union has established regulatory levels for this mycotoxin in cereals, grapes and coffee to minimize toxin contamination of these products (European Commission, 2006). Specifically, the permissible limit of OTA in dried vine fruit is 10 μg kg^{-1}, while for grape juice and wine it is 2 μg kg^{-1}. OTA has also been reported as the main mycotoxin of concern in grape products (Leong et al., 2006a, 2006c). The most contributing species for OTA contamination in wine and table grapes belong to the *Aspergillus* section *Nigri* group and particularly *Aspergillus carbonarius* (Cabañes et al., 2002; Abarca et al., 2003), although other grape-associated fungal species may be able to produce OTA but in negligible amounts (Valero et al., 2005).

In an ecological system, fungi inescapably come into contact with each other as they grow (Magan et al., 2007) and the presence of some non-toxigenic fungi may play an important role in the field prior to harvest. Some atoxigenic fungal species may interact beneficially with toxigenic fungi and influence the OTA content derived from the latter species. Therefore, these atoxigenic species could be considered as a biological control for OTA contamination in grapes. The outcome of interactions between different fungal species or strains combined with different environmental factors is in a state of flux, as reported by Magan and Aldred (Magan et al., 2007), and until now, few studies have examined the competitiveness between fungal species or strains on *in vitro* colony interactions (Marín et al., 1998a; Valero et al., 2006a). These researchers reported that the relative growth rate of toxigenic strains was influenced by the presence of the competing species, and toxin production was either inhibited or stimulated. Recently, the influence of mixed fungal spore populations on aflatoxin B1 production in a range of different ecological factors has been

reported (Mohale et al., 2013), indicating that the toxin was inhibited more than 80% *in vitro* at 0.99 and 0.96 a_w when toxigenic and atoxigenic strains of *Aspergillus flavus* were grown in different proportions of mixed spore suspensions.

The aim of the present study was to investigate the potential role of two non-toxigenic species of the *Aspergillus* section *Nigri* group, namely, *Aspergillus tubingensis* and *Aspergillus japonicus*, grown in mixed spore suspensions together with three toxigenic isolates of *A. carbonarius* on OTA production. Fungi were allowed to grow on a Synthetic Grape-juice Medium (SGM) at diverse mixed spore ratios (0:100, 25:75, 50:50, 75:25 and 100:0) in different temperatures (15, 20 and 25 °C), water activity (a_w) levels (0.95 and 0.98), and incubation time (7, 14, 21 and 28 days). The results obtained from this work could be useful in elucidating the role of atoxigenic fungal species and their ability to control toxigenic isolates/strains and reduce OTA production.

3.2 Materials and methods

3.2.1 Fungal species

Three isolates of *A. carbonarius* (Ac-28, Ac-29 and Ac-33), and two species of *Aspergillus* section *Nigri*, namely, *A. tubingensis* (At-4) and *A. japonicus* (Aj-1), were used in this study. The fungi have been previously isolated from Greek wine grapes during the 2012 harvesting period and screened for their capability to produce OTA on Czapek yeast extract agar, after incubation for 7 days at 25 °C (Pitt and Hocking, 2009). The ochratoxigenic potential of these species was determined by high-pressure liquid chromatography (HPLC) analysis according to the method of Bragulat et al. (2001). The selected *A. carbonarius* isolates were characterized as high OTA producers (>12 mg g^{-1}), based on a previous work undertaken in our laboratory (Kizis et al., 2014), while *Aspergillus* section *Nigri* strains as OTA-negative producers. The selection of *A. tubingensis* and *A. japonicus* in this work was based on these species being commonly isolated from Greek vineyards, as reported in a previous study of the same research group (Kizis et al., 2014). All isolates were held in the fungal collection of the Laboratory of Food Microbiology and Biotechnology (Agricultural University of Athens, Athens, Greece) and kept at 4 °C.

3.2.2 Media and inoculum preparation

A synthetic grape-juice medium (SGM) similar to the composition of grapes between véraison and ripeness, was prepared, as reported elsewhere (Magan et al., 2010). The medium consisted of: 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; distilled water, ca 1000 mL. The a_w of this basal medium was measured by an AquaLab LITE (Degacon, USA) water activity meter at 25 °C and modified to the required levels of 0.95 and 0.98 by adding glycerol (Mitchell et al., 2004). Finally, the pH of the medium was adjusted to 3.5-4.0 with 2M KOH. SGM was autoclaved for 20 min at 121 °C and then *ca.* 20 mL of the medium were poured into 9-cm petri dishes. Spore suspensions were prepared from 7-day-old cultures on malt extract agar (Biolab, Budapest, Hungary) incubated at 25 °C. The conidia were rinsed with 10 mL of sterile distilled water containing 0.05% Tween 80 (Merck, Haar, Germany) and collected by scratching the fungal colony surface with a sterile spatula. The final spore suspension was adjusted to 10^5 spores mL^{-1} with the aid of a Neubauer counting chamber (Brand, Wertheim, Germany) and a compound microscope (N-400, Optika, Italy).

3.2.3 Treatment application and incubation conditions

Fungal pairs of the three toxigenic isolates of *A. carbonarius* and the two non-toxigenic species of *A. tubingensis* and *A. japonicus* were prepared at different mixed spore ratios. Thus, proportions of 100% of each toxigenic and atoxigenic species and ratios of 75:25, 50:50 and 25:75 spore inoculum were prepared by adding the required volume of spore suspension in test tubes to obtain the different mixed spore ratios. Subsequently, from the final spore mixtures, 200 mL of each proportion were taken with a pipette, placed into the Petri dish containing solidified SGM medium and spread with a sterile glass rod. After spreading, all Petri dishes with the same a_w level were sealed into polyethylene bags and incubated at 15, 20 and 25 °C. Bags were opened daily to avoid any changes of the atmospheric conditions due to fungal respiration (i.e., oxygen depletion, carbon dioxide accumulation) that could affect growth. The experiment was conducted twice with three replicates per treatment each time (n = 6).

3.2.4 OTA extraction and quantification

OTA production was evaluated at 7, 14, 21 and 28 days of incubation according to the method of Bragulat et al. (2001). From each Petri dish 6 agar plugs (6 mm diameter each) were removed with the aid of a sterile cork borer. Samples were weighed, placed into a 1.5 mL eppendorf tube, 1 mL of methanol was added and vortexed for 5 min. Finally, the samples were left still for 1 h, filtered through a 0.2-μm-syringe-driven filter unit (Millex, Millipore Co., Bedford, MA) and stored at 4 °C until analysis. Moreover, known concentrations of OTA (50 and 500 ppb) were spiked on SGM, and recovery rates were estimated by HPLC analysis, giving satisfactory recovery percentages of 92.0 and 79.0%, respectively.

The HPLC consisted of 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, Maryland, USA). The samples were eluted 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, *vol/vol/vol*) through a Waters spherisorb C18 analytical column, 5 μm ODS2 (4.6 × 250 mm; Resteck Co., Pinnacle II, Bellefonte, USA). Run time for samples was 20 min, with OTA being detected at about 11 min. The limit of quantification was 2.0 ng OTA g^{-1} SGM (ppb), while the limit of detection was 1.0 ng OTA g^{-1} SGM.

3.2.5 Statistical analysis

The effect of the different parameters (temperature, a_w, time and fungal isolate) on OTA production of the three ochratoxigenic *A. carbonarius* isolates was evaluated by analysis of variance (ANOVA). The significance of mixed spore ratios was assessed by using Tukey's Studentized Range (HSD) test at a significance level of $P<0.05$, after revealing a significant effect between OTA production and ratios using a single factor ANOVA. All statistical analyses were performed by JMP ver. 8 package (SAS Institute Inc., Cary, NC, USA).

3.3 Results and discussion

3.3.1 Effect of different environmental factors on OTA production by the three isolates of *A. carbonarius*

Three high OTA producing isolates of *A. carbonarius* were investigated for their efficacy to produce toxin on SGM at different environmental factors for up to 28 days (Table 3.1). Analysis of variance revealed that the different toxigenic isolates, a_w levels, temperature, and their interactions, had a significant effect on OTA accumulation ($P < 0.05$). The effect of incubation time on OTA accumulation, as well as the interaction term of time with the other parameters, was not statistically significant, with the exception of temperature × time interaction term that showed significant effect on OTA accumulation.

Regarding the optimum conditions for OTA production by the toxigenic isolates when grown alone, two different patterns between Ac-28 (Fig. 3.1A through 3.1D), Ac-29 (Fig. 3.2A through 3.2D) and Ac-33 (Fig. 3.3A through 3.3D) were evident. Maximum amounts of OTA for Ac-28 (3.21 μg g^{-1}) and Ac-29 (7.69 μg g^{-1}) were detected at 20 °C/0.98 a_w and (9.13 μg g^{-1}) at 15 °C/0.95 a_w for Ac-33, indicating that the latter isolate could produce toxin in lower temperature and water availability conditions. Previous studies examined the impact of environmental factors on OTA production by different strains of *A. carbonarius* isolated from table and wine grapes from different geographical areas (Abarca et al., 2003; Bellí et al., 2004b; Mitchell et al., 2004; Bellí et al., 2005b) and reported that higher water activities (0.98 a_w) favored OTA production by most members of *Aspergillus* section *Nigri* group. Similar results were obtained in this study for Ac-28 and Ac-29, while for Ac-33, OTA was favored at a lower a_w level (0.95). With respect to optimum temperature conditions for OTA production, our findings are in line with previous studies that reported that the optimum temperature is near 20 °C in most cases (Bellí et al., 2005b; Valero et al., 2006a). Thus, for Ac-28 and Ac-29 higher amounts of OTA were measured at 20 °C, whereas for Ac- 33 a lower temperature (15 °C) was necessary, which is also in agreement with Lasram et al. (2010) who reported that the optimum temperature for OTA production is in the range 15 to 25 °C, with a marked decrease at temperatures higher than 25 °C.

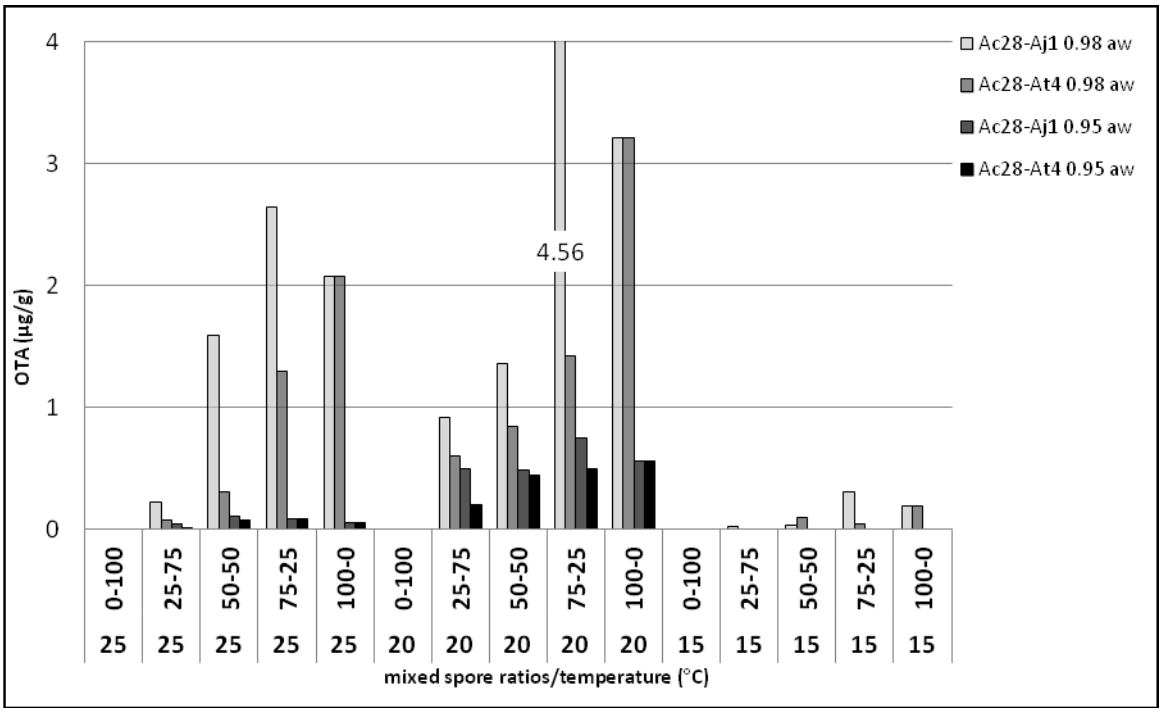
Table 3.1: Analysis of variance for the effect of water activity (a_w), temperature (T), fungal isolate, time and their interactions on OTA production of the three *A. carbonarius* isolates on SGM medium.

Effect	Mean Square	F value
isolate	28.2080	7.55*
T	10.4268	5.58*
a_w	17.2903	9.26*
time	3.5909	1.92$^{n.s.}$
isolate*T	19.0796	5.11*
isolate*a_w	40.7619	10.91*
isolate*time	4.5077	1.2$^{n.s.}$
T*a_w	10.3481	5.54*
T*time	13.0197	6.97*
a_w*time	2.6586	1.42$^{n.s.}$

*Significant for $P<0.05$; n.s.: not significant.

With reference to toxigenic controls (100%) tested, maximum toxin levels were detected at day 7 for Ac-28 and Ac-29, followed by a decrease on the 14th day and a steady production for the remaining days, while for Ac-33, high OTA amounts were detected from the 21st day onwards (Fig. 3.4). Bellí et al. (2005b) suggested that the highest OTA amount of Spanish and Italian isolates of *A. carbonarius* was detected at the 5th day of incubation, while other studies (Bellí et al., 2004c) found that the time between 5 and 10 days is crucial for OTA diffusion.

Figure 3.1 (A)



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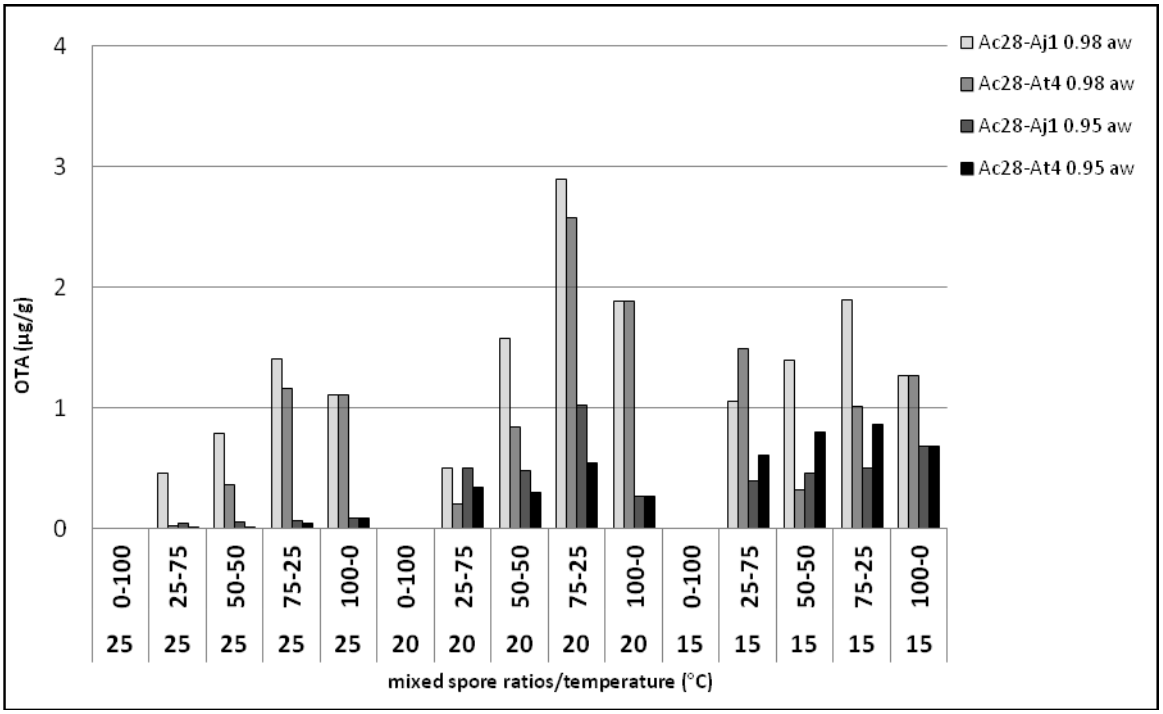


Figure 3.1 (C)

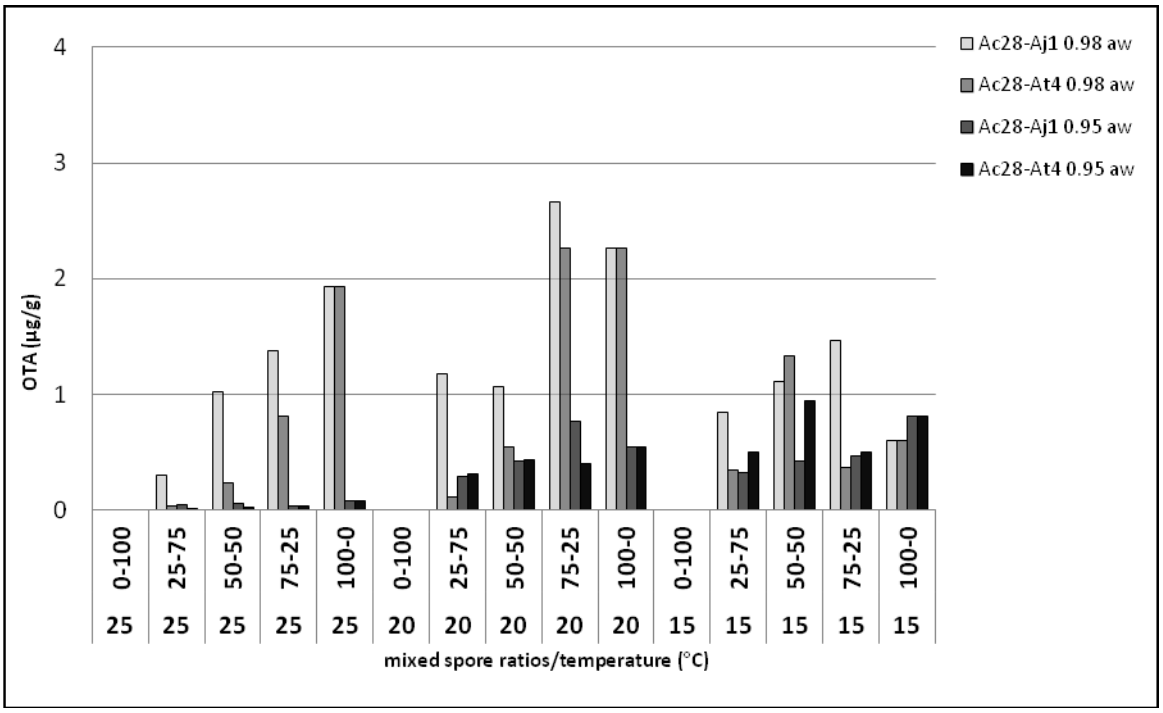


Figure 3.1 (D)

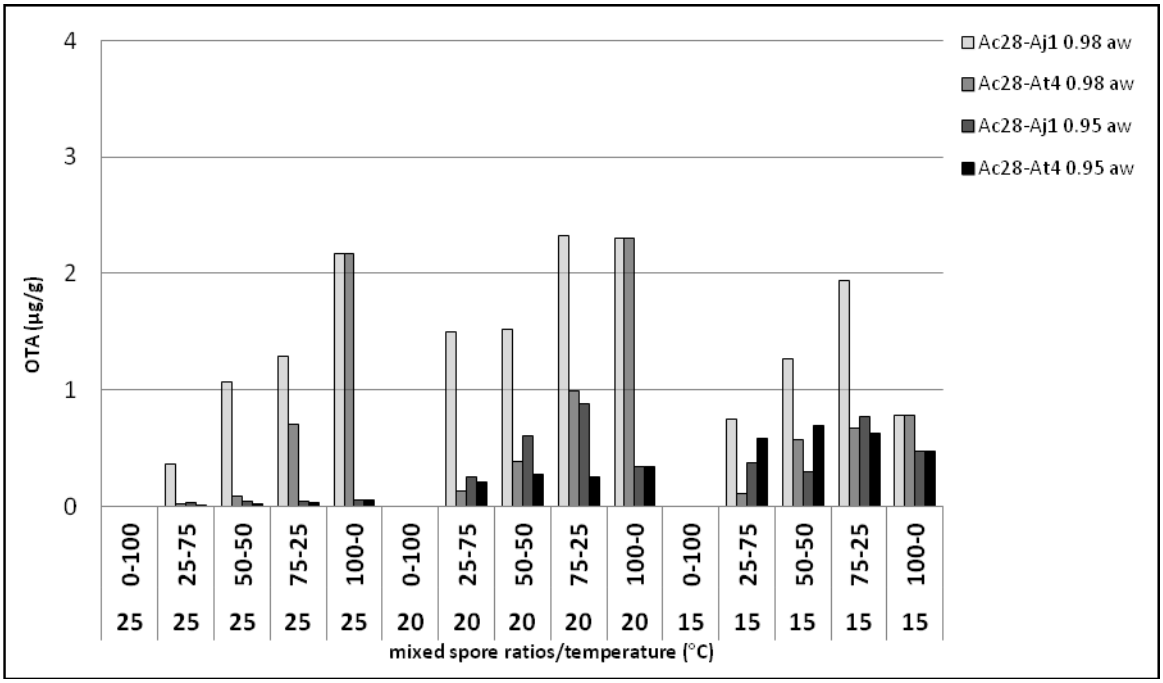
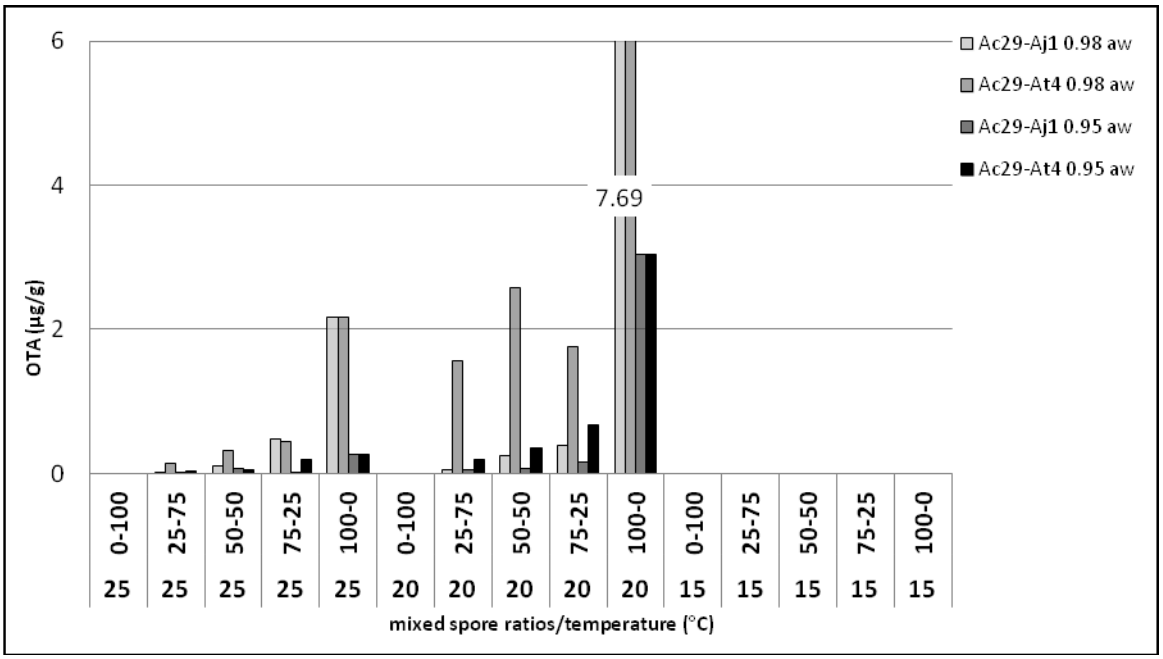


Figure 3.1: Effect of two non-toxigenic species and environmental factors on OTA production by *A. carbonarius* (Ac-28) when grown together in mixed spore populations of different ratios after incubation of (A) 7 days, (B) 14 days, (C) 21 days and (D) 28 days.

Figure 3.2 (A)



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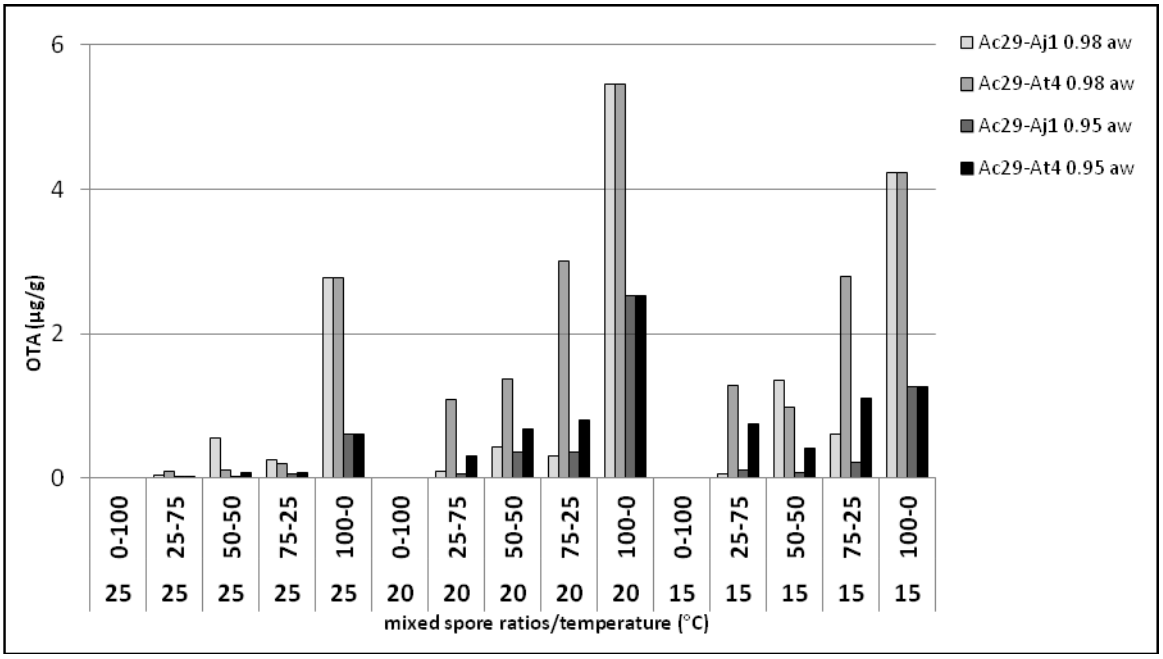

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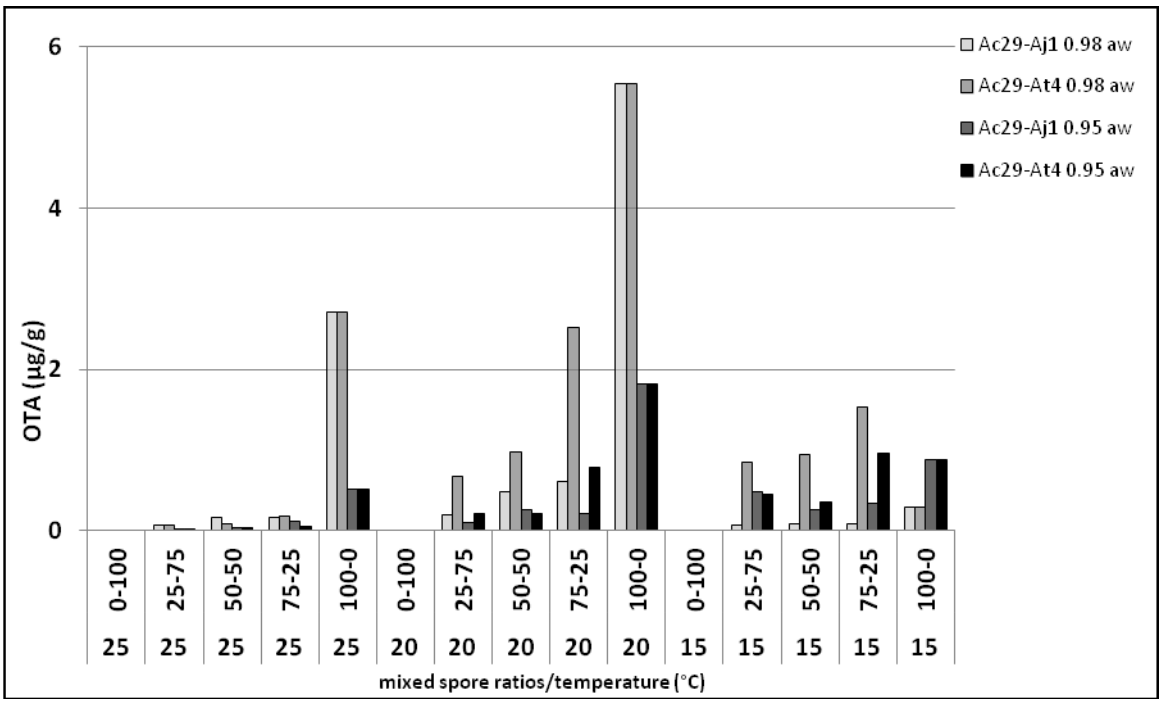

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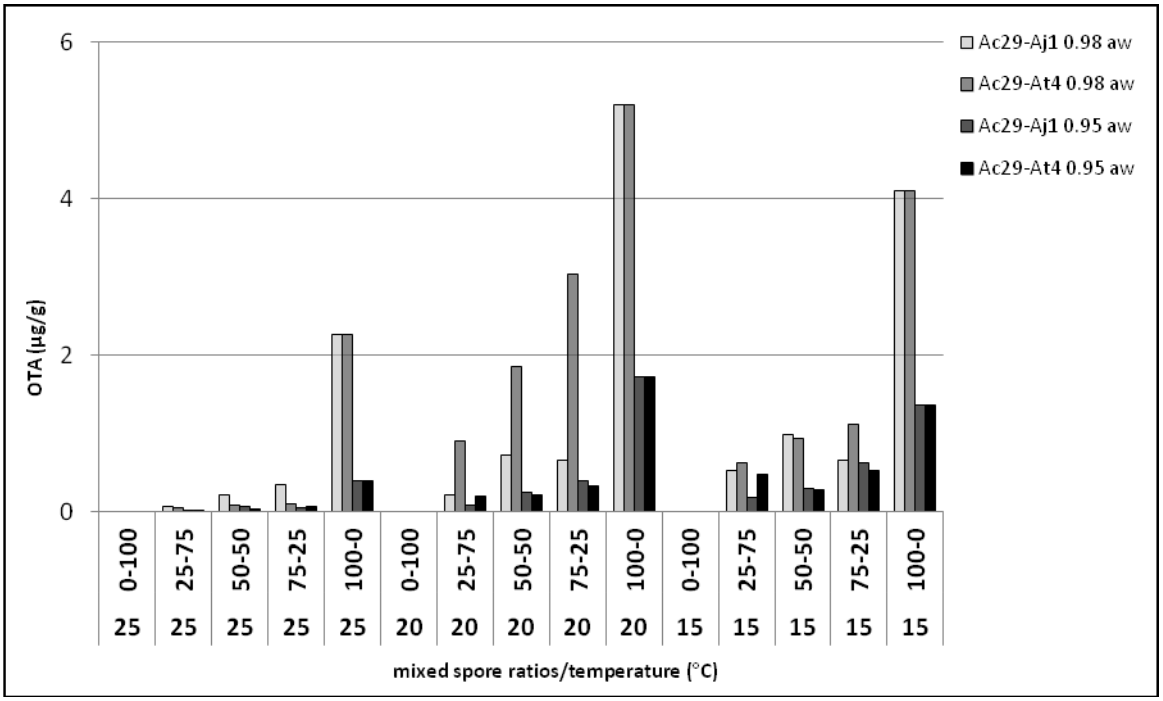


Figure 3.2: Effect of two non-toxigenic species and environmental factors on OTA production by *A. carbonarius* (Ac-29) when grown together in mixed spore populations of different ratios after incubation of (A) 7 days, (B) 14 days, (C) 21 days and (D) 28 days.

3.3.2 Effect of different environmental factors and ratios of mixed spore suspensions on OTA production

The effect of temperature, a_w, fungal pairs and mixtures of atoxigenic and toxigenic species on OTA production are shown in Table 3.2. Since all the non toxigenic proportions (0 %) presented no OTA accumulation, the toxin evaluation was reported to be statistically different from the 100% proportion of the toxigenic isolate. In most cases, the intermediate inoculum ratios (25:75, 50:50 and 75:25 %) were found not to be statistically significant when compared with the concentration of 100% of the toxigenic isolate, regardless of competing species and ecological factors. However, at the proportions in which the concentration of the toxigenic isolate was higher (75:25 %), the OTA amount was found to be in higher amounts as well.

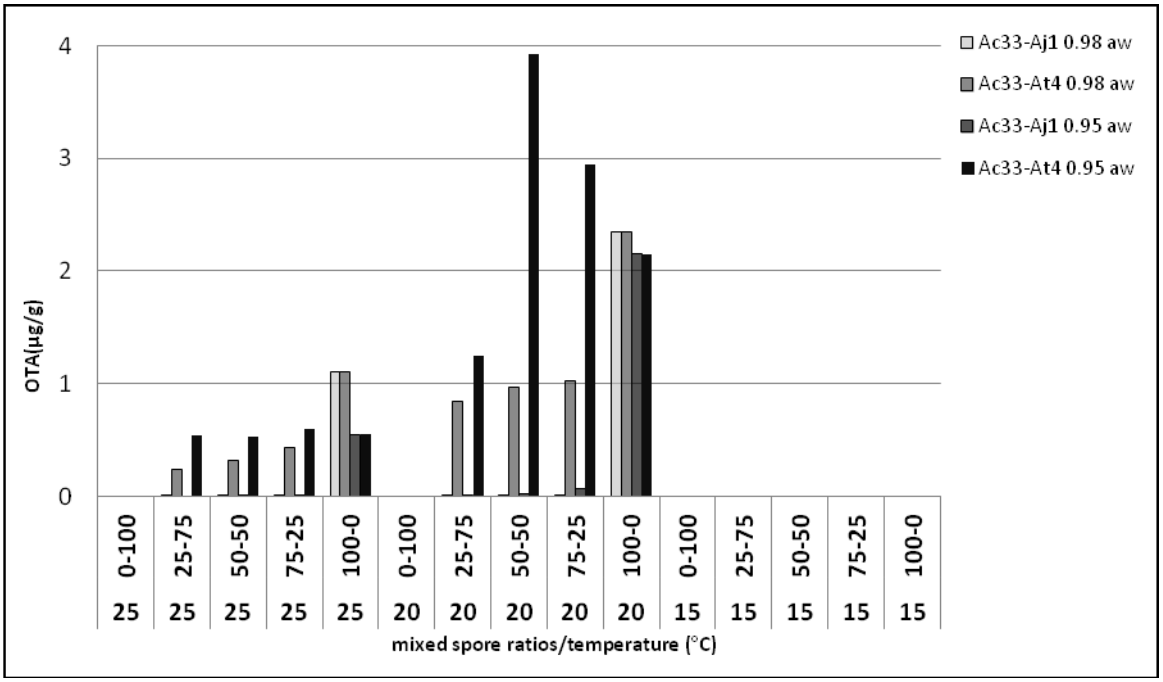
To our knowledge, only few relevant reports have been published regarding mixed inoculum studies for *A. carbonarius* and/or other fungal species on *in vitro* studies to elucidate the potential role of competing species on OTA production. Nevertheless, studies on mixed inoculums within different *A. carbonarius* strains have been conducted (Romero et al., 2010), reporting that the highest amount of OTA was observed at 15 °C/0.95 a_w after 28 days of incubation, which is in good agreement with the strain Ac-33 used in the present work when co-cultured with the two non-toxigenic *Aspergillus* section *Nigri* species. Yet, these studies did not include mixed spore interactions between the different fungal species. Valero et al. (2007a) also worked on mixed fungal combinations of *A. carbonarius* with different fungal strains on in situ studies for healthy and injured grapes. In healthy grapes, OTA production by *A. carbonarius* was mostly reduced when co-cultured with the OTA-negative *A. niger* aggregate. Similar results were obtained in the present study for Ac-28 and Ac-29 at optimum for OTA conditions when co-cultured with *A. tubingensis* (Figs. 3.1A through 3.1D and 3.2A through 3.2D), although for Ac-33 OTA reduction was not always evident by the presence of the non toxigenic species (Fig. 3.3A through 3.3D).

A similar study has examined the effect of mixed spore populations of non-toxigenic and toxigenic *A. flavus* species on aflatoxin B1 production on maize at different environmental conditions (Mohale et al., 2013). It was reported that aflatoxin production was significantly reduced by the presence of the atoxigenic species, regardless of a_w levels. Regarding our results, toxin reduction by the presence of different atoxigenic populations was not significant when results were assessed by the Tukey's studentized range test. Exceptions

were observed only for Ac-28 at 20 and 25 °C/0.98 a_w, where the reduction in OTA was statistically significant regardless of the antagonist (Table 3.2).

With regard to the two atoxigenic species used in this study, co-culturing with *A. tubingensis* did not result in a significant OTA reduction by the toxigenic isolates, regardless of the different mixed spore ratios at both water activities. Thus, OTA production was gradually increased from 25:75 of toxigenic/atoxigenic mixture until 100% proportion of the toxigenic isolates in most of the cases studied (Figs. 3.1A through 3.1D, 3.2A through 3.2D and 3.3A through 3.3D). On the other hand, *A. japonicus* had a slightly inhibitory effect on OTA production when co-cultured at different combinations of mixed spores with Ac-29 and Ac-33 toxigenic isolates (Figs. 3.2A through 3.2D and 3.3A through 3.3D). Therefore, OTA concentration by the latter isolate had a greater decrease by the presence of *A. japonicus* and the highest amounts of OTA were mainly detected at 100% ratios of the toxigenic isolates.

Figure 3.3 (A)



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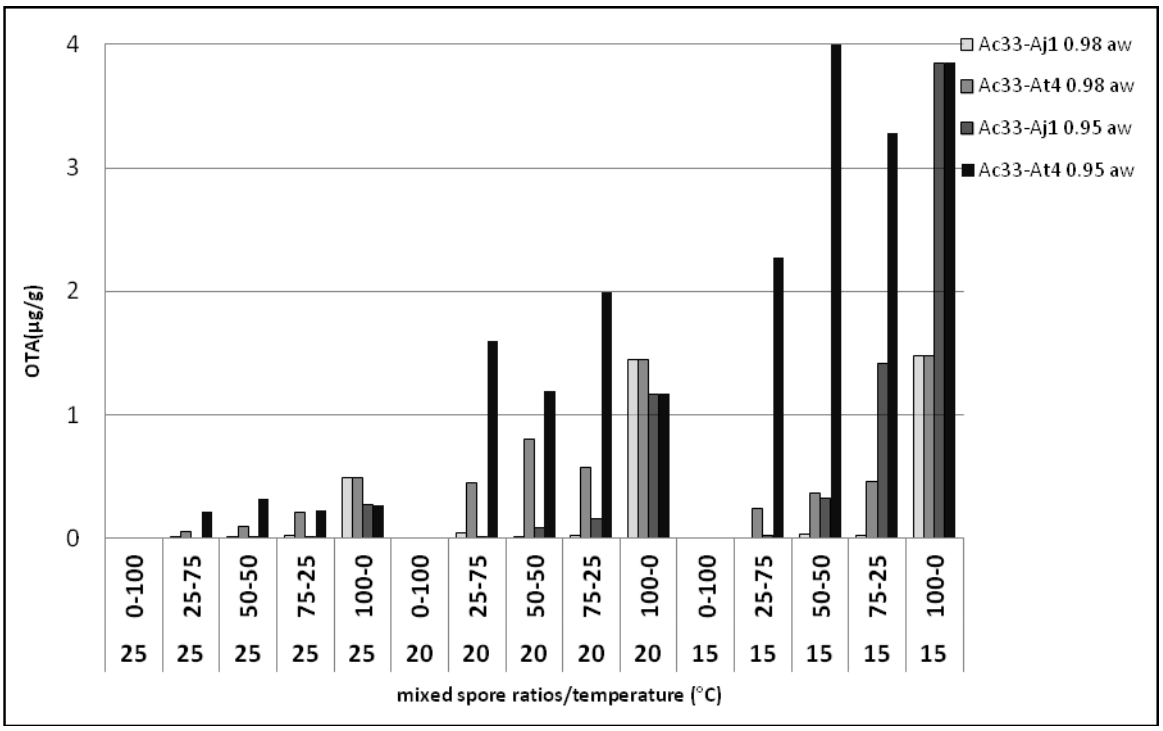

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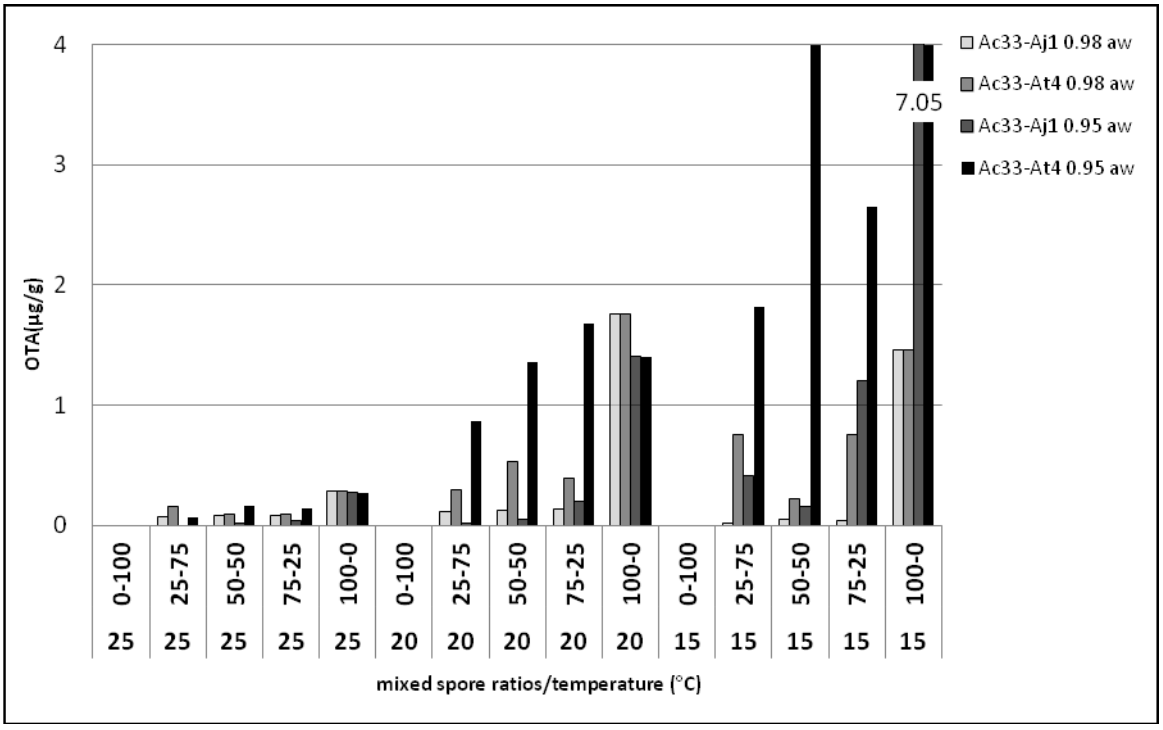

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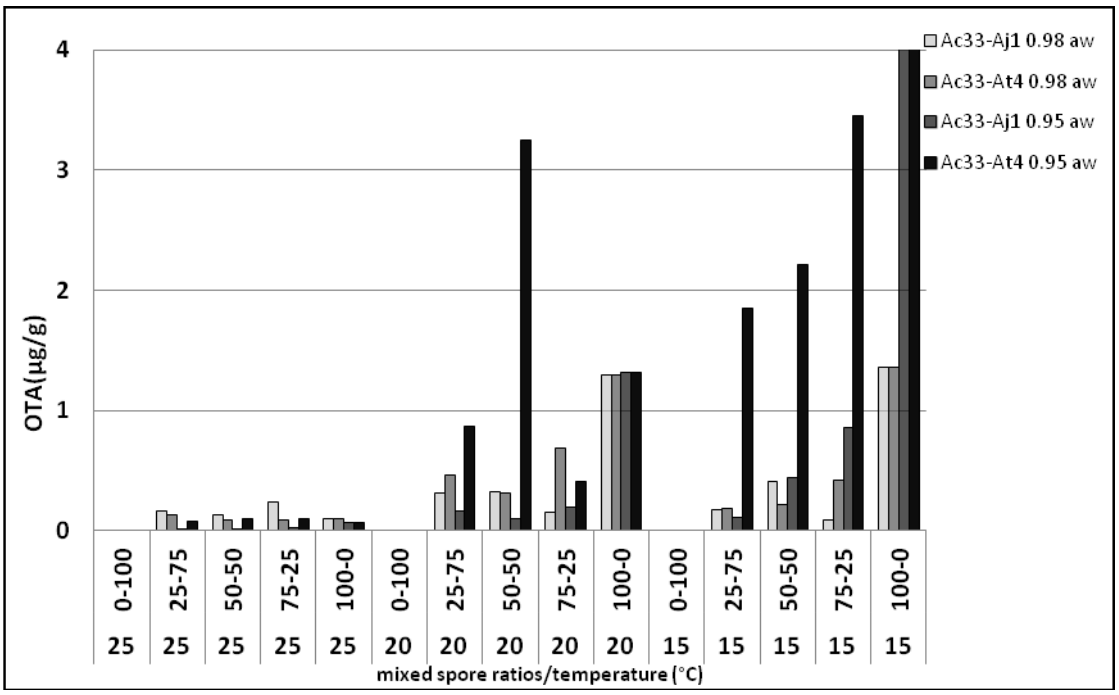

Figure 3.3: Effect of two non-toxigenic species and environmental factors on OTA production by *A. carbonarius* (Ac-33) when grown together in mixed spore populations of different ratios after incubation of (A) 7 days, (B) 14 days, (C) 21 days and (D) 28 days.

In contrast, when Ac-28 was grown together with *A. japonicus,* OTA production seemed to be affected positively, since stimulation was observed from 100% were OTA content was 3.21 µg g^{-1} to 75:25 % proportions of the toxigenic isolates were OTA content increased to 4.56 µg g^{-1} at the 7th day of incubation. Similar response was observed for the rest days of sampling (Fig. 3.1A through 3.1D).

Very few studies have focused on interactions between fungal strains or species under various environmental conditions and how these factors can affect toxin concentration (Valero et al., 2006a; Valero et al., 2007c; Magan and Aldred, 2007). Magan et al. (2007) found that the effect of interactions between spot-inoculated colonies of *A. carbonarius* and other fungi on OTA production was higher at 20 °C and 0.987 a_w and stimulated after interactions with other species. In the same study, lower a_w levels (0.93 and 0.95) reduced OTA production after interaction between different fungal strains. Compared with our study, OTA diffusion was found to be stimulated at higher a_w levels in most toxigenic strains after interaction with mixed spore cultures.

In conclusion, this study focused on OTA evaluation after interspecific interactions of different mixed spore ratios between three *A. carbonarius* toxigenic isolates and two non-toxigenic species of *Aspergillus* section *Nigri*. Overall, the different non-toxigenic *Aspergillus* section *Nigri* species, in some cases, inhibited toxin production by *A. carbonarius*, while in others, stimulated it. Therefore, understanding the effect of the inoculum relative ratio levels on OTA production will help to assess the potential application of non-toxigenic species as bio-control agents in the field. Finally, a great variation in OTA production between the three ochratoxigenic isolates of *Aspergillus carbonarius* and the competing non-toxigenic *Aspergillus* section *Nigri* species was observed. Further studies are required to examine the combined effect of temperature, a_w, and fungal interactions between toxigenic and atoxigenic species to determine the ability of the latter species on OTA diffusion. Finally, other species of the *Aspergillus* section *Nigri* group or other fungi involved in grape mycobiota could also be used in mixed spore populations together with *A. carbonarius* strains/isolates to better understand their influence on OTA production.

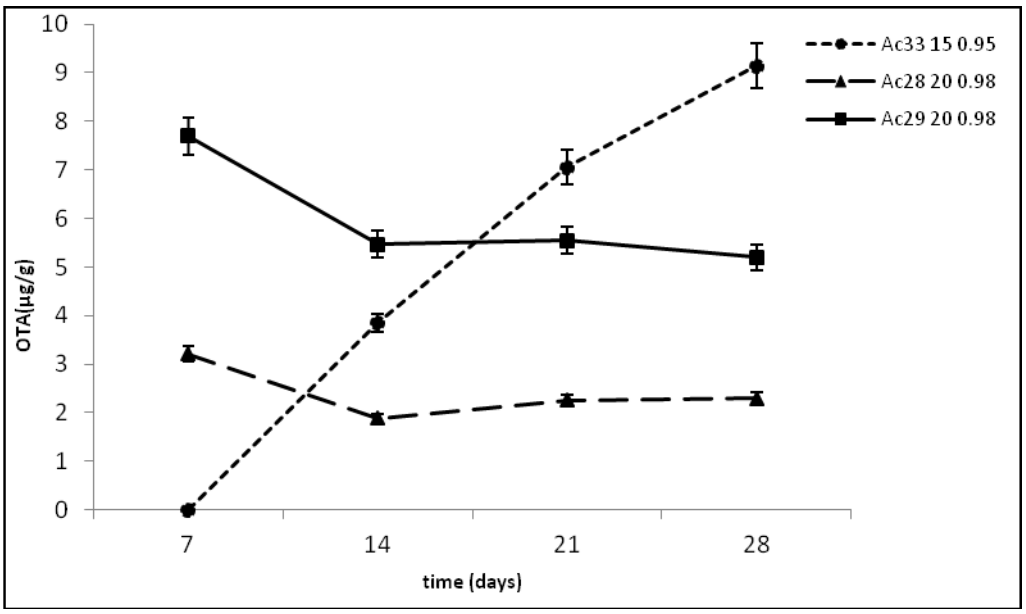

Figure 3.4: OTA production of the three *A. carbonarius* controls (100% proportion) Ac-28(▲), Ac-29(■) and Ac-33(●) in terms of time under the optimum for toxin production conditions (for Ac-28 and Ac-29 isolates 20 °C/0.98 a_w, for Ac-33 isolate 15 °C/0.95 a_w). Bars indicate standard errors of the mean.

Table 3.2: Effect of water activity (a_w), temperature (T), fungal pairs and mixed spore ratios on OTA production by three *A. carbonarius* isolates.

Temperature		25 °C		20 °C		15 °C	
Pairs	**ratios**	**0.98 a_w**	**0.95 a_w**	**0.98 a_w**	**0.95 a_w**	**0.98 a_w**	**0.95 a_w**
Ac28-At4	100-0 %	1.82[a]	0.06[a]	2.41[a]	0.42[a]	0.71[a]	0.61[a]
Ac28-At4	75-25 %	0.99[b]	0.04[ab]	1.81[a]	0.42[a]	0.57[a]	0.49[a]
Ac28-At4	50-50 %	0.24[c]	0.03[abc]	0.65[b]	0.36[a]	0.51[a]	0.48[a]
Ac28-At4	25-75 %	0.03[c]	0.01[bc]	0.26[b]	0.26[a]	0.48[a]	0.42[a]
Ac28-At4	0-100 %	0.00[c]	0.00[c]	0.00[b]	0.00[b]	0.00[a]	0.00[a]
Ac28-Aj1	100-0 %	1.86[a]	0.13[a]	3.11[ab]	1.13[a]	1.40[a]	0.48[a]
Ac28-Aj1	75-25 %	1.67[a]	0.06[a]	2.41[a]	0.85[a]	0.94[ab]	0.43[a]
Ac28-Aj1	50-50 %	1.11[ab]	0.05[a]	1.38[bc]	0.49[a]	0.71[ab]	0.29[a]
Ac28-Aj1	25-75 %	0.33[bc]	0.03[a]	1.02[cd]	0.38[a]	0.66[ab]	0.27[a]
Ac28-Aj1	0-100 %	0.00[c]	0.00[a]	0.00[d]	0.00[a]	0.00[b]	0.00[a]
Ac29-At4	100-0 %	2.47[a]	1.46[a]	2.96[a]	2.27[a]	3.02[a]	0.87[a]
Ac29-At4	75-25 %	0.23[b]	0.16[b]	0.96[b]	0.64[b]	1.36[ab]	0.64[ab]
Ac29-At4	50-50 %	0.14[b]	0.09[b]	0.63[b]	0.36[b]	0.71[ab]	0.41[ab]
Ac29-At4	25-75 %	0.08[b]	0.05[b]	0.39[b]	0.22[b]	0.68[ab]	0.26[ab]
Ac29-At4	0-100 %	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.00[b]
Ac29-Aj1	100-0 %	2.47[a]	1.46[a]	2.96[a]	2.27[a]	3.02[a]	0.87[a]
Ac29-Aj1	75-25 %	0.30[b]	0.18[b]	0.28[b]	0.27[b]	0.69[b]	0.29[ab]
Ac29-Aj1	50-50 %	0.25[b]	0.15[b]	0.25[b]	0.23[b]	0.50[b]	0.19[b]
Ac29-Aj1	25-75 %	0.04[b]	0.03[b]	0.06[b]	0.07[b]	0.23[b]	0.15[ab]
Ac29-Aj1	0-100 %	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.00[b]
Ac33-At4	100-0 %	0.49[a]	0.39[a]	0.83[a]	2.43[a]	1.07[a]	5.01[a]
Ac33-At4	75-25 %	0.20[ab]	0.23[ab]	0.38[ab]	1.75[ab]	0.40[ab]	2.61[ab]
Ac33-At4	50-50 %	0.14[ab]	0.21[ab]	0.35[b]	1.14[ab]	0.29[b]	2.34[ab]
Ac33-At4	25-75 %	0.14[ab]	0.18[ab]	0.29[b]	1.00[ab]	0.19[ab]	1.48[ab]
Ac33-At4	0-100 %	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.00[b]
Ac33-Aj1	100-0 %	0.49[a]	0.39[a]	0.83[a]	1.00[a]	1.07[a]	5.01[a]
Ac33-Aj1	75-25 %	0.08[ab]	0.05[b]	0.06[b]	0.15[b]	0.12[b]	0.86[b]
Ac33-Aj1	50-50 %	0.06[ab]	0.03[b]	0.06[b]	0.06[b]	0.04[b]	0.22[b]
Ac33-Aj1	25-75 %	0.05[ab]	0.03[b]	0.05[b]	0.04[b]	0.03[b]	0.13[b]
Ac33-Aj1	0-100 %	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.00[b]

[a] Ac, *A. carbonarius*; At, *A. tubingensis*; Aj, *A. japonicus*.

[b] Values with different letters within the same column are statistically significant ($P < 0.05$).

CHAPTER 4

Effect of interaction between Aspergillus carbonarius and non-ochratoxigenic grape-associated fungal isolates on growth and Ochratoxin A production at different water activities and temperatures

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Abstract

The effect of water activity (0.90, 0.94 and 0.98 a_w) and temperature (15, 20 and 25 °C) on the *in vitro* interactions between three ochratoxigenic strains of *Aspergillus carbonarius* (Ac-28, Ac-29 and Ac-33) and eleven non-ochratoxigenic grape-associated fungal strains was assessed in this study. Fungal strains were allowed to grow in dual cultures on Synthetic Grape-juice Medium (SGM) for 15 days and fungal interactions were given a numerical score to obtain an Index of Dominance (I_D) for each fungus. Results showed that in most cases *A. carbonarius* toxigenic strains were dominant against other fungal species. However, *A. carbonarius* presented mutual antagonism with *A.* section *Nigri* strains regardless of water activity (a_w) and temperature. Moreover, interactions with *Penicillium spinulosum* and *Cladosporium* spp. at 15 °C, as well as *Botrytis cinerea* at 20 °C, showed that the antagonists were more competitive against *A. carbonarius*. In some cases, growth rates of *A. carbonarius* strains were either slightly stimulated or inhibited after interaction in dual cultures, depending on temperature, a_w and competing species. Regarding OTA production, the presence of other species sometimes decreased the production or slightly enhanced it, depending on fungal competitor and environmental conditions. Overall, OTA production was higher at 15 °C/0.98 a_w and 20 °C/0.98 a_w for all target strains and at 20 °C/0.94 a_w for Ac-33 strain only, but decreased at higher temperatures regardless of a_w and interacting species.

Keywords: Fungal interactions, Index of Dominance, environmental factors, grape mycobiota, Aspergillus carbonarius, Ochratoxin A

4.1 Introduction

Wine grapes can be contaminated by several fungal species at preharvest period and during harvesting or grape processing (Valero et al., 2007c). Isolates from wine and table grapes belonging to the genera *Alternaria*, *Aspergillus*, *Botrytis*, *Cladosporium*, *Epicoccum*, *Eurotium*, *Fusarium* and *Rhizopus* have been found to be responsible for grape infection (Abrunhosa et al., 2001; Bellí et al., 2004a; Kizis et al., 2014). Some species belonging in *Aspergillus* and *Penicillium* genera have been found as the main source of Ochratoxin A (OTA) and in particular *Aspergillus carbonarius* as the major OTA producer in grapes and their derivatives (Bellí et al., 2002; Cabañes et al., 2002; Ostry et al., 2002; Soufleros et al., 2003; Stefanaki et al., 2003). OTA in wine was first detected by Zimmerli and Dick several years ago (1995). It is a fungal secondary metabolite that has been reported as a mycotoxin with nephrotoxic, hepatotoxic, genotoxic, teratogenic and immunotoxic impact to humans and animals (IARC, 1993; Castegnaro et al., 1998). Additionally, it has been reported that OTA is a disease determinant of Balkan endemic nephropathy (Krogh, 1978). According to the annual report of Rapid Alert System for Food and Feed (European Commission, 2012), OTA is the second more frequent mycotoxin found in food products after aflatoxins.

Although, numerous studies have examined the ecophysiology of *A. carbonarius* at different environmental conditions (Bellí et al., 2004a; Tassou et al., 2009; Spadaro et al., 2010), only few focused on the interactions between *A. carbonarius* and other fungal species (Valero et al., 2007c; Magan et al., 2010). Magan and Lacey (1984, 1985) investigated the competitiveness of different fungal species under diverse environmental conditions and classified these interactions by giving a numerical score to each species. Thus, for mutual intermingling of hyphae between two species the score 1-1 was assigned, for mutual antagonism on contact or distance the score 2-2 and 3-3 was given, respectively, and for dominance by one species over another the score 4-0 was given when species were in contact, and 5-0 when in distance. The second number in the score always indicates the antagonist strain. The score for each species can be added to obtain an overall Index of Dominance (I_D). The I_D has been reported to change significantly with water availability (a_w), temperature and nutritional status and it has been employed as an index to compare the variations under different environmental conditions in food-based ecosystems (Marín et al., 1998a; Lee and Magan, 1999; Magan and Aldred, 2007; Magan et al., 2010). Moreover, the interaction and competition between *A. carbonarius* and other fungal species has an influence on OTA

production (Lee and Magan, 2000). It is thus necessary to extend our knowledge on the outcome of interactions between *A. carbonarius* and other grape-related species under diverse environmental conditions, in an attempt to elucidate the potential of growth and OTA production in these products.

The aim of this study was to examine the effect of different environmental factors (a_w and temperature) and fungal interactions on the (a) growth rates, (b) OTA production and (c) profile of hyphae intermingling, between three *A. carbonarius* strains and eleven grape colonizing related fungi.

4.2 Materials and methods

4.2.1 Fungal strains and media preparation

The black aspergilli strains used in this study were three *A. carbonarius* OTA-positive producers (Ac-28, Ac-29, Ac-33), an *A. carbonarius* OTA-negative (Ac-27), *Aspergillus niger* (An-1), *Aspergillus tubingensis* (At-4), *Aspergillus japonicus* (Aj-1) and *Aspergillus ibericus* (Ai-1). Other grape-associated fungal strains used were *Alternaria alternata*, *Fusarium oxysporum*, *Cladosporium* spp., *Botrytis cinerea*, *Penicillium spinulosum* and *Aspergillus flavus*. All the strains have been previously isolated from grapes collected from different geographical areas of Greece and characterized at species level by molecular methods (Kizis et al., 2014). They were tested for their potential to produce OTA on Czapek yeast extract agar (CYA), after incubation at 25 °C for 7 days, as described by Bragulat et al. (2001) and characterized by high OTA production (>12 mg g^{-1}). All isolates were held in the fungal collection of the Laboratory of Microbiology and Biotechnology of Food (Agricultural University of Athens) and kept at 4 °C.

The growth medium used was a Synthetic Grape-juice Medium (SGM), similar to grape composition between véraison and ripeness (Delfini, 1982). It consisted of: 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; distilled water, *ca.* 1000 mL. The a_w of this basal medium was 0.98, measured by an AquaLab LITE (Degacon, USA) water activity meter at 25 °C. The required water activity levels of SGM (0.90 and 0.94 a_w) were adjusted by adding different amounts of glycerol (Mitchell et al., 2004). The pH of the medium was adjusted to 3.5-4.0 with KOH (2 M).

4.2.2 Inoculation and incubation conditions

All fungal strains were grown on malt extract agar (MEA; Biolab, Hungary) medium in 9 cm Petri dishes for 7-10 days at 25 °C to obtain high sporulating cultures. Spore suspensions were prepared by adding 10 mL of sterile distilled water containing 0.05% Tween 80 (Merck, Schuchardt, Germany) and scratching the colony surface with a sterile spatula. The final concentration of spores was assessed by a Neubauer counting chamber (Brand, Wertheim, Germany) and adjusted to 10^5 spores mL^{-1}, approximately. Petri dishes containing *ca.* 20 mL of the solidified SGM growth medium were inoculated in dual cultures at a distance of 45 mm with 1 mL pipette resulting in 100 spores inoculum. However, in more restricting conditions of a_w and temperature, where growth was slower, inoculation occurred at 30 mm distance. Plates with the same a_w were sealed in a polyethylene bag to minimize moisture loss and maintained at different temperatures (15, 20 and 25 °C). Controls of the three *A. carbonarius* strains were inoculated centrally on SGM plates and incubated at the same temperature conditions. Cultures were allowed to grow for 15 days at marginal, suboptimal and optimal conditions (0.90, 0.94, 0.98 a_w and 15, 20, 25 °C). All experiments were carried out with three replicates per treatment and repeated twice.

4.2.3 Growth assessment, Index of Dominance and OTA analysis

Growth of *A. carbonarius* strains was recorded daily by two diameter measurements of the mycelium at right angles to each other with the aid of a ruler. Estimates of the growth rates (mm d^{-1}) were obtained by plotting colony's radius increase against time and fitting the experimental data with linear regression. The interactions between fungal mycelia were observed macroscopically with the aid of a stereoscope (*STMPRO*, BEL Photonics, Italy) and each fungus was given a numerical score according to the modified method of Magan and Lacey (1984) as described in the introduction. All scores were added to obtain an overall Index of Dominance (I_D) as a measure of the competitiveness of individual fungal species against the three *A. carbonarius* strains under different environmental conditions.

OTA production was assessed after 15 days of incubation according to the method of Bragulat et al. (2001). Up to 6 agar plugs (6 mm diameter each) were removed with a sterile cork borer, across the diameter of the mycelium in the direction of the interaction zone. Samples were weighed, placed into a 1.5 mL eppendorf tube, 1mL of methanol added and shaken for 5 min. Then, they were left still for 1 h and filtered through a 0.2 mm syringe-

driven filter unit (Millex, Millipore Co., Bedford, Mass.) and stored at 4 °C until HPLC analysis.

The HPLC consisted of 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 samples were eluted 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, *v/v/v*) through a Waters spherisorb C18 analytical column, 5 mm ODS2 (4.6 × 250 mm) (Resteck Co., Pinnacle II, Bellefonte, USA). Run time for samples was 20 min with OTA being detected at about 11 min.

4.2.4 Statistical analysis

The effect of a_w, temperature, fungal strain and their interactions on growth of toxigenic *A. carbonarius* was evaluated by analysis of variance (ANOVA), while the effect of competing fungi was furthermore estimated by applying the Tukey's Studentized Range (HSD) test at a significance level of $p<0.05$. All statistical analyses were performed using the statistical package JMP®8 (SAS Institute Inc., Cary, NC, USA).

4.3 Results and discussion

4.3.1 Effect of a_w and temperature on Index of Dominance (I_D)

In this study, the effect of the competitiveness of grapes' mycobiota against three high OTA-producing strains of at marginal, sub-optimum and optimum conditions has been examined. Until now, only few studies focused on interactions between *A. carbonarius* strains and other fungal species which are associated with grape mycobiota and particularly interactions with black aspergilli species (Valero et al., 2007c; Magan et al., 2010). The numerical interaction scores and indices of dominance were the same for the three examined strains of *A. carbonarius* (Table 4.1). All *A. carbonarius* ochratoxigenic strains showed a mutual antagonism (2-2) when grown together with *A.* section *Nigri* strains and *A. flavus* (Fig. 4.1a and b), regardless of a_w and temperature conditions. Nevertheless, for 0.90 a_w/15 °C where no fungal growth was observed for all strains, and for the cases of 0.94 a_w/15 °C and 0.90 a_w/20 °C where the growth rate for both competing and target strains was slow, no observations were conducted and thus these interactions were not examined.

However, when toxigenic *A. carbonarius* strains were co-cultured with *P. spinulosum*, *F. oxysporum*, *A. alternata*, *Cladosporium* spp. and *B. cinerea*, significant competitiveness by the target strains was observed in most of the examined cases (dominance on contact, 4-0). In contrast, at 15 °C interactions with *P. spinulosum* and *Cladosporium* spp. showed that the antagonists were more competitive against *A. carbonarius*. Increased competitiveness of *B. cinerea* against *A. carbonarius* was also observed at 20 °C. Specifically, the score for the interaction with *P. spinulosum* at 0.98 a_w/15 °C changed from dominance on contact by one species (4-0) to mutual antagonism on contact (2-2). Additionally, the score for *Cladosporium* spp. (Fig. 4.2a and b) and *B. cinerea* (Fig. 4.3a and b) at lower temperature conditions, changed from dominance on contact by *A. carbonarius* (4-0) to dominance on contact or distance by the other species (0-4, 0-5). This was also supported by growth rates of the target strains which were also reduced in appearance of *B. cinerea*.

Table 4.1: Effect of water activity (a_w) and temperature (T) on interaction scores and Index of Dominance (I_D) for ochratoxigenic *A. carbonarius* and paired species, after 15 days of incubation. In all cases, the first score is for *A. carbonarius*. Bold scores indicate the changes in competitiveness which may occur.

Water activity (a_w)	**0.98**	**0.94**	**0.90**	***I_D***	**0.98**	**0.94**	**0.90**	***I_D***	**0.98**	**0.94**	**0.90**	***I_D***
strains / T	**25 °C**				**20 °C**				**15 °C**			
A. carbonarius Ac-27	2-2	2-2	2-2	***6/6***	2-2	2-2	n.e	***4/4***	2-2	n.e	n.g	***2/2***
A. tubingensis At-4	2-2	2-2	2-2	***6/6***	2-2	2-2	n.e	***4/4***	2-2	n.e	n.g	***2/2***
A. ibericus Ai-1	2-2	2-2	2-2	***6/6***	2-2	2-2	n.e	***4/4***	2-2	n.e	n.g	***2/2***
A. niger An-1	2-2	2-2	2-2	***6/6***	2-2	2-2	n.e	***4/4***	2-2	n.e	n.g	***2/2***
A. japonicus Aj-1	2-2	2-2	n.e	***4/4***	2-2	2-2	n.e	***4/4***	2-2	n.e	n.g	***2/2***
P. spinulosum	4-0	4-0	n.e	***8/0***	4-0	4-0	n.e	***8/0***	2-2	n.e	n.g	***2/2***
A. flavus	2-2	2-2	2-2	***6/6***	2-2	2-2	n.e	***4/4***	2-2	n.e	n.g	***2/2***
Fusarium oxysporum	4-0	4-0	n.e	***8/0***	4-0	4-0	n.e	***8/0***	4-0	n.e	n.g	***4/0***
Alternaria alternata	4-0	4-0	n.e	***8/0***	4-0	4-0	n.e	***8/0***	4-0	n.e	n.g	***4/0***
***Cladosporium* spp.**	4-0	4-0	n.e	***8/0***	4-0	4-0	n.e	***8/0***	**0-5**	n.e	n.g	***0/5***
Botrytis cinerea	4-0	4-0	n.e	***8/0***	**0-4**	**0-4**	n.e	***0/8***	**0-4**	**0-4**	n.g	***0/8***
Total ID	*32/12*	*32/12*	*10/10*	***74/34***	*28/16*	*28/16*	-	***56/32***	*22/23*	*0/4*	-	***22/27***

*n.e., not examined, *n.g., no growth.

These results suggest that *A. carbonarius* is very competitive against the other species not only at optimum, but also at suboptimum, and partially at marginal for growth conditions. Moreover, the other representative fungi of *Aspergillus* genera, presented mutual antagonism with toxigenic *A. carbonarius* in all studied cases. This reveals that competitiveness characterizes not only *A. carbonarius* but aspergilli in general. Our findings are in agreement with previous researchers (Valero et al., 2006a; Magan et al., 2010), who reported pronounced dominance of black aspergilli on other grape associated fungi. Interestingly, when Valero et al. (2007c) examined the competitiveness of *A. carbonarius* at 20 °C/0.97 a_w against *A. alternata*, they observed dominance on contact of the target strain (4-0). Similar results for *A. alternata* at 20 °C/0.98 a_w were obtained from the I_D values in the present work.

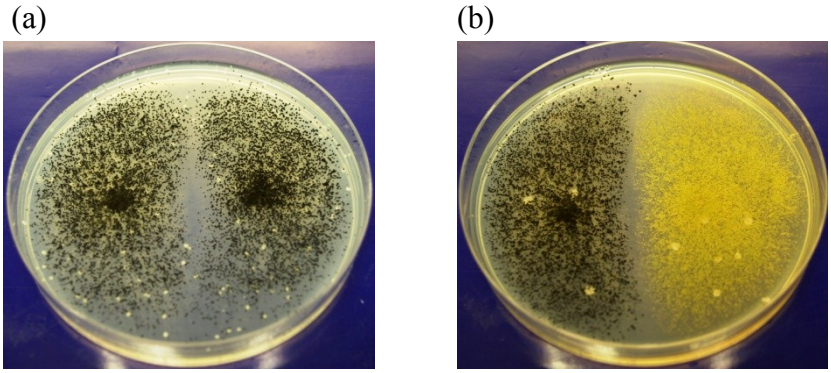

Figure 4.1: *Aspergillus carbonarius* (Ac-28) after 15 days of growth in dual cultures, with a non-toxigenic *Aspergillus carbonarius* strain (Ac-27) (a) and *A. flavus* (b), at 25 °C and 0.98 a_w. The target strain is illustrated on the left side of the Petri dish and the competitors on the right side.

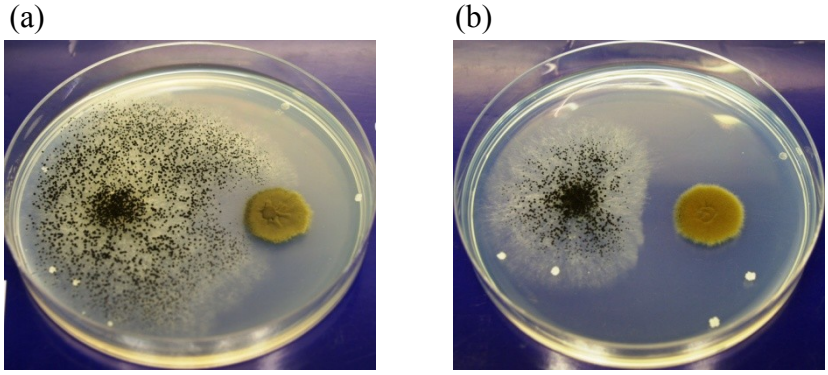

Figure 4.2: *Aspergillus carbonarius* (Ac-33) after 15 days of growth in dual cultures, with *Cladosporium* spp. at 25 °C/0.98 a_w (a) and 15 °C/0.98 a_w (b), respectively. The target strain is illustrated on the left side of the Petri dish and the competitors on the right side.

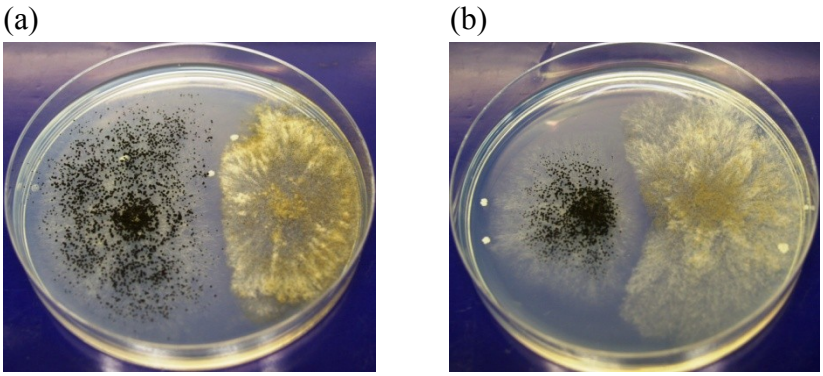

Figure 4.3: *Aspergillus carbonarius* (Ac-33) after 15 days growth in dual cultures, with *Botrytis cinerea* at 25 °C/0.94 a_w (a) and 20 °C/0.94 a_w (b), respectively. The target strain is illustrated on the left side of the Petri dish and the competitors on the right side.

Competitiveness of *A. carbonarius* over the grape associated fungi was furthermore justified by the total I_D score which was higher for optimal and suboptimal conditions for growth. Marín et al. (1998a) also reported higher I_D values for *Aspergillus* spp. when competing with other maize associated fungi on potato dextrose agar, showing also reduced competitiveness at lower levels of a_w and temperature. In our work, the total I_D score presented an increase with temperature increment from 15 °C to 25 °C but this difference was also a_w dependent. Thus, the highest I_D scores were observed at 0.94 and 0.98 a_w with the exception of 15 °C where total I_D denoted the greatest competitiveness of grape associated fungi against *A. carbonarius* (I_D: 22/27).

4.3.2 Effect of a_w, temperature and interactions on growth rate of *A. carbonarius* strains

All factors studied (T, a_w, target strains) and their interactions were found to be statistically significant according to the analysis of variance (Table 4.2). Additionally, effects of temperature, a_w and paired species on *A. carbonarius* growth rate are shown in Table 4.3. Among the competing species, a slight, but statistically significant, inhibitory effect on growth rates of Ac-28 and Ac-33 was observed by *A. ibericus* (Ai-1), regardless of a_w and temperature levels, and by *A. tubingensis* to a lesser extent. Regarding competition of Ac-29, growth rate was inhibited in most cases, but stimulated at 15 °C/0.98 a_w, by the same species. Compared with the I_D (Table 4.1), growth rate of all the target strains, was mainly inhibited in cases where mutual antagonism (2-2) observed. In general, the different fungal genera rarely presented considerable effect on growth rates of *A. carbonarius*, with main exceptions those reported previously for I_D, namely *B. cinerea* and *Cladosporium* spp. Thus, when competitiveness of these antagonists increased, resulting in a greater I_D for them, a decrease on targets' growth rate was also observed. Overall, growth rates of Ac-28 and Ac-29, which were previously isolated from Crete (Kizis et al., 2014) were decreased at 20 °C/0.94 a_w, while for Ac-33, originating from Attica, decrease was observed at 25 °C/0.98 & 0.94 a_w, as well as at 15 °C/0.98 a_w. Stimulation of *A. carbonarius* growth occurred in few cases and only for Ac-29 and Ac-33. Eventually, results showed that isolates derived from these two geographical areas presented a different fungal behaviour due to inter-specific antagonism. Similar conclusions are presented for antagonism on *A. carbonarius* by other researchers. Magan and Aldred (2008) and Marín et al. (1998a) reported that abiotic factors play a major role in influencing both the competitive ability of dominance (I_D) and growth rate in an ecological niche. To sum up, the growth rate of the target strains did not change considerably

when compared with the growth rate of the controls. Nonetheless, in some cases where mutual antagonism occurred, an inhibition of growth by the targets was observed. Yet, growth rate was not always directly related to dominance and I_D index.

Table 4.2: Analysis of variance of the effect of a_w, temperature, strain and their interactions on the growth rate of the three strains of *A. carbonarius* after 15 days of incubation on SGM medium.

Effect	Mean Square	F value
Strain	2.5192	454.70*
T	42.0350	7587.17*
a_w	55.3538	9991.18*
Strain × T	0.3473	62.70*
Strain × a_w	0.2808	50.69*
T × a_w	1.1995	216.50*
Strain × T × a_w	0.0742	13.38*

* Significant for $p<0.00001$.

4.3.3 Effect of interactions between *A. carbonarius* and other mycobiota on OTA production at different environmental conditions

Regarding OTA determination, nine different environmental conditions and interactions with eleven different species were studied. Overall, higher OTA quantities observed at 15 and 20 °C/0.98 a_w for all the target strains and at 20 °C/0.94 a_w only for Ac-33. This interspecies difference probably denotes the more xerophilous profile of Ac-33 in contrast with the other two species. Generally, OTA was decreased at higher temperatures and lower water activity levels. The lowest OTA production was observed at 25 °C/0.90 a_w. Several studies report as optimum for OTA production the suboptimal temperatures, and the intermediate and higher a_w levels for growth (Bellí et al., 2005b; Leong et al., 2006b; Magan et al., 2010; Valero et al., 2006a).

Table 4.3: Effect of water activity (a_w), temperature (T) and competing species in mean growth rates of *A. carbonarius* strains. Values with different letters within the same column are statistically significant at $P<0.05$.

Temperature	25 °C			20 °C			15 °C		
Strains / a_w	**0.98**	**0.94**	**0.90**	**0.98**	**0.94**	**0.90**	**0.98**	**0.94**	**0.90**
Control Ac-28	6.08^{ab}	4.14^{abc}	1.92^{ab}	4.22^{ab}	2.67^{ab}	n.e.	2.52^{a}	n.e.	n.g.
***A. carbonarius* Ac-27**	6.13^{ab}	3.66^{bcd}	2.00^{a}	3.90^{c}	2.25^{fg}	n.e.	2.49^{a}	n.e.	n.g.
***A. tubingensis* At-4**	5.89^{abc}	3.52^{d}	1.59^{c}	4.25^{ab}	2.29^{ef}	n.e.	2.34^{bcd}	n.e.	n.g.
***A. ibericus* Ai-1**	5.44^{c}	3.60^{d}	1.71^{bc}	3.98^{bc}	2.34^{e}	n.e.	2.28^{d}	n.e.	n.g.
***A. niger* An-1**	6.12^{ab}	3.64^{cd}	2.00^{a}	3.90^{c}	2.18^{gh}	n.e.	2.30^{cd}	n.e.	n.g.
***A. japonicus* Aj-1**	5.56^{bc}	4.16^{ab}	n.e.	3.55^{d}	2.52^{cd}	n.e.	2.33^{bcd}	n.e.	n.g.
P. spinulosum	6.07^{ab}	4.43^{a}	n.e.	3.99^{bc}	2.66^{ab}	n.e.	2.44^{ab}	n.e.	n.g.
A. flavus	6.36^{a}	3.96^{abcd}	2.12^{a}	3.84^{c}	2.23^{fg}	n.e.	2.30^{cd}	n.e.	n.g.
F. oxysporum	5.65^{bc}	3.71^{bcd}	n.e.	3.90^{c}	2.11^{h}	n.e.	2.49^{a}	n.e.	n.g.
A. alternata	5.77^{abc}	4.34^{a}	n.e.	4.35^{a}	2.61^{bc}	n.e.	2.50^{a}	n.e.	n.g.
***Cladosporium* spp.**	5.90^{abc}	3.95^{abcd}	n.e.	4.24^{b}	2.75^{a}	n.e.	2.41^{abc}	n.e.	n.g.
B. cinerea	5.73^{abc}	4.34^{a}	n.e.	4.01^{bc}	2.47^{d}	n.e.	2.31^{cd}	n.e.	n.g.
Control Ac-29	4.78^{bc}	3.61^{a}	1.56^{a}	2.93^{ab}	2.19^{a}	n.e.	2.13^{d}	n.e.	n.g.
***A. carbonarius* Ac-27**	4.50^{bc}	3.33^{abc}	1.35^{c}	2.93^{ab}	2.09^{b}	n.e.	2.01^{de}	n.e.	n.g.
***A. tubingensis* At-4**	4.92^{abc}	3.04^{bc}	1.45^{b}	2.72^{cde}	2.07^{b}	n.e.	2.49^{ab}	n.e.	n.g.
***A. ibericus* Ai-1**	4.74^{bc}	3.11^{bc}	1.51^{ab}	2.78^{bc}	1.87^{d}	n.e.	2.57^{a}	n.e.	n.g.
***A. niger* An-1**	5.14^{ab}	3.07^{bc}	1.27^{c}	3.09^{a}	2.09^{b}	n.e.	1.80^{e}	n.e.	n.g.
***A. japonicus* Aj-1**	4.75^{bc}	2.88^{c}	n.e.	2.75^{cd}	1.96^{c}	n.e.	2.10^{d}	n.e.	n.g.
P. spinulosum	4.68^{bc}	3.39^{ab}	n.e.	2.86^{bc}	2.12^{b}	n.e.	2.23^{bcd}	n.e.	n.g.
A. flavus	4.68^{bc}	3.20^{abc}	1.58^{a}	2.81^{bc}	2.09^{b}	n.e.	1.97^{de}	n.e.	n.g.
F. oxysporum	5.25^{ab}	3.09^{bc}	n.e.	2.73^{cde}	1.97^{c}	n.e.	1.97^{de}	n.e.	n.g.
A. alternata	5.25^{ab}	3.48^{ab}	n.e.	2.61^{de}	1.92^{cd}	n.e.	2.07^{de}	n.e.	n.g.
***Cladosporium* spp.**	5.52^{a}	3.43^{ab}	n.e.	2.81^{bc}	1.74^{e}	n.e.	2.14^{cd}	n.e.	n.g.
B. cinerea	5.27^{ab}	3.62^{a}	n.e.	2.56^{e}	2.11^{b}	n.e.	2.41^{abc}	n.e.	n.g.
Control Ac-33	5.38^{ab}	4.24^{bc}	1.77^{bc}	3.62^{ab}	2.59^{bcd}	n.e.	2.49^{a}	n.e.	n.g.
***A. carbonarius* Ac-27**	5.08^{bc}	3.89^{f}	1.71^{c}	3.68^{ab}	2.74^{ab}	n.e.	2.22^{bcd}	n.e.	n.g.
***A. tubingensis* At-4**	4.66^{de}	3.99^{ef}	1.83^{ab}	3.26^{cd}	2.46^{de}	n.e.	2.07^{d}	n.e.	n.g.
***A. ibericus* Ai-1**	4.43^{e}	3.51^{g}	1.80^{bc}	3.71^{ab}	2.70^{abc}	n.e.	2.23^{bcd}	n.e.	n.g.
***A. niger* An-1**	4.49^{de}	4.12^{cd}	1.92^{a}	3.44^{ab}	2.53^{d}	n.e.	2.24^{bcd}	n.e.	n.g.
***A. japonicus* Aj-1**	5.06^{bc}	3.58^{g}	n.e.	3.86^{a}	2.32^{ef}	n.e.	2.36^{ab}	n.e.	n.g.
P. spinulosum	4.79^{cd}	4.15^{bcd}	n.e.	3.72^{ab}	2.44^{def}	n.e.	2.28^{bc}	n.e.	n.g.
A. flavus	5.43^{a}	3.51^{g}	1.58^{d}	3.93^{a}	2.82^{a}	n.e.	2.34^{abc}	n.e.	n.g.
F. oxysporum	4.71^{de}	4.20^{bcd}	n.e.	3.86^{a}	2.28^{f}	n.e.	2.39^{ab}	n.e.	n.g.
A. alternata	4.61^{de}	4.08^{de}	n.e.	3.80^{a}	2.07^{g}	n.e.	2.21^{bcd}	n.e.	n.g.
***Cladosporium* spp.**	5.14^{ab}	4.78^{a}	n.e.	3.92^{a}	2.55^{cd}	n.e.	2.18^{cd}	n.e.	n.g.

*n.e.: not examined; n.g.: no growth

The effect of interaction between *A. carbonarius* strains and the other fungi on OTA production of the former is shown in Figs. 4.4-4.6. OTA production by *A. carbonarius* was mainly decreased by the competitors in all cases compared with the controls. Similar results were obtained by Valero et al. (2007c). Few exceptions were observed with some of the competing strains resulting in OTA stimulation by the target species. Specifically, *A. tubingensis* at 15 °C/0.98 a_w increased the OTA production of Ac-28, while at 20 °C/0.98 a_w a considerable decrease occurred when Ac-28 and Ac-29 were cultured with *P. spinulosum* and *A. ibericus*, respectively.

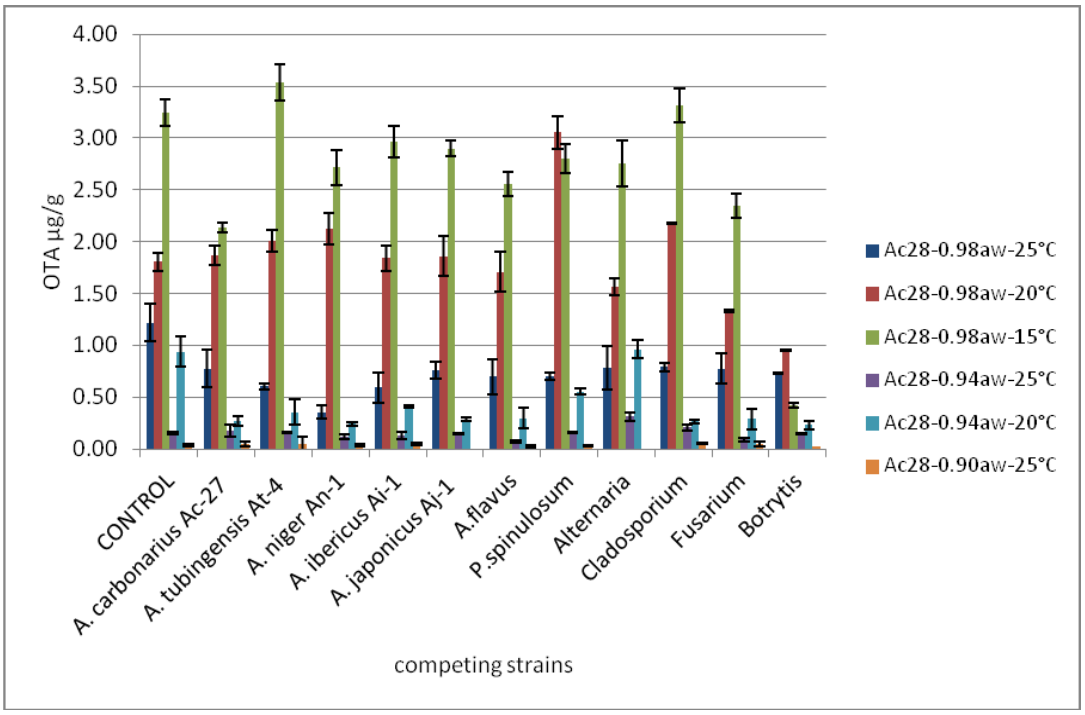

Figure 4.4: Ochratoxin A production by *A. carbonarius* (Ac-28) when grown against other grape-associated fungal strains. Error bars indicate standard deviation of 3 replicates.

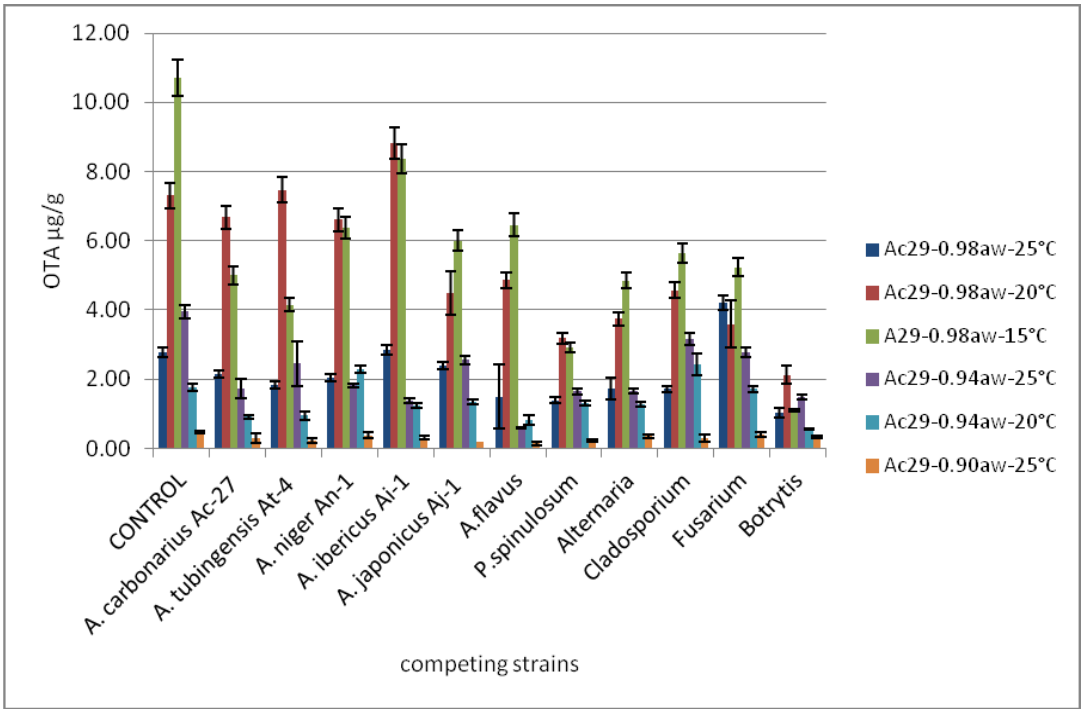

Figure 4.5: Ochratoxin A production by *A. carbonarius* (Ac-29) when grown against other grape-associated fungal strains. Error bars indicate standard deviation of 3 replicates.

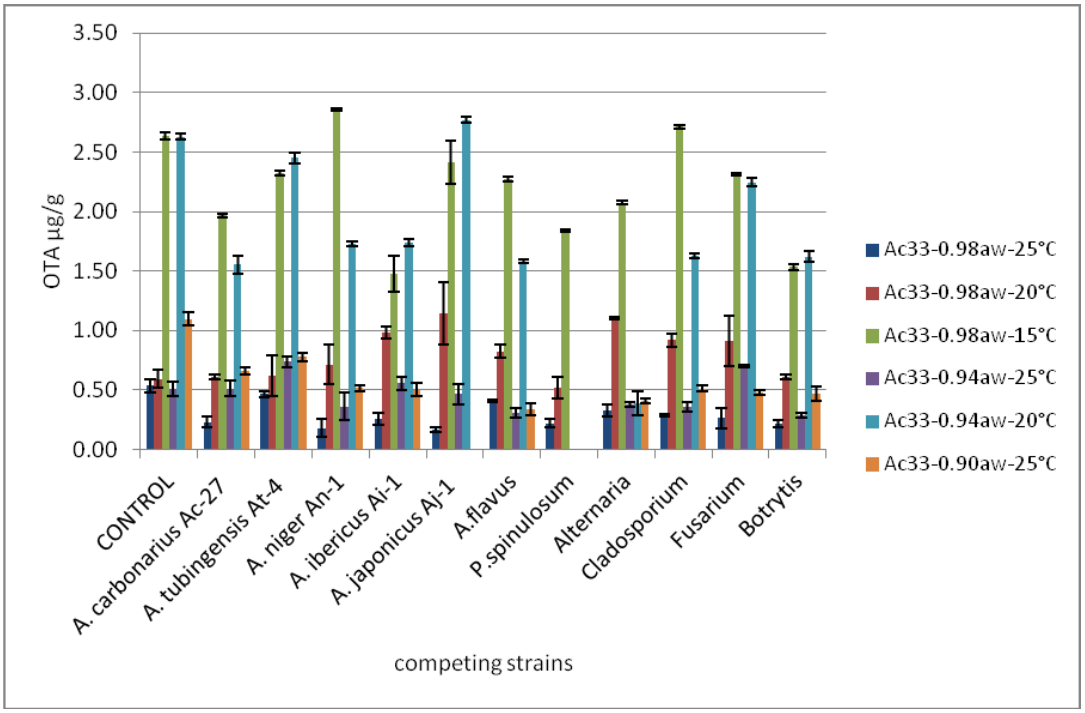

Figure 4.6: Ochratoxin A production by *A. carbonarius* (Ac-33) when grown against other grape-associated fungal strains. Error bars indicate standard deviation of 3 replicates.

For the same conditions, Ac-33 presented stimulation in OTA production when co-cultured with *A. japonicus*, *A. alternata*, *Cladosporium* spp. and *F. oxysporum*. The latter species also stimulated OTA of Ac-28 at 25 °C/0.98 a_w. Valero et al. (2007c) reported that only *Penicillium janthinellum* and *Eurotium amstelodami* resulted in stimulation of OTA production, while Magan et al. (2010) concluded that in some cases *A. alternata* and *Cladosporium* spp. Stimulated OTA production at dual cultures with *A. carbonarius*. Nevertheless, in contrast to our results, the latter study reveals OTA stimulation at 20 °C/0.98 a_w by all competitors examined. Generally, for target strains, even at conditions where the growth rate was stimulated by the competitors, OTA concentration was decreased. In all conditions, *B. cinerea* had the most inhibitory effect on OTA accumulation by both Ac-28 and Ac-29. In contrast, Magan et al. (2010) reported that OTA was increased after interaction between *A. carbonarius* and *B. cinerea* at 20 °C regardless of a_w assayed and at 25 °C/0.95 a_w and 30 °C/0.98 a_w. However, our observations are particularly important given that *B. cinerea* belongs to the autochthonous mycobiota of grapes and could be an obstacle in the growth and OTA production of *A. carbonarius* in the field. Moreover, it is known that *B. cinerea* is producing large amounts of hydrolytic enzymes and therefore this might be an important factor to consider for OTA accumulation (Magan et al., 2010). Abrunhosa et al. (2002) found that a range of fungi isolated from grapes were able to degrade OTA from *A. carbonarius* strains. Overall, this work illustrated that fungal interactions were most inhibitory and less stimulatory to OTA production by three high OTA producing *A. carbonarius* strains. It is clear that the influence of changes in climate conditions compared to fungal interactions might be a crucial parameter and play a significant role in the study of mycotoxin control.

CHAPTER 5

Modelling the effect of natamycin, pine-resin and environmental factors on the growth and OTA production by Aspergillus carbonarius using response surface methodology

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Abstract

The effect of two antifungal compounds (natamycin, pine-resin), temperature and water activity, on the growth rate, lag phase duration and Ochratoxin A (OTA) production by three *Aspergillus carbonarius* isolates (Ac-28, Ac-29 and Ac-33), was studied by means of Response Surface Methodology (RSM) based on a Central Composite Design (CCD). Two different experimental designs were performed as a function of temperature (16.6-33.4 °C), water activity (0.90-0.97 a_w), natamycin (0-1000 ng mL^{-1}) or pine-resin (0-2.61%, *w/v*) on a Synthetic Grape-juice Medium (SGM). OTA production was analyzed after 5, 10 and 15 days of incubation. A second order polynomial model was fitted to each response parameter to assess the growth and OTA potential of all fungal isolates. Results showed that natamycin, a_w and temperature had significant effects on the lag phase duration of all isolates, as well as on OTA accumulation after 10 days of incubation for Ac-29 and 15 days for Ac-28 and Ac-33 isolates. The same results were obtained for OTA production after treatment with pine-resin. However, fungal growth rates were not statistically significant in both experiments, with the exception of Ac-29 and Ac-33 after treatment with pine-resin. Overall, high natamycin concentrations (800 and 1000 ng mL^{-1}) delayed fungal growth depending on the environmental factors assayed. Moreover, treatment with pine-resin at 16.6 °C/0.94 a_w/1.1% *w/v*, as well as at 25 °C/0.90 a_w/1.1% *w/v*, completely inhibited fungal growth up to 15 days of incubation.

Keywords: natamycin, pine-resin, A. carbonarius, response surface methodology, central composite design

Chemical compounds studied in this article: Natamycin (PubChem CID: 5284447).

5.1 Introduction

Aspergillus carbonarius, a mold naturally occurring on grapes, has been reported as the major fungal species responsible for the presence of Ochratoxin A (OTA) in wine and grape derivatives (Magan and Aldred, 2005). The toxin has teratogenic, nephrotoxic and immunotoxic properties and it has been classified as a possible human carcinogen (Group 2B) (IARC, 1993). It has being legislated by the European Union with a permitted amount of 2.0 μg kg^{-1} for wine, must and grape juice (European Commission, 2006), due to its high toxicity and common detection in wines worldwide (Battilani, Giorni, Bertuzzi, Formenti and Pietri, 2006b; Chulze, Magnoli and Dalcero, 2006; Tjamos, Antoniou and Tjamos, 2006; Chiotta, Ponsone, Combina, Torres and Chulze, 2009). For this reason, appropriate agricultural management in the field and the use of fungicides at permissible doses, must be considered in order to prevent growth of mycotoxin-producing fungi and therefore to minimize the consumer exposure in such natural occurring toxins.

Controlling fungal disease and toxin production on grapes using fungicides is quite complex since recent evidence indicates that the use of some fungicides, under certain conditions, may stimulate the production of mycotoxins (Bellí, Marín, Sanchis and Ramos, 2006b; Medina, Jiménez, Mateo and Magan, 2007). Several studies have previously examined the effectiveness of various commercial fungicides, such as Carbendazim and Switch, against fungal growth and OTA production by *A. carbonarius* (Tjamos et al., 2004; Bellí et al., 2006b; Medina et al., 2007b; García-Cela, Ramos, Sanchis and Marín, 2011).

Nowadays, a strict legislation concerning the use of chemicals and maximum residue levels of pesticides (MRLs) on the field has been established from the European Union for many products, including grapes, in order to ensure the lowest possible consumer exposure (European Commission, 2005b). Therefore, there is a growing interest in examining alternative antifungal compounds against the appearance of *A. carbonarius* on grapes and grape products (Medina et al., 2007a). Natamycin is an attractive natural antifungal agent which is produced by the fermentation of the bacterium *Streptomyces natalensis*. It is allowed for use in many countries as a food additive and it is commonly used in dairy products, especially at the surface of cheese and dry sausages, to eliminate mold contamination at these products (European Commission, 1995). Additionally, the European Food Safety Authority (EFSA) has published a favorable scientific opinion on the use of natamycin as a food additive (EFSA, 2009). Studies revealed that only a minimum amount of natamycin (≤1 μg

L^{-1}) is required to inhibit almost completely OTA production by *Aspergillus ochraceus*, and also to reduce other mycotoxins produced by several food-borne fungal species (Stark and Tan, 2003b). Natamycin has a broad spectrum of activity against toxigenic molds such as *Aspergillus flavus* (aflatoxin), *Aspergillus niger* (ochratoxin) and other (Rusul and Marth, 1988; Stark, 2003a). On the other hand, another natural substance, namely pine-resin, which is produced from the hydrocarbon secretion of conifer trees, has been traditionally employed in Greece in the production of resinated wine, but it has never been studied for its efficacy to control fungal growth and OTA production by *A. carbonarius*.

Additionally, the range of environmental factors was selected in order to take into account the marginal growth regimes of *A. carbonarius* and enable the modelling of these parameters. Besides, the moisture content of grapes may vary according to processing conditions, e.g. sun-dried grapes. Grapes intended for raisins and sweet wines production are exposed to sun-drying conditions for extended periods of time, where diurnal temperatures can be higher than 40 °C in Mediterranean areas, and water activity of grapes drops gradually even under 0.75 due to their high sugar content and water losses (Valero, Sanchis, Ramos and Marín, 2007c).

The aim of the present study was to employ a response surface methodology based on a central composite experimental design to investigate the effect of two antifungal compounds (natamycin and pine-resin) combined to varied environmental factors (temperature and a_w) on the growth rate (μ_{max}), lag phase duration (λ), and OTA accumulation by three *A. carbonarius* isolates on a synthetic grape juice medium (SGM) simulating must during processing. The developed models could be useful to evaluate the risk of contamination by *A. carbonarius* under the effect of natamycin and pine-resin as alternative antifungal agents.

5.2 Materials and methods

5.2.1 Fungal isolates

Three isolates of *A. carbonarius* were used in this study (Ac-28, Ac-29 and Ac-33). All of them have been previously isolated from grapes of Greek vineyards and identified at species level by molecular techniques (Kizis, Natskoulis, Nychas and Panagou, 2014). They were also tested for their ochratoxigenic potential on Czapek Yeast extract Agar (CYA) after incubation at 25 °C for 7 days, and evaluated as high OTA producing species (> 12 mg g^{-1}).

All isolates were kept in the fungal collection of the Laboratory of Microbiology and Biotechnology of Foods, Agricultural University of Athens, at −20 °C until use.

5.2.2 Medium preparation and formulation of natamycin & pine-resin

A Synthetic Grape-juice Medium (SGM) was employed in this study as a simulation of grape composition between véraison and ripeness (Magan, Aldred, Hope and Mitchell, 2010). The a_w of the medium was measured by an AquaLab LITE (Degacon, USA) a_w meter at 25 °C and modified with glycerol at different a_w levels (0.90-0.97) as provided by the CCD. Finally, the pH of the medium was adjusted after sterilization to 3.5-4.0 with KOH (2 M).

Natamycin formulation was Delvocid (50% a.i., Heerlen, Netherlands), whereas a stock solution of 10 μg mL^{-1} was diluted in sterile distilled water (pH 6.5) and kept in darkness until dilution with the media in order to avoid any loss of activity (Clark, Shirk and Kline, 1964). Pine-resin was kindly provided from Megara resins industry (Fanis Anastassios S.A., Megara, Greece) and a stock solution of 1 g mL^{-1} was diluted in ethanol (100% purity). Afterwards, the solution was filtrated through a sterile release membrane filter of 0.25 μm mean pore diameter (PES, Millex, Millipore Co., Bedford, Mass.) and finally both antifungals were diluted to the required range of concentrations according to the CCD. Natamycin was added into the autoclaved medium and poured into 9-cm Petri dishes at concentrations ranged from0 to 1000 ng mL^{-1}. In contrast, pine-resin was impossible to incorporate into the medium due to its low solubility in the water. However, resin could be dissolved in ethanol, and the later was used as solvent to prepare the appropriate pine-resin concentrations. Sterilized cellophane membranes (P400, Cannings Ltd., Bristol) were soaked into the pine-resin after the dilution with ethanol (1:1, *w/v*) to obtain concentrations between 0 and 2.61% and placed on top of the culture media before spore inoculation. It has been shown that membrane allows fungus to obtain nutrients from the substrate without affecting significantly its growth (Ramos, Magan and Sanchis, 1999). Preliminary studies were carried out in order to examine the effect of ethanol on the inhibition of the fungus. It was found that ethanol did not have any adverse effect on fungal growth due to rapid evaporation before inoculation.

5.2.3 Inoculation and incubation conditions

All isolates of *A. carbonarius* were initially 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) medium into 9 cm Petri dishes for 7 days at 25 °C. Spore suspensions were prepared by adding 10 mL of sterile distilled water containing 0.05%Tween 80 wetting agent onto the media (Merck, Schuchardt, Germany) and scratching the mycelium with a sterile stainless steel spatula. The final spore concentration was determined by a Neubauer counting chamber (Brand, Wertheim, Germany) and adjusted to 3×10^5 spores mL^{-1}. Finally, Petri dishes containing *ca.* 20 mL of the solidified medium, at different water activities, were centrally inoculated with 3 μL of spore suspension and incubated in a range of temperature conditions between 16.6-33.4 °C as defined by the CCD. Plates with the same a_w were maintained into the same polyethylene bags to minimize moisture fluctuation.

5.2.4 Experimental design

Response surface methodology (RSM) is an effective experimental tool in statistics, where many factors and their interactions can be simultaneously varied, providing an effective methodology to investigate the aspects affecting the desired response (Box and Wilson, 1951). The Central Composite Design (CCD) is the most widely used experimental design with center and marginal points, parameter interaction, rotatability, high quality predictions and reduced number of experimental runs (Myers and Montgomery, 2002). This experimental design has been widely used in process optimization for the production of secondary metabolites and absorption of heavy metals by different fungal species (Sergent, Parra and Dantigny, 2013).

As two different antifungals were used (natamycin and pine-resin), two different experimental designs were also implemented. The effect of natamycin, pine-resin and environmental factors were evaluated on the μ_{max}, λ and OTA production of the 3 fungal isolates using a CCD experiment). The CCD was a 2^3 full factorial design with three variables (X1, temperature; X2, a_w; X3, natamycin or pine-resin), three center points, two replicates and a response surface methodology.

For each experiment, three coded variables at five levels were determined as follows: −1.68, −1, 0, +1 and +1.68, where −1.68 and +1.68 are indicated as low and high star points,

respectively, −1 is the cube point responding to the low level of each factor, 0 is the central point (intermediate level) and +1 is the cube point representing the high level of every factor.

The actual level of each factor was calculated using the following equation (Neter, Kutner, Nachtsheim and Wasserman, 1996):

$$\text{Actual level} = \text{Coded value} * (\text{high level} - \text{low level})/2 + (\text{high level} + \text{low level})/2 \quad (1)$$

Relationships between coded and actual levels are listed in Table 5.1. This experimental design was performed using the statistical package JMP®8 (SAS Institute Inc., Cary, NC, USA).

Table 5.1: Relationship among actual and coded values of the variables used in the central composite design (CCD).

Point	star	low	center	high	star
Coded values	**−1.68**	**−1**	**0**	**+1**	**+1.68**
Temperature[1,2]	16.6	20	25	30	33.4
Water activity[1,2]	0.90	0.92	0.94	0.96	0.97
Natamycin (ng mL^{-1})[1]	0	200	500	800	1000.4
Pine-resin (%) [a,2]	0	0.20	1.10	2.00	2.61

[1]CCD with three variables: natamycin, a_w and T

[2]CCD with three variables: pine-resin, a_w and T

[a] (pine-resin % *w/v*)

5.2.5 Fungal growth and OTA analysis

Radial μ_{max} was assessed by measuring the diametric extension (in millimeters) of the mycelium at right angles for up to 15 days. The mean value of the radii were used to determine the radial μ_{max} and λ by applying the Baranyi's primary model (Baranyi and Roberts, 1995), modified accordingly to adapt to fungal growth (Marín, Cuevas, Ramos and Sanchis, 2008):

$$R_{(t)} = \mu_{max} A - \log\left\{1 + \frac{[\exp(\mu_{max} A) - 1]}{\exp(R_{max})}\right\} \quad (2)$$

and

$$A = t + \frac{1}{\mu_{max}} \log[\exp(-\mu_{max}t) + \exp(-\mu_{max}\lambda) - (\mu_{max}t - \mu_{max}\lambda)] \quad (3)$$

where R(t) indicates the changes in colony radius versus time (mm), μ_{max} is the maximum growth rate (mm day^{-1}), and λ is the lag phase duration (days).

Subsequently, the effect of the three independent variables (i.e., temperature, a_w, natamycin or pine-resin concentration) on the μ_{max}, λ and OTA production was modelled using the following quadratic polynomial equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_{11} X_1^2 + \beta_2 X_2 + \beta_{22} X_2^2 + \beta_3 X_3 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (4)$$

where X1, X2 and X3 are the coded values of the factors listed in Table 5.1, and the parameters $\beta 0 \ldots \beta 23$ are the coefficients of the polynomial model.

OTA production was analyzed after 5, 10 and 15 days of incubation for each case assayed. It needs to be noted that OTA accumulation did not show a linear trend over time and therefore the actual OTA concentrations were modelled individually for each sampling day. The methodology for OTA determination was proposed by Bragulat, Abarca and Cabañes (2001). Specifically, up to 6 agar plugs with the mycelium (6 mm diameter each) were removed from the media by means of a sterile cork borer and transferred into a 2 mL pre-weighed eppendorf tube. One milliliter of methanol was added and the tubes were placed into the rotating shaker for 5 min. Then, they were left still for 1 h, filtered through a 0.2 μm syringe-driven filter unit (Millex, Millipore Co., Bedford, Mass.) and analyzed by HPLC as described elsewhere (Kogkaki, Natskoulis, Magan and Panagou, 2015).

The limit of quantification was 2.0 ng OTA g^{-1} SGM and the limit of detection was 1.0 ng OTA g^{-1} SGM. Additionally, known concentrations of OTA (50 and 500 ng mL^{-1}) were spiked on SGM and a recovery assay for the HPLC method was assessed with satisfactory recovery percentages of 82-110%.

5.2.6 Statistical analysis

The regression coefficients of the linear, quadratic and interaction terms were determined by ANOVA analysis. Moreover, the correlation between the response and independent variables was illustrated by means of response surface plots showing the simultaneous interaction of two variables on the responses assayed. All data were analyzed using the statistical package JMP®8 (SAS Institute Inc., Cary, NC, USA). Finally, the

performance of the polynomial models was determined computationally by the calculation of the coefficient of determination (R^2) and the root mean squared error (RMSE), and also graphically by plotting the observed against the predicted values of the response variables.

5.3 Results and discussion

5.3.1 Effect of natamycin and environmental factors on the growth and OTA production by *A. carbonarius*

The analysis of variance revealed that all single factors (T, a_w, natamycin) had significant effects on the λ, as well as the interaction terms of T × natamycin, T × T and T × a_w, except the latter term for Ac-33. Additionally, natamycin was statistically significant for OTA accumulation after 10 days of growth for Ac-29 and 15 days for Ac-28 and Ac-33 (Table 5.2). Regarding the fungal μ_{max}, natamycin was not statistically significant for any of the 3 isolates assayed in this work, contrary to the factors of T and a_w that presented high statistical significance for all the examined isolates. Regards to the interaction terms of the factors studied, only natamycin × natamycin for Ac-29 and T × a_w for Ac-33 were found to have a significant effect on μ_{max}. According to the CCD, five temperature conditions were selected (16.6, 20, 25, 30 and 33.4 °C), whereas at the lowest condition tested (16.6 °C) fungal growth was completely inhibited for all isolates at 0.94 a_w and 500 ng mL^{-1} natamycin.

The response surface plots of λ of *A. carbonarius* as a function of natamycin concentration and T at 0.94 a_w are shown in Fig. 5.1a through c for Ac-28, Ac-29, and Ac-33, respectively. The plots showed a similar trend for all isolates indicating that high concentrations of natamycin (≥800 ng mL^{-1}) combined with low T levels (≤20 °C) could prolong the lag phase duration and hence delay the growth of the fungus. This effect was less pronounced as the level of T progressively increased even for high concentrations of natamycin. Moreover, the experimental data could be appropriately fitted into a second-order polynomial model with a coefficient of determination (R^2) close to 0.90 for all responses (0.92 for Ac-28, 0.91 for Ac-29 and 0.89 for Ac-33) (Table 5.2). The model adequacy could be also graphically observed by plotting the observed versus predicted lag times (Fig. 5.2). The observed response values agreed well with the predicted response values. The latter values distributed normally on the straight line approving the satisfaction of normality assumption. Thus, a linear distribution of the predicted response values indicating a well-fitting model.

Treatments included in the CCD indicated that the lag time was extended when natamycin concentration was increased, yet lag time was shorter at those cases with high a_w levels and relatively high temperatures (25 °C/0.97 a_w/500 ng mL^{-1}, 30 °C/0.96 a_w/200 & 800 ng mL^{-1}). Additionally, high temperature conditions combined with intermediate a_w levels (30 °C/0.92 a_w/200 & 800 ng mL^{-1}, 30 °C/0.96 a_w/200 & 800 ng mL^{-1}, 33.5 °C/0.94 a_w/500 ng mL^{-1}) were noticed for the short lag time as well, regardless of natamycin concentration (Supplementary Table 5.1). Conclusively, treatment with natamycin delayed spore germination for *A. carbonarius* under the range of environmental factors studied. Therefore, the effect of this agent, on the time elapsed between inoculation and visible fungal growth was found to be of high importance. The response surface plot of the interaction of natamycin and a_w at 25 °C for the variable of μ_{max} is shown in Supplementary Fig. 5.1. It is graphically clear that only a_w affected the μ_{max} of the fungus, whereas natamycin did not play a significant role in reducing the fungal growth rate. The associated regression statistics are shown in Table 5.2. The experimental data fitted satisfactorily into the second-order polynomial model with a coefficient of determination (R^2) of 0.90 for Ac-28 and even higher for the other two *A. carbonarius* isolates.

Fig. 5.3 presents the OTA accumulation by the three *A. carbonarius* isolates as a function of temperature, a_w and natamycin concentration after 5, 10 and 15 days of incubation. As expected, OTA production was lower at higher temperature levels regardless of natamycin concentration. Furthermore, OTA accumulation by all the examined isolates was reduce at the 15th day of incubation in the highest natamycin concentration, with the exception of 20 °C/0.92 a_w/800 ng mL^{-1} for Ac-28. At 25 °C/0.94 a_w/500 & 1000 ng mL^{-1}, OTA production was reduced compared to the control (25 °C/0.94 a_w/0 ng mL^{-1}) for all *A. carbonarius* isolates. Modelling the OTA production showed some difficulties since the percentage of variation (R^2) explained by the models was relatively low (0.78 for Ac-28 after 15 days, 0.77 for Ac-29 after 10 days, and 0.72 after 15 days for Ac-33). Supplementary Fig. 5.2 presents the response surface profiles for OTA production after 10 days of incubation for Ac-29 and 15 days for Ac-28 and Ac-33, as a function of a_w and natamycin concentration at 25 °C. Overall, OTA production was higher at marginal temperature conditions for fungal growth (20 °C) in most cases, regardless of natamycin or a_w level. Particularly, Ac-29 produced the highest OTA amounts at 25 °C/0.97 a_w/500 ng mL^{-1}, that reached 6.28 followed by 5.05, and 3.19 μg g^{-1} after 5, 10, and 15 days of growth, respectively. Maximum OTA levels were also observed at the condition of 25 °C/0.90 a_w/500 ng mL^{-1}, probably due to low a_w level, a stressful condition for fungal growth, and hence the toxigenic potential of *A.*

carbonarius might be increasing under these conditions (Bellí, Ramos, Coronas, Sanchis and Marín, 2005b). The lowest OTA production was detected at high temperature conditions combined with intermediate a_w and relatively high natamycin concentrations, i.e., 30 °C/0.92 a_w/800 ng mL^{-1} and 33.4 °C/0.94 a_w/500 ng mL^{-1}, for all *A. carbonarius* isolates.

Natamycin is a fungicide of the polyene macrolide group and its antifungal activity is due to its ability of binding to the cell membrane sterols, particularly ergosterol which is the principal sterol in fungal membranes. The physical state of the membrane is changing due to a hydrophobic region which complexes with ergosterol and small ions such as K+, H+, amino acids and other metabolites can freely pass, disrupting the cell's ionic control and finally kill the cell (Hamilton-Miller, 1974; Deacon, 1997). The fact that prolonged lag phases were observed in this study indicates that natamycin may be able to delay *A. carbonarius* germination and thus decelerate the contamination in concentrations up to 1 μg mL^{-1}. Gourama and Bullerman (1988) discussed the effect of natamycin on the growth and ochratoxin production by *A. ochraceus* in olive paste and found that the inhibitory effect of natamycin was more pronounced on toxin production than on fungal growth. These findings are in agreement with our study since natamycin had no significant effect on the fungal growth rate in contrast with OTA production. Medina et al. (2007a), also studied the potential of natamycin to reduce fungal growth and OTA production by *A. carbonarius*. They reported that the presence of natamycin prevented fungal growth and OTA production at 20 ng mL^{-1} in a freshly prepared red grape extract medium produced by organic table grapes.

For the present study, low levels of natamycin were used, up to 1 μg mL^{-1}, corresponding to the lower limit of permitted concentrations (EFSA, 2009). However, despite the restricted use of natamycin on the surface of cheeses and sausages, it is important to carry out research in order to investigate the effectiveness of this compound in other foods and ways of application. The new data derived hereby could broaden the use of natamycin as an interesting alternative to sodium metabisulphite that is widely applied today in musts and currants. Given the concern of authorities for the adverse effects of SO_2 on the health of consumers, natamycin could have potential as a preservative for the wine industry.

Table 5.2: Parameter estimates of the polynomial model for λ, μ_{max} and OTA production by *A. carbonarius* isolates (Ac-28, Ac-29 and Ac-33) after treatment with natamycin.

	Lag phase			Growth rate			OTA production		
Parameter	**Ac-28**	**Ac-29**	**Ac-33**	**Ac-28**	**Ac-29**	**Ac-33**	**Ac-28 (15 days)**	**Ac-29 (10 days)**	**Ac-33 (15 days)**
Intercept	*3.166±0.364****	*2.658± 0.386****	*3.002±0.425****	*3.486±0.223****	*3.071±0.168****	*2.820±0.131****	*0.096±0.202*	*1.358±0.319****	*0.719±0.255***
T	*2.904±0.171****	*-2.894±0.181****	*-2.889±0.199****	*1.597±0.104****	*1.456±0.078****	*1.069±0.061****	*-0.444±0.094****	*-0.594±0.150****	*-0.213±0.119*
a_w	*-0.807±0.171****	*-0.882±0.181****	*-0.631±0.199***	*1.134±0.104****	*0.768±0.078****	*0.893±0.061****	*-0.400±0.094****	*1.209±0.150****	*-0.664±0.119****
Natamycin	*0.900±0.171****	*1.051±0.181****	*1.090±0.199****	*-0.065±0.104*	*-0.013±0.078*	*-0.044±0.061*	*0.211±0.094**	*-0.488±0.150***	*-0.281±0.119**
T * a_w	*0.473±0.223**	*0.589±0.237**	*0.490±0.260*	*0.134±0.137*	*0.204±0.102*	*0.201±0.080**	*0.442±0.123****	*-0.929±0.196****	*0.202±0.156*
T * natamycin	*-1.107±0.223****	*-1.101±0.237****	*-1.457±0.260****	*-0.228±0.137*	*0.081±0.102*	*-0.079±0.080*	*-0.460±0.123****	*0.295±0.196*	*0.059±0.156*
a_w* natamycin	*-0.06107±0.223*	*-0.169±0.237*	*-0.112±0.260*	*0.126±0.137*	*-0.048±0.102*	*0.152±0.080*	*-0.461±0.123****	*-0.045±0.196*	*0.308±0.156*
T *T	*1.630±0.188****	*1.752±0.199****	*1.537±0.219****	*0.045±0.115*	*0.027±0.086*	*-0.063±0.068*	*-0.046±0.104*	*-0.480±0.165***	*-0.429±0.132***
a_w* a_w	*-0.027±0.188*	*0.043±0.199*	*-0.030±0.219*	*-0.040±0.115*	*-0.169±0.086*	*-0.005±0.068*	*0.738±0.104****	*0.402±0.165**	*0.753±0.132****
Natamycin*natamycin	*-0.187±0.188*	*0.101±0.199*	*0.037±0.219*	*0.107±0.115*	*0.185±0.086**	*0.110±0.068*	*0.076±1.104*	*0.234±0.165*	*0.049±0.132*
RMSE	*1.095*	*1.162*	*1.278*	*0.671*	*0.504*	*0.396*	*0.607*	*0.961*	*0.768*
R^2	*0.92*	*0.91*	*0.89*	*0.90*	*0.92*	*0.93*	*0.78*	*0.77*	*0.72*

* Significant at $P < 0.05$
** Significant at $P < 0.01$
*** Significant at $P < 0.001$

Table 5.3: Parameter estimates of the polynomial model for λ, μ_{max} and OTA production by *A. carbonarius* (Ac-28, Ac-29 and Ac-33) after treatment with pine-resin.

	Lag phase			Growth rate			OTA production		
Parameter	**Ac-28**	**Ac-29**	**Ac-33**	**Ac-28**	**Ac-29**	**Ac-33**	**Ac-28 (15 days)**	**Ac-29 (10 days)**	**Ac-33 (15 days)**
Intercept	*3.061±0.973***	*3.256±1.000***	*3.833±0.916****	*0.842±0.148****	*0.604±0.146****	*0.553±0.120****	*6.094±0.611****	*30.786±0.873****	*83.188±4.963****
T	*-1.755±0.457****	*-1.148±0.469**	*-2.117±0.430****	*0.523±0.069****	*0.323±0.068****	*0.429±0.056****	*-1.173±0.287****	*-1.078±0.410***	*0.112±2.330*
a_w	*-2.177±0.457****	*-2.585±0.469****	*-2.043±0.430****	*0.626±0.069****	*0.551±0.068****	*0.514±0.056****	*0.543±0.287*	*2.075±0.410****	*3.330±2.330*
Pine-resin	*0.368±0.457*	*1.302±0.469***	*0.332±0.430*	*-0.082±0.069*	*-0.181±0.068**	*-0.136±0.056**	*-0.702±0.287**	*-1.849±0.410****	*5.148±2.330**
T * a_w	*-0.184±0.597*	*-1.782±0.614***	*0.409±0.562*	*0.554±0.091****	*0.286±0.090***	*0.439±0.073****	*-0.288±0.375*	*-2.619±0.535****	*6.479±3.045**
T * pine-resin	*0.172±0.597*	*1.457±0.614**	*-0.228±0.562*	*-0.093±0.091*	*-0.278±0.090***	*0.005±0.073*	*0.345±0.375*	*0.399±0.535*	*-0.606±3.045*
a_w* pine-resin	*-0.106±0.597*	*-1.519±0.614**	*-0.141±0.562*	*-0.157±0.091*	*0.034±0.090*	*-0.126±0.073*	*-0.655±0.375*	*-0.891±0.535*	*1.072±3.045*
T *T	*1.247±0.503**	*1.441±0.517***	*0.948±0.473*	*-0.010±0.076*	*0.029±0.075*	*0.091±0.062*	*-1.928±0.316****	*-10.567±0.451****	*-31.285±2.565****
a_w* a_w	*1.094±0.503**	*1.406±0.517***	*0.991±0.473**	*0.041±0.076*	*0.020±0.075*	*0.085±0.062*	*-1.553±0.316****	*-9.729±0.451****	*-22.457±2.565****
Pine-resin*pine-resin	*-1.164±0.503**	*-0.661±0.517*	*-0.803±0.473*	*0.124±0.076*	*0.470±0.075****	*0.223±0.062****	*-0.675±0.316**	*-7.766±0.451****	*-10.682±2.565****
RMSE	*2.927*	*3.008*	*2.755*	*0.446*	*0.441*	*0.361*	*1.838*	*2.625*	*14.92*
R^2	*0.59*	*0.67*	*0.60*	*0.82*	*0.79*	*0.83*	*0.66*	*0.96*	*0.82*

*Significant at $P < 0.05$
** Significant at $P < 0.01$
*** Significant at $P < 0.001$

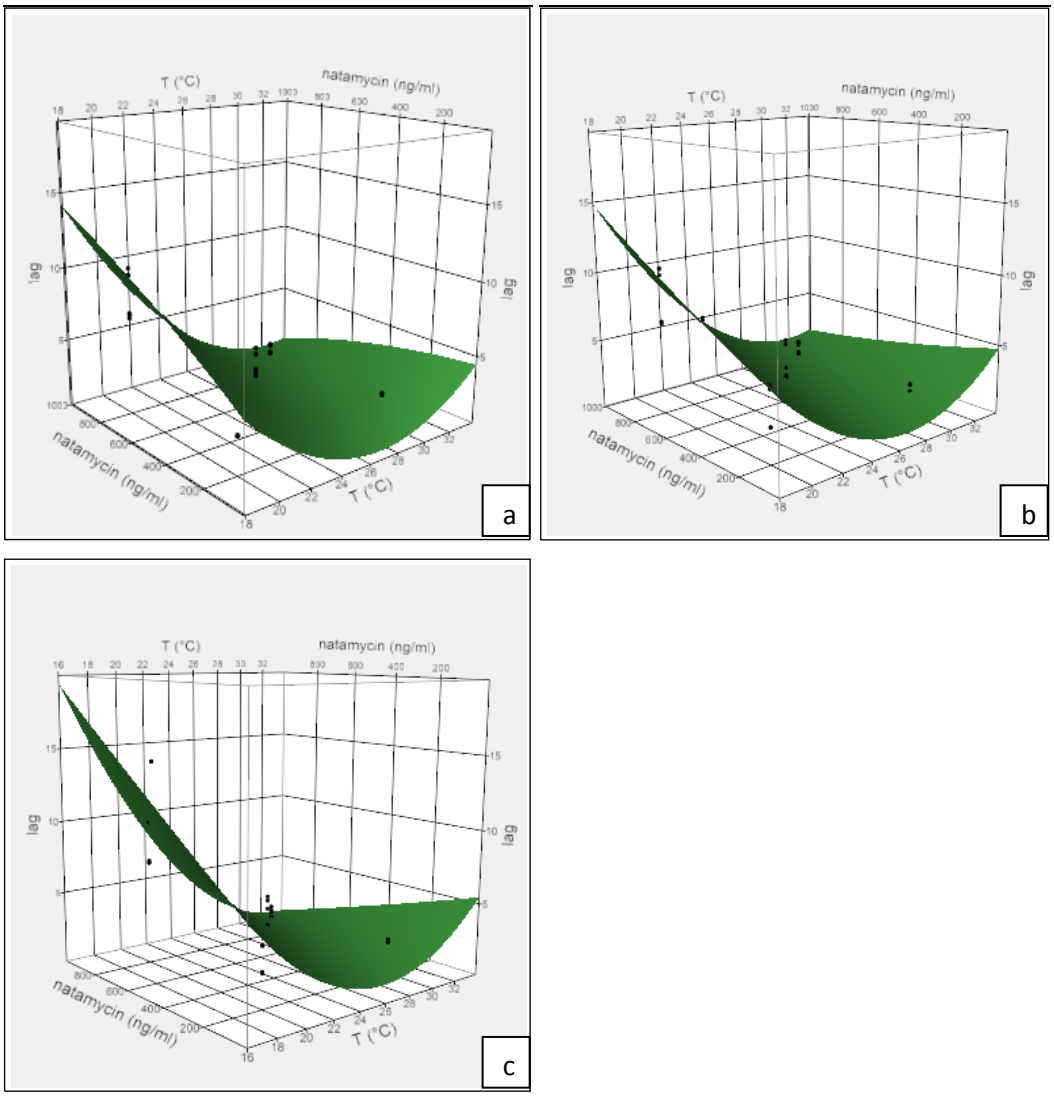


Figure 5.1: Response surface for λ (days) of *A. carbonarius* Ac-28 (a), Ac-29 (b) and Ac-33 (c) as a function of temperature (T) and natamycin concentration at 0.94 a_w.

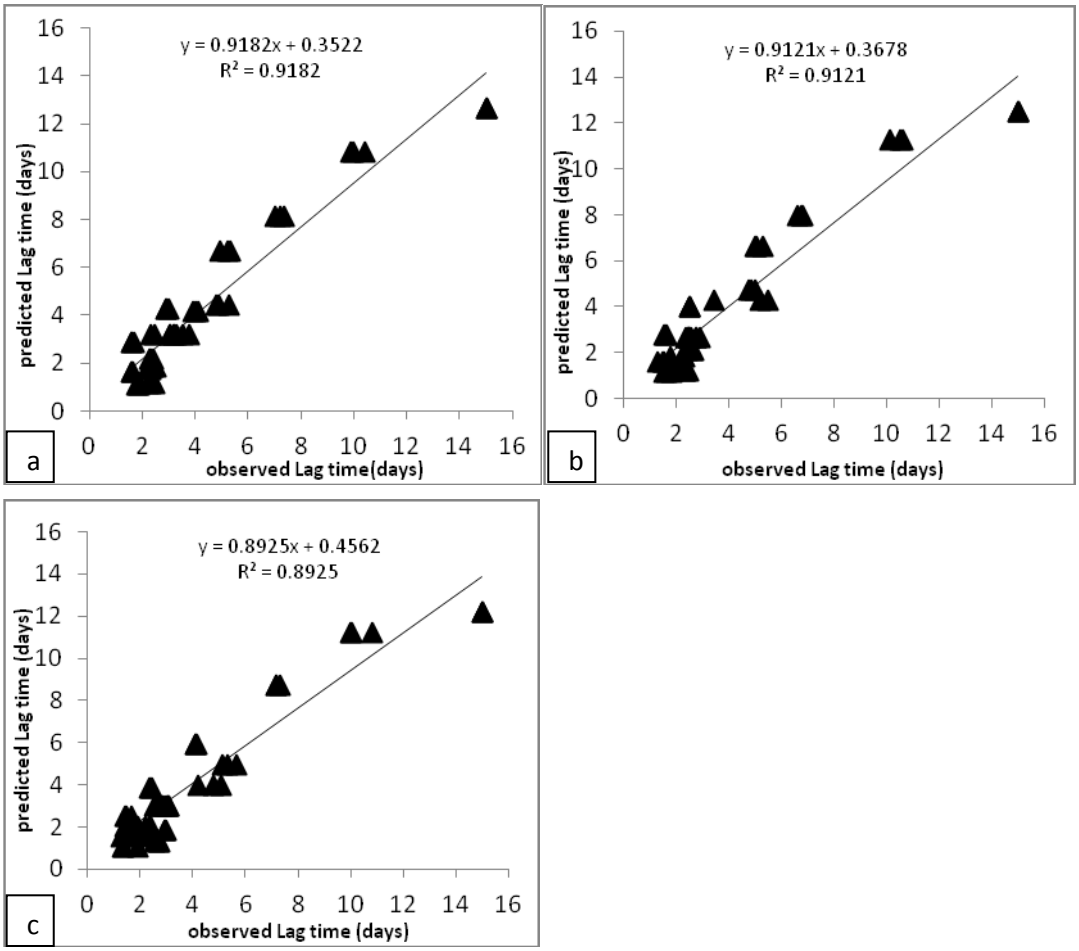


Figure 5.2: Comparison between observed and predicted lag times obtained from the quadratic polynomial model for the fungal isolates Ac-28 (a), Ac-29 (b) and Ac-30 (c) after treatment with natamycin.

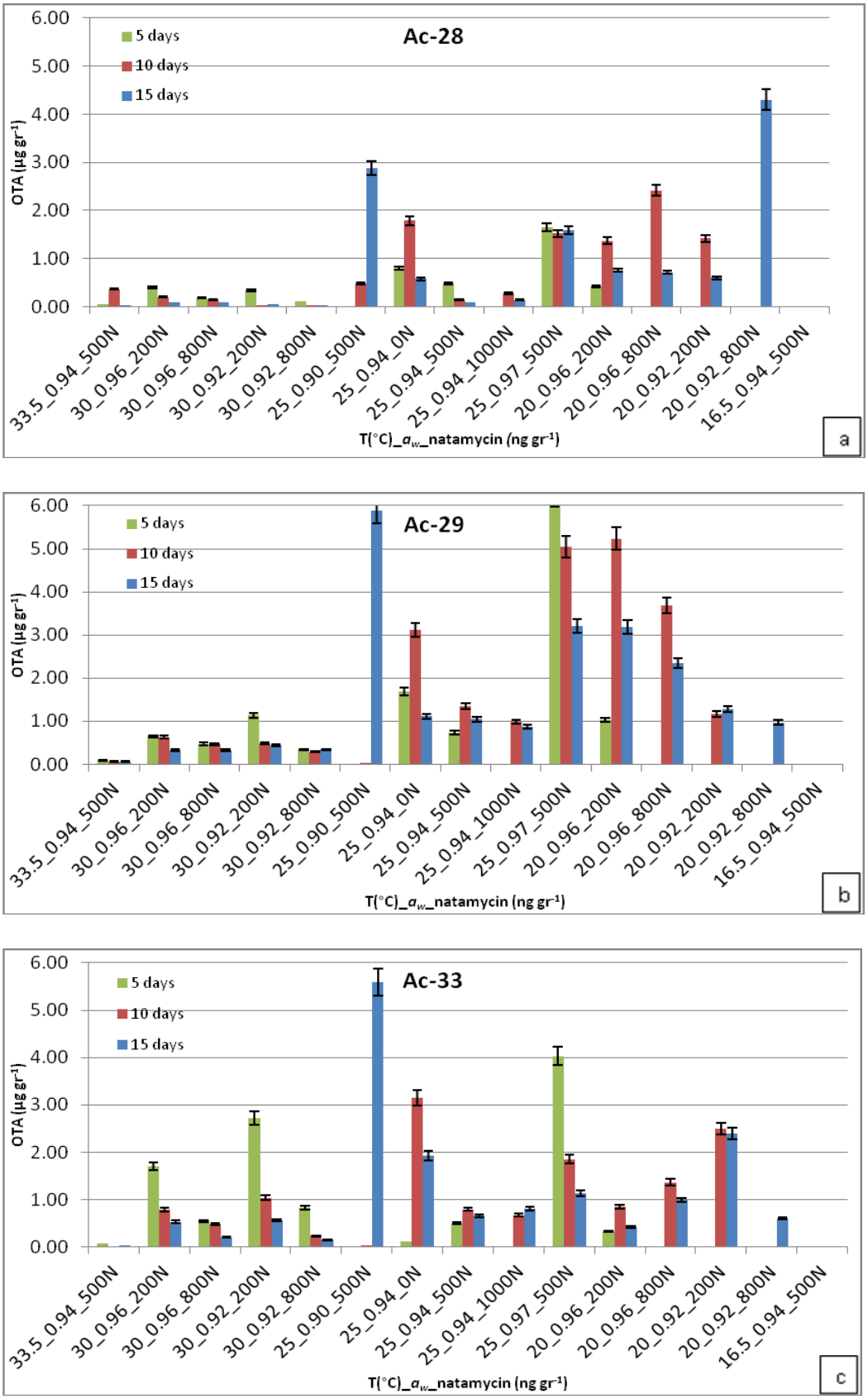


Figure 5.3: OTA production by *A. carbonarius* Ac-28 (a), Ac-29 (b), and Ac-33 (c) at varied temperature *T), water activity (a_w) and natamycin concentration after 5, 10 and 15 days of incubation. Error bars indicate standard deviation of 3 replicates.

5.3.2 Effect of pine-resin and environmental factors on the growth and OTA production by *A. carbonarius*

All of the processed variables (T, a_w, pine-resin) investigated in this study are indicated in Table 5.1. The recorded values of all the experimental runs for the growth response parameters under the conditions included in the experimental design are presented in Supplementary Table 5.2.

The statistical analysis of the data revealed that the environmental factors (T and a_w) were significant for lag phase duration and growth rate of all fungal isolates, whereas pine-resin factor significantly affected the lag phase duration of only Ac-29 and the μ_{max} of Ac-29 and Ac-33. These results indicated that pine-resin was the least influential factor, whereas T and a_w were the most significant (Table 5.3).

Response surface plots for the lag phase of *A. carbonarius* Ac-29 as a function of the interaction of T and pine-resin concentration at 0.94 a_w (a), a_w and pine-resin concentration at 25 °C (b) and μ_{max} of Ac-29 as a function of T and pine-resin concentration at 0.94 a_w (c) are presented in Fig. 5.4. It can be concluded that the lag phase of Ac-29 was extended when the concentration of pine-resin was increased at low T conditions and a_w levels. Additionally, μ_{max} of Ac-29 was increased as T and pine-resin concentration was also increased. Modelling of λ and μ_{max} for Ac-29 was difficult since R^2 was as low as 0.670 and 0.791, respectively. Pine-resin was mostly effective at lower temperature conditions and a_w levels, since it completely inhibited mycelium growth for up to 15 days of incubation at the condition of 16.6 °C/0.94 a_w/1.1% *w/v* along side with 25 °C/0.90 a_w/1.1% *w/v*, whereas μ_{max} was generally increased at higher a_w levels. Comparison between the observed and predicted values in cases where pine-resin coefficient revealed to have a significant effect on the growth parameters through the analysis of variance, are presented in Fig. 5.5. The R^2 of these models were 0.67, 0.79 and 0.83 for the λ and μ_{max} of Ac-29, as well as the μ_{max} of Ac-33, respectively.

Regards to OTA concentration, it is obvious that the low levels of toxin was detected during the 5th day of fungal growth and increased thereafter, particularly at the temperatures of 20 and 25 °C (Fig. 5.6). At higher temperature conditions (30 °C) the production of OTA decreased within the same environmental conditions after treatment with pine-resin. The only exception was observed at 30 °C/0.96 a_w/0.2% *w/v* for Ac33, whereas OTA increased at the 5th day of incubation at the higher pine-resin treatment. However, OTA content was reduced at the 10^{th} and 15^{th} day at higher pine-resin concentrations at 30 °C/0.96 and 30 °C/0.92 a_w conditions. Regarding lower temperature conditions (20 °C), OTA was stimulated at the 15^{th}

day of growth, with the exception of 20 °C/0.96 a_w/2% *w/v* for Ac-28 and Ac-33 where OTA production was reduced with increasing pine-resin concentration. Additionally, at 25 °C/0.90 a_w/1.1% *w/v* and 16.5 °C/0.94 a_w/1.1% *w/v* OTA was not detected since fungal growth was entirely inhibited at these conditions for up to 15 days. Supplementary Fig. 5.3 presents the response surface for OTA production by *A. carbonarius* Ac-28, Ac-33 after 15 days and Ac-29 after 10 days of incubation at 25 °C. The R^2 statistic indicates that the fitted model explained 65.77%, 95.72% and 82.23% of the variability in OTA accumulation for Ac-28, Ac-29 and Ac-33, respectively (Table 5.3).

Several chemical compounds of resins may be responsible for either stimulation or inhibition of fungal growth and toxin production. Monoterpenes represent one of the most important constituents of the resin canal extractives and exudates of softwoods, either as hydrocarbons or their derivatives. Viscous oleoresin of conifer trees contains approximately equal quantities of monoterpenoids and diterpene acids and small quantities of sesquiterpenoids. Mono- and sesquiterpenoid oleoresin components are volatile compounds and evaporated, but diterpene resin acids are non-volatile compounds. The most common resin acids in softwood are tricyclic terpenoids, and they are classified into pimarane and abietane types. The knots of pine normally contain a higher concentration of extractives compared to the stem of pine. Additionally, monoterpenes, e.g. α- pinene, 3-carene and β-pinene, have been identified in the roots of Scots pine. The antimicrobial activity of knotwood and plant extracts from Scots pine has been tested against food-associated microorganisms, e.g. Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi (Yang and Jaakkola, 2012). As a result, they concluded that plant extracts of scots-pine had no antifungal activity on growth of *A. niger*. Ćosić, Vrandečić, Postić, Jurković and Ravlić (2010), also studied the *in vitro* antifungal activity of scots-pine (*Pinus sylvestris L.*), on the growth of several phytopathogenic fungi. In fact, scots-pine had no antifungal activity against most of the researched fungi. Furthermore, they noticed that scots-pine stimulated the mycelium growth of *Diaporthe helianthi* and *Helminthosporium sativum* when compared to the controls. These results are in agreement with our work since pine-resin stimulated the fungal growth of *A. carbonarius* especially at the high concentrations assayed.

Jung, Yoo, Moon and Lee (2007), reported the effect of pine needle extracts for their antifungal effect against *Alternaria mali*. They reported that *Alternaria mali* growth was inhibited on malt extract agar (MEA) media with pine needle extract. Above all chemicals examined in this study, furfural was the most effective ingredient to be considered as a substitute for antifungal reagents. Finally, pine-resin revealed to have no effect on fungal

growth of the fungus and a low effect on toxin production, except the high temperature conditions where OTA was reduced. Until now, it is not recommended as an antifungal agrochemical to prevent *A. carbonarius* in the field and more studies are needed to examine its efficacy.

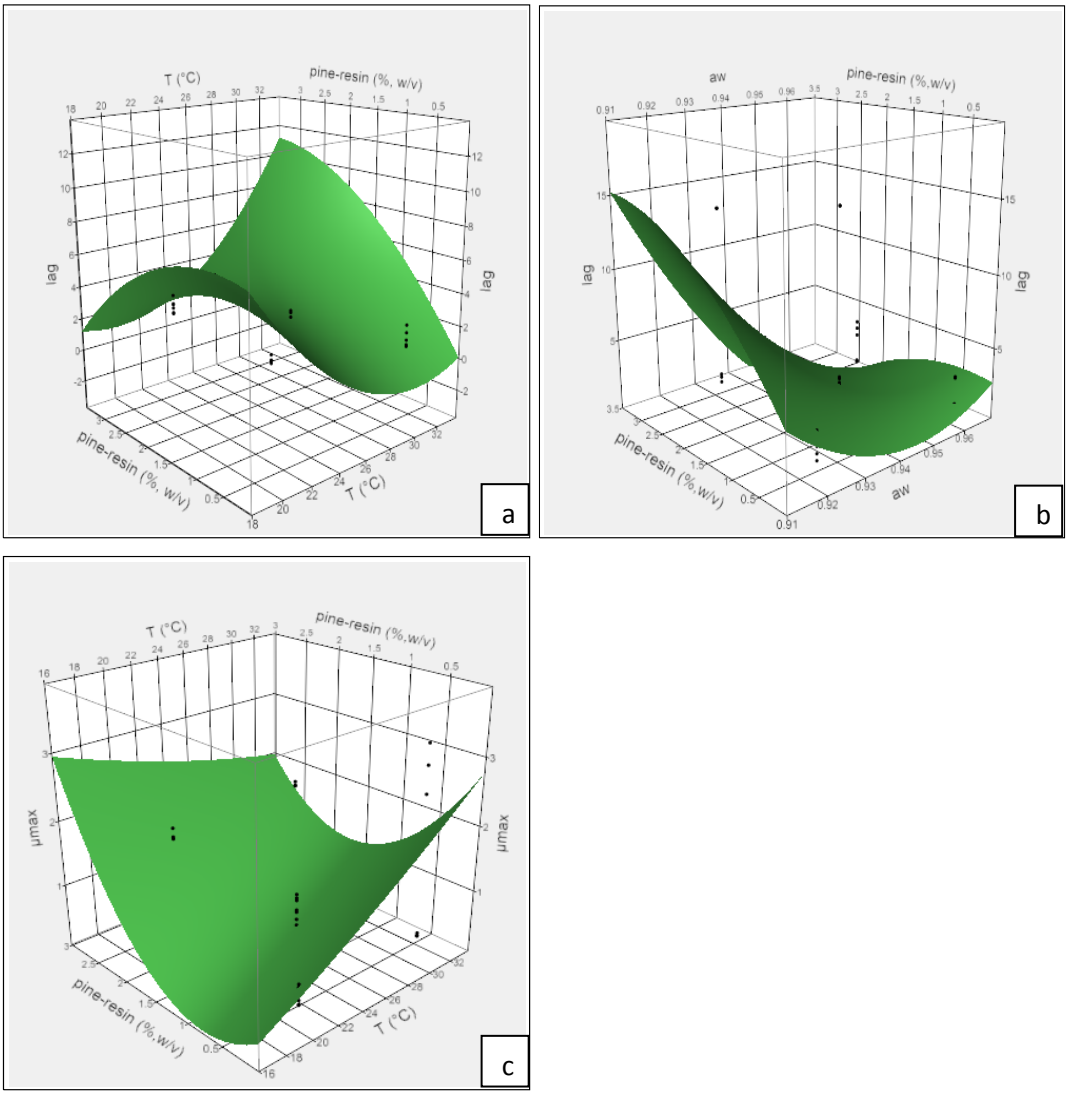


Figure 5.4: Response surface for lag phase of *A. carbonarius* Ac-29 as a function of T and pine-resin concentration at 0.94 a_w (a), water activity (a_w) and pine-resin concentration at 25 °C (b) and μ_{max} (mm d^{-1}) of *A. carbonarius* Ac-29 as a function of T and pine-resin concentration at 0.94 a_w (c).

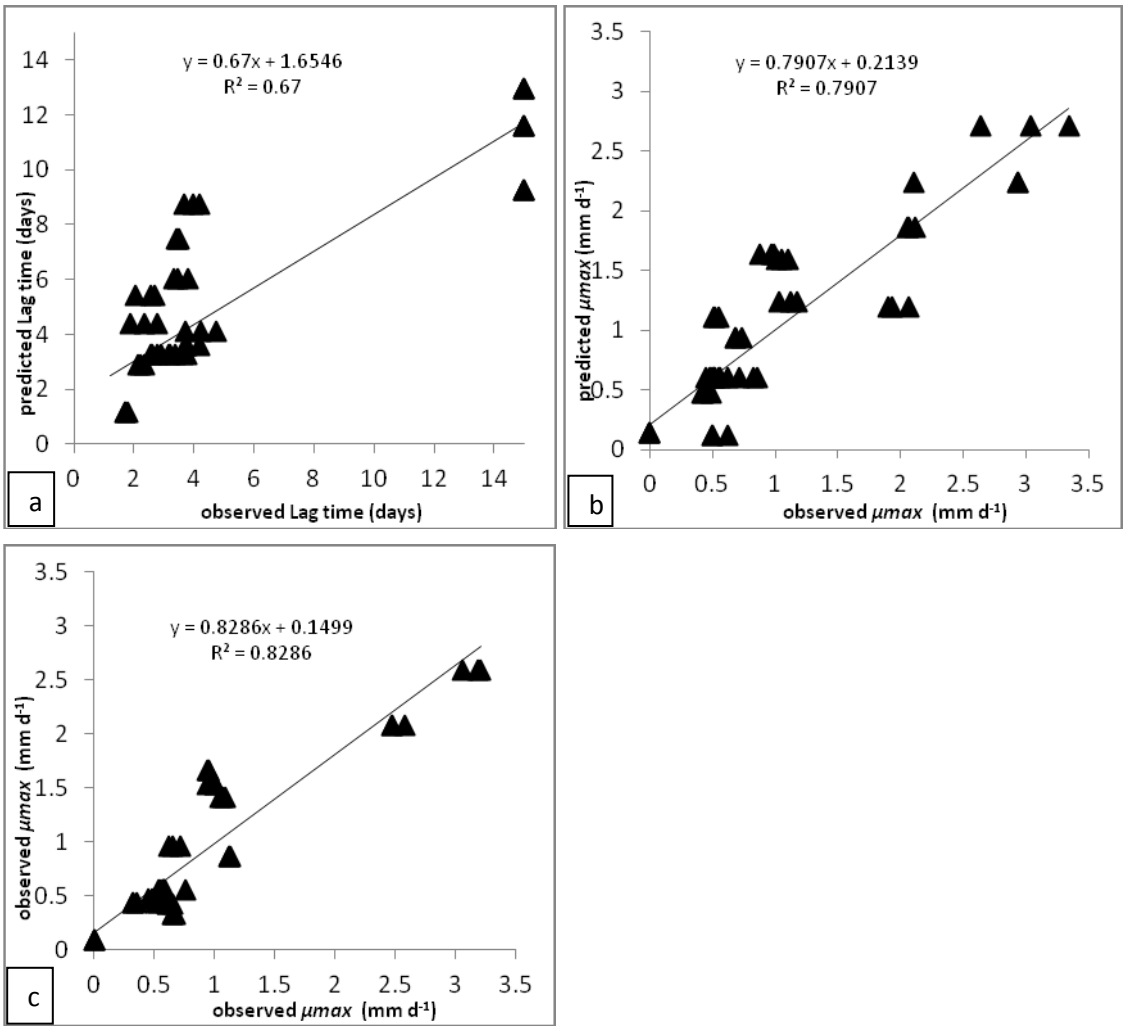


Figure 5.5: Comparison between observed and predicted values obtained from the quadratic polynomial model for the lag time of Ac-29 (a), growth rate of Ac-29 (b) and growth rate of Ac-33 (c), after treatment with pine-resin.

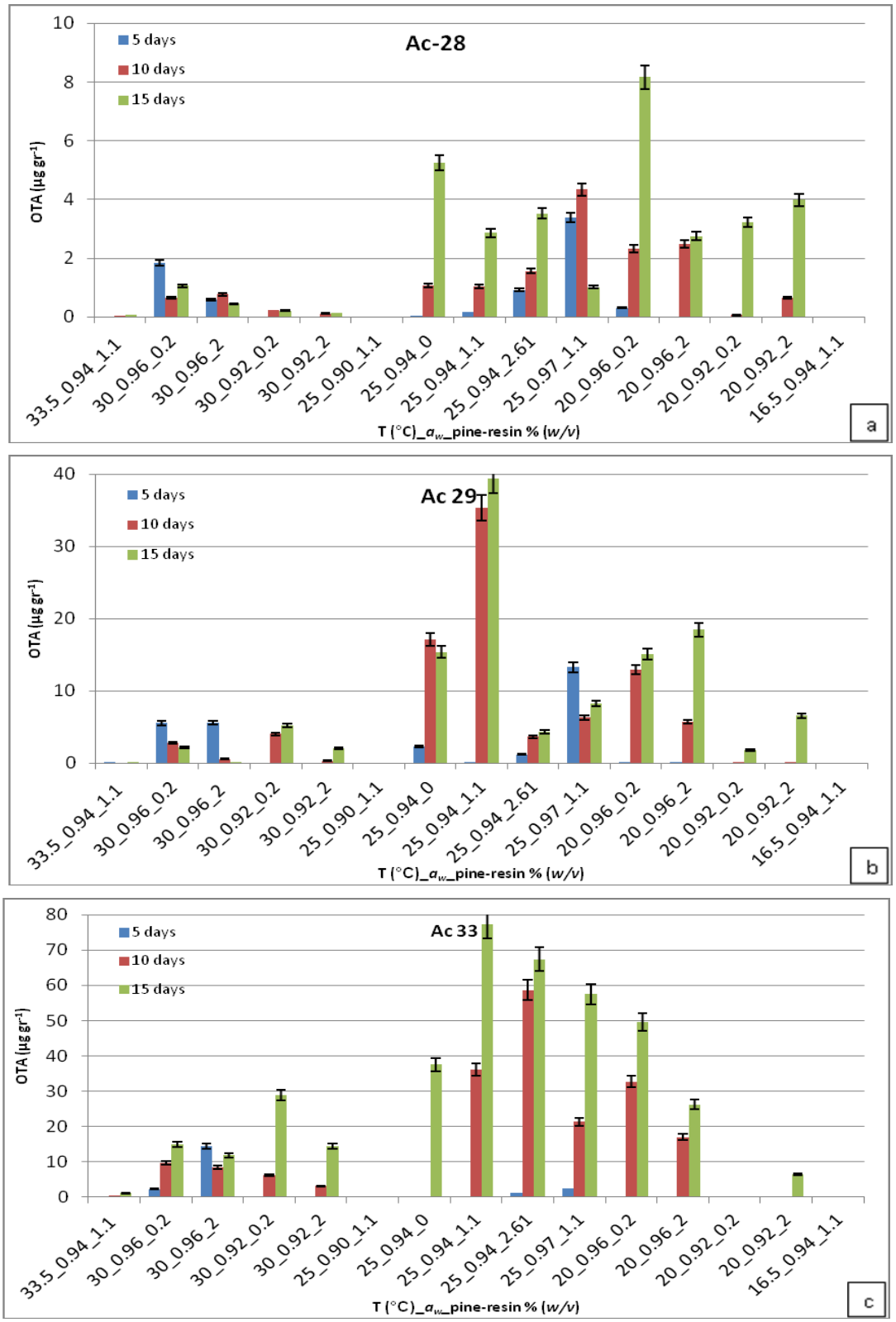


Figure 5.6: OTA production by *A. carbonarius* Ac-28 (a), Ac-29 (b) and Ac-33 (c) at varied temperature (T), water activity (a_w) and pine-resin concentrations after 5, 10 and 15 of incubation. Error bars indicate standard deviation of 3 replicates

5.4 Conclusion

Natamycin was able to increase the lag phase duration and reduce OTA accumulation at intermediate temperature conditions (20 and 25 °C). On the other hand, pine-resin had no effect on the growth rate and increased OTA levels at the same conditions assayed. However, at high temperatures (30 °C) OTA production was detected at lower levels after treatment with both antifungals. Both treatments completely inhibited fungal growth at 16 °C/0.94 a_w condition. Although natamycin was effective in delaying fungal growth by extending the lag time in all cases, treatment with pine-resin affected the lag time of only one isolate of *A. carbonarius* and the growth rate of two of them. Overall, natamycin concentration up to 1 μg mL^{-1} is recommended for use as a possible fungicidal agent against *A. carbonarius*. Conclusively, more studies are needed in order to investigate the efficacy of pine-resin on *A. carbonarius* growth parameters and OTA accumulation.

5.5 Supplementary Files

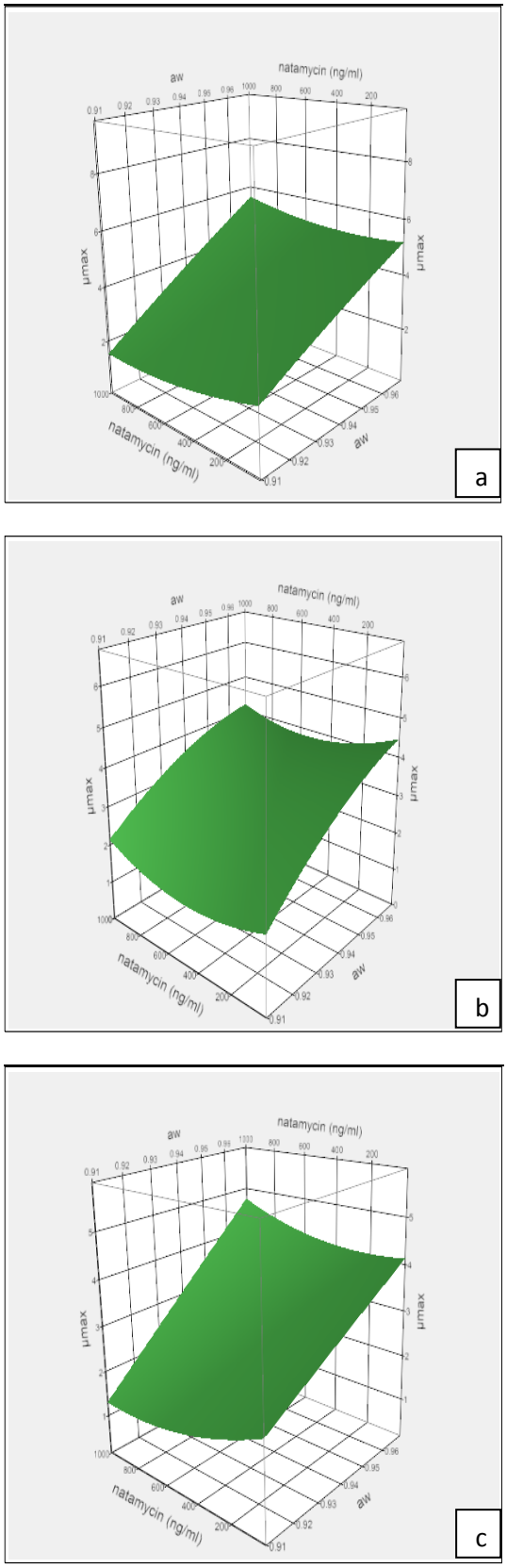
Supplementary Table 5.1

Matrix of the central composite design experiment and data obtained for the growth kinetic response variables of the three *A. carbonarius* isolates (Ac-28, Ac-29 and Ac-33) after treatment with natamycin. Values are the average (± standard deviation) of three replicates.

	Factors			λ (d)			μ_{max} (mm d^{-1})		
Run	**T**	**a_w**	**natamycin**	**Ac-28**	**Ac-29**	**Ac-33**	**Ac-28**	**Ac-29**	**Ac-33**
1	16.591	0.94	500	15.00 (0.00)[a]	15.00 (0.00)[a]	15.00 (0.00)[a]	0.00 (0.00)[a]	0.00 (0.00)[a]	0.00 (0.00)[a]
2	20	0.92	200	5.18 (0.20)	5.12 (0.15)	4.12 (0.04)	1.83 (0.03)	1.73 (0.00)	1.70 (0.05)
3	20	0.92	800	10.08(0.28)	10.42(0.27)	10.29(0.46)	1.43 (0.08)	1.51 (0.08)	1.51 (0.02)
4	20	0.96	200	2.93 (0.05)	2.53 (0.00)	2.40 (0.04)	2.99 (0.06)	2.69 (0.02)	2.76 (0.02)
5	20	0.96	800	7.21 (0.18)	6.72 (0.09)	7.21 (0.08)	4.14 (0.15)	2.53 (0.09)	2.91 (0.15)
6	25	0.90	500	4.99 (0.25)	4.71 (1.09)	4.70 (0.43)	0.67 (0.01)	0.57 (0.02)	0.84 (0.08)
7	25	0.94	0	3.54 (0.23)	2.65 (0.21)	3.02 (0.09)	3.79 (0.11)	3.25 (0.08)	2.89 (0.05)
8	25	0.94	500	2.34 (0.12)	2.38 (0.06)	2.65 (0.09)	3.24 (0.08)	3.05 (0.05)	2.82 (0.07)
9	25	0.94	500	2.86 (0.38)	2.56 (0.18)	2.81 (0.26)	3.43 (0.13)	3.08 (0.00)	2.85 (0.06)
10	25	0.94	500	2.91 (0.38)	2.53 (0.21)	2.86 (0.18)	3.49 (0.13)	3.07 (0.00)	2.81 (0.09)
11	25	0.94	1000	4.00 (0.09)	4.87 (0.12)	5.38 (0.28)	2.91 (0.07)	3.01 (0.10)	2.89 (0.02)
12	25	0.974	500	2.26 (0.08)	2.22 (0.11)	2.95 (0.03)	4.64 (0.12)	3.48 (0.08)	4.21 (0.09)
13	30	0.92	200	2.34 (0.07)	2.12 (0.28)	2.32 (0.10)	4.52 (0.03)	3.83 (0.14)	3.25 (0.03)
14	30	0.92	800	2.44 (0.07)	2.57 (0.12)	1.75 (0.45)	4.24 (0.049)	4.19 (0.14)	2.47 (0.07)
15	30	0.96	200	1.61 (0.01)	1.45 (0.11)	1.65 (0.25)	7.25 (0.207)	5.86 (0.11)	4.85 (0.14)
16	30	0.96	800	1.84 (0.05)	1.67 (0.14)	1.54 (0.31)	6.45 (0.11)	5.77 (0.07)	4.95 (0.09)
17	33.409	0.94	500	1.63 (0.04)	1.59 (0.04)	1.51 (0.13)	5.79(0.16)	5.17 (0.09)	4.73 (0.17)

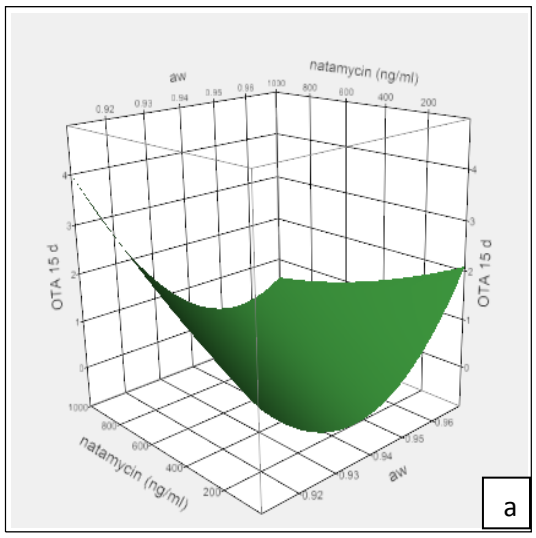
[a] No growth was observed for all fungal isolates and thus μ_{max} and λ were considered as 0.00 (mm d^{-1}) and 15 days, respectively.

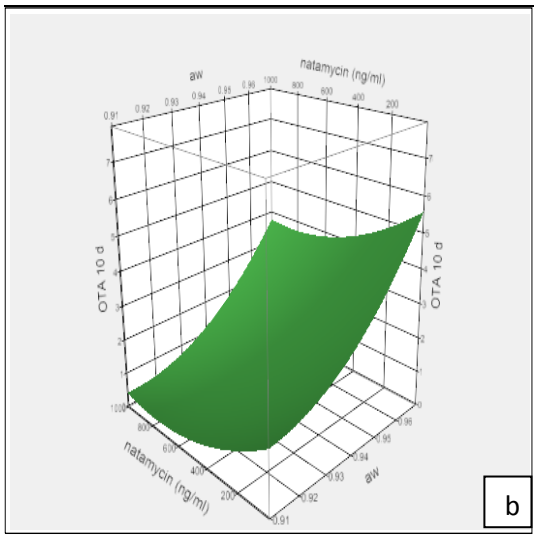
Supplementary Figure 5.1

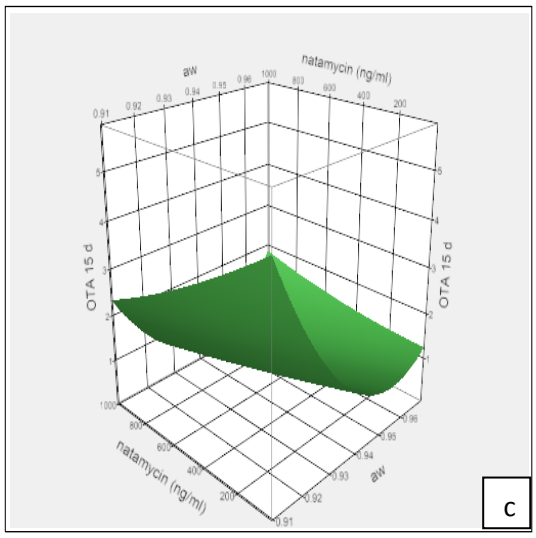


Response surface for growth rate (μ_{max}, mm d^{-1}) of *A. carbonarius* Ac-28 (a), Ac-29 (b) and Ac-33 (c) as a function of water activity (a_w) and natamycin concentration for a temperature condition of 25 °C.

Supplementary Figure 5.2







Response surface for OTA production (μg g^{-1}) by *A. carbonarius* Ac-28 (a), Ac-33 (c) after 15 days and Ac-29 (b) after 10 days of incubation, as a function of water activity (a_w) and natamycin concentration for a temperature condition of 25 °C.

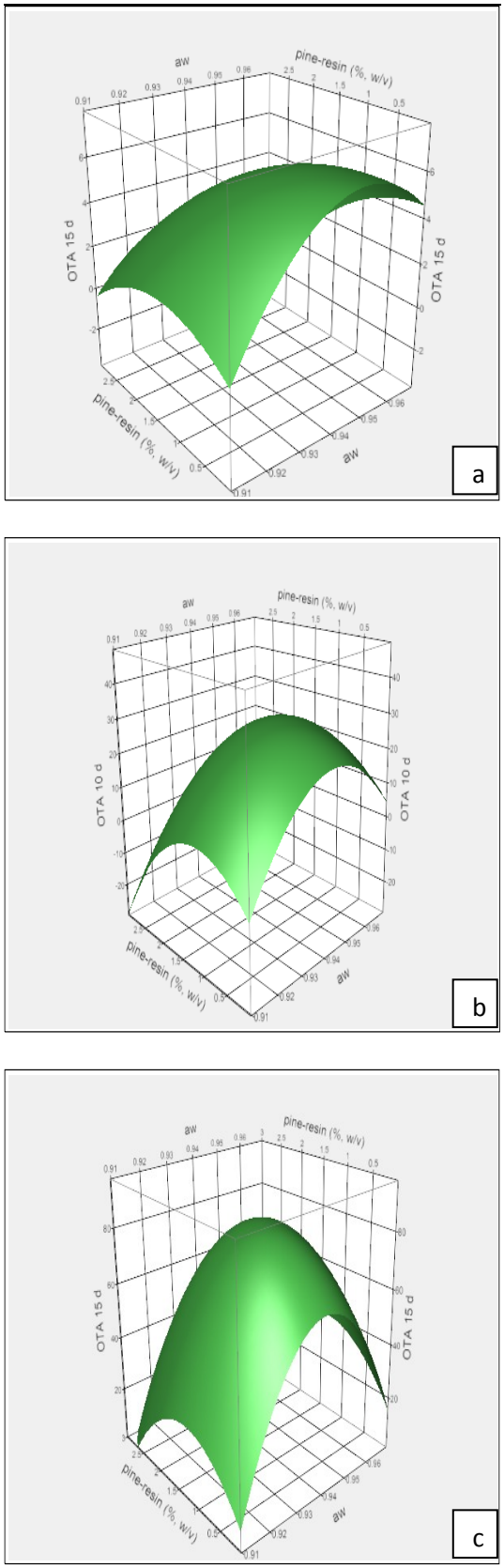
Supplementary Table 5.2

Matrix of the central composite design experiment and data obtained for the growth kinetic response variables of the three *A. carbonarius* isolates (Ac-28, Ac-29 and Ac-33) after treatment with pine-resin. Values are the average (± standard deviation) of three replicates.

	Factors			λ(d)			μ_{max} (mm d^{-1})		
Run	**T**	**a_w**	**pine-resin**	**Ac-28**	**Ac-29**	**Ac-33**	**Ac-28**	**Ac-29**	**Ac-33**
1	16.591	0.94	1.1	15.00 (0.00)[a]	15.00 (0.00)[a]	15.00 (0.00)[a]	0.00 (0.00)[a]	0.00 (0.00)[a]	0.00 (0.00)[a]
2	20	0.92	0.2	3.35 (0.22)	3.52 (0.23)	4.89 (0.66)	0.28 (0.00)	0.44 (0.04)	0.49 (0.04)
3	20	0.92	2	4.19 (0.14)	3.94 (0.26)	6.56 (0.09)	0.28 (0.01)	0.49 (0.05)	0.34 (0.01)
4	20	0.96	0.2	2.63 (0.36)	3.46 (0.04)	2.55 (0.08)	0.83 (0.02)	0.70 (0.02)	1.12 (0.00)
5	20	0.96	2	2.37 (0.16)	4.22 (0.50)	3.31 (0.61)	0.76 (0.08)	1.97 (0.08)	0.66 (0.01)
6	25	0.90	1.1	15.00 (0.00)[a]	15.00 (0.00)[a]	15.00 (0.00)[a]	0.00 (0.00)[a]	0.00 (0.00)[a]	0.00 (0.00)[a]
7	25	0.94	0	3.25 (0.09)	1.64 (0.36)	3.52 (0.09)	1.76 (0.00)	2.66 (0.47)	1.07 (0.01)
8	25	0.94	1.1	3.10 (0.24)	3.42 (0.29)	3.57 (0.92)	0.72 (0.18)	0.50 (0.01)	0.57 (0.02)
9	25	0.94	1.1	2.89 (0.47)	3.00 (0.55)	3.72 (0.80)	0.96 (0.01)	0.56 (0.05)	0.64 (0.10)
10	25	0.94	1.1	2.51 (0.58)	2.88 (0.31)	3.39 (0.53)	0.95 (0.09)	0.80 (0.07)	0.57 (0.01)
11	25	0.94	2.61	2.46 (0.30)	3.88 (0.25)	4.43 (0.36)	0.77 (0.05)	0.94 (0.06)	0.67 (0.04)
12	25	0.974	1.1	1.22 (0.17)	2.22 (0.10)	3.11 (0.41)	1.22 (0.00)	1.06 (0.04)	0.95 (0.00)
13	30	0.92	0.2	3.15 (0.32)	2.33 (0.44)	2.57 (0.10)	0.48 (0.00)	0.52 (0.02)	0.56 (0.04)
14	30	0.92	2	4.01 (0.36)	15.00 (0.00)[a]	2.99 (0.27)	0.67 (0.02)	0.54 (0.06)	0.62 (0.02)
15	30	0.96	0.2	1.01 (0.18)	1.56 (0.04)	1.53 (0.16)	3.81 (0.14)	3.01(0.35)	3.15 (0.08)
16	30	0.96	2	2.12 (0.28)	1.73 (0.05)	1.72 (0.19)	2.80 (0.05)	2.08 (0.03)	2.51 (0.06)
17	33.409	0.94	1.1	2.08 (0.10)	2.42 (0.32)	2.86 (0.14)	0.93 (0.02)	1.11 (0.07)	0.98 (0.03)

[a] No growth was observed for all fungal isolates and thus μ_{max} and λ were considered as 0.00 (mm d^{-1}) and 15 days, respectively.

Supplementary Figure 5.3



Response surface for OTA production (μg g^{-1}) by *A. carbonarius* Ac-28 (a), Ac-33 (c) after 15 days and Ac-29 (b) after 10 days of incubation, as a function of water activity (a_w) and pine-resin concentration for a temperature condition of 25 °C.

CHAPTER 6

Differentiation and identification of grape-associated black aspergilli using Fourier transform infrared (FT-IR) spectroscopic analysis of mycelia

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Abstract

The purpose of this study was to evaluate the potential of FT-IR spectroscopy as a high-throughput method for rapid differentiation among the ochratoxigenic species of *Aspergillus carbonarius* and the non-ochratoxigenic or low toxigenic species of *Aspergillus niger* aggregate, namely *A. tubingensis* and *A. niger* isolated previously from grapes of Greek vineyards. A total of 182 isolates of *A. carbonarius*, *A. tubingensis*, and *A. niger* were analyzed using FT-IR spectroscopy. The first derivative of specific spectral regions (3002-2801 cm^{-1}, 1773-1550 cm^{-1}, and 1286-952 cm^{-1}) were chosen and evaluated with respect to absorbance values. The average spectra of 130 fungal isolates were used for model calibration based on Discriminant analysis and the remaining 52 spectra were used for external model validation. This methodology was able to differentiate correctly 98.8 % in total accuracy in both model calibration and validation. The per class accuracy for *A. carbonarius* was 95.3% and 100% for model calibration and validation, respectively, whereas for *A. niger* aggregate the per class accuracy amounted to 100% in both cases. The obtained results indicated that FT-IR could become a promising, fast, reliable and low-cost tool for the discrimination and differentiation of closely related fungal species.

Keywords: Aspergillus carbonarius, *Aspergillus niger* aggregate, *Aspergillus niger, Aspergillus tubingesnis, black aspergilli, differentiation, FT-IR spectroscopy*

6.1 Introduction

Fungal members of *Aspergillus* section *Nigri* group (black aspergilli), saprophytes generally considered ubiquitous in nature, are responsible for food spoilage and Ochratoxin A (OTA) production in several food commodities (Ostry et al., 2002; Palumbo et al., 2010; Copetti et al., 2012; Hayrettin et al., 2012; Perrone et al., 2013). Black-spored species belonging to this group, particularly the biseriate species *A. carbonarius* and species of *Aspergillus niger* aggregate (*A. niger* and *A. tubingensis*), are the most frequently isolated species from grapes, raisins and wine (Chulze et al., 2006; Gómez et al., 2006; Moss, 2007; Chiotta et al., 2009). Several studies have clarified that the main OTA producer from this group is *A. carbonarius* which produces this mycotoxin very consistently and to a lesser extent the so-called *Aspergillus niger* aggregate, particularly *A. niger* and *A. tubingensis* (Magnoli et al., 2003; Bellí et al., 2005b; Perrone et al., 2006a; Samson et al., 2007; Lasram et al., 2012). OTA is a secondary metabolite with nephrotoxic, immunosuppressive, teratogenic, and carcinogenic properties which has been classified as group 2B carcinogen by the International Agency for Research on Cancer (IARC, 1993). It has been reported as the first mycotoxin commonly found in grape products and therefore the European Union has set permissible limits of OTA intake from dried vine fruit to 10 μg kg^{-1}, and from grape juice and wine to 2 μg kg^{-1} (European Commission, 2005a).

In food mycology, identification schemes are based on phenotypic and genotypic characterization. The former methods are mainly focused on fungal growth on different agar media, followed by carefully scrutinizing the morphological characteristics of the mycelium, and on microscopic observations. Additionally, physico-chemical reaction patterns can be also used as phenotypic methods for fungal identification (Samson et al., 2007; Meijer et al., 2011). Genotypic methods concern the polymerase chain reaction (PCR) of amplified DNA, using conserved sequences of mitochondrial and ribosomal genes and internal transcribed spacer regions (ITS) (Martínez-Culebras et al., 2009; Schoch et al., 2012).

The characterization of black aspergilli is considered one of the most confusing and difficult task due to indistinguishable differences among the species. Thus, fungal taxonomy of *Aspergillus* section *Nigri* group is unclear and many attempts have been undertaken to find appropriate taxonomic criteria (Dachoupakan et al., 2009; Meijer et al., 2011; Silva et al., 2011; Kizis et al., 2014). Although some species, such as *A. carbonarius* and uniseriate species, namely *A. aculeatus* and *A. japonicus*, can be easily recognized based on

morphological criteria and microscopic observations, identification at species level remains problematic and insufficient (Abarca et al., 2004; Samson et al., 2007). Molecular characterization and phylogenetic analysis of ITS and 5.8S rRNA gene region of representative black aspergilli based on Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) has been proposed as the most accurate method to identify fungal species (Martínez-Culebras and Ramón, 2007). Even though the RFLP-based studies for black aspergilli species are precise, molecular techniques remain fastidious, expensive and demand special laboratory skills and facilities (Rozynek et al., 2004).

Fourier Transform Infrared Spectroscopy (FT-IR) is a rapid method with high sensitivity, robustness and ease of use for the identification and differentiation of microorganisms causing food spoilage (Garon et al., 2010; Kaya-Celiker et al., 2015). The mid-FT-IR spectroscopy is a simple instrumental technique that can give evidence for the presence of various functional groups whereby vibrational motions of molecules either absorb or reflect radiation at wavenumbers of 4000-400 cm^{-1}. The absorption changes at specific frequencies allow the determination of which molecular groups are present, and how they are arranged or interact by means of an FT-IR spectrum. The spectral profile provides information about important macromolecules like proteins, lipids, phosphate-containing compounds, phospholipids and carbohydrates present in cells. Classification is based on the analysis of the "molecular fingerprint" which is obtained by this spectrum (Naumann, 2000).

FT-IR spectroscopy has been successfully applied for the identification of filamentous fungi. Specifically, Garon et al. (2010) used this technique for the discrimination of closely related *Aspergillus* species (*A. flavus*, *A. fumigatus* and *A. parasiticus*) which have been previously collected from feed and bioaerosols in agricultural environment, and concurrently for the differentiation among aflatoxigenic and non-aflatoxigenic isolates. Tralamazza et al. (2013) have also examined the ability of FT-IR as an alternative technique to molecular procedures for fungal identification. Particularly, they investigated the potential of FT-IR to discriminate and classify three environmental *Aspergillus* species (*A. niger*, *A. ochraceus*, and *A. westerdijkiae*) isolated from coffee beans and highlighted the ability of FT-IR to distinguish *Aspergillus* species as an alternative to molecular procedures. In another study, FT-IR spectroscopy was successfully employed for the differentiation of 16 isolates belonging to five *Fusarium* species (Nie et al., 2007). Other researchers also reported that FT-IR can be used for the identification and characterization of filamentous fungi and yeasts (Fischer et al., 2006; Santos et al., 2010; Shapaval et al., 2012; Zervakis et al., 2012; Lecellier et al., 2014, 2015; Kaya-Celiker et al., 2015). All these studies indicated that FT-IR

spectroscopy can be effectively used as a rapid routine method of high specificity, reliability, and with minimal sample preparation for fungal taxonomy. The diffuse reflectance absorbance (DRIFT) spectroscopy, employed in this study for the analysis of fungal mycelia, is an alternative FT-IR spectroscopy method that was developed by Goodacre et al. (1996) for the spectroscopic analysis of powders and materials with rough surfaces and offers the advantage of simple sample preparation and the potential to analyze non-transparent materials.

The purpose of this study was to evaluate the potential of FT-IR spectroscopy as a high-throughput method for rapid differentiation among the ochratoxigenic species of *A. carbonarius* and the non-ochratoxigenic or low toxigenic species of *A. niger* aggregate, namely *A. niger* and *A. tubingensis*. This method was used to establish a comprehensive database of taxonomically well-defined black aspergilli species to aid in their taxonomy. Compared to previous studies (Tralamazza et al., 2013) the importance of this work is based on the differentiation of closely related species of *Aspergillus* section *Nigri* group using a high-performing technique. To our knowledge this is the first attempt to discriminate black aspergilli species using FT-IR spectroscopy.

6.2 Materials and methods

6.2.1 Fungal isolates

A total of 182 fungal isolates were analyzed in this study from which 91 isolates have been identified as *Aspergillus carbonarius* and 91 as *Aspergillus niger* aggregate (of which 80 identified as *Aspergillus tubingensis* and 11 as *Aspergillus niger*). All of them have been previously isolated from grapes of Greek vineyards. Specifically, 115 fungi were taken from the culture collection of the Laboratory of Food Microbiology and Biotechnology of the Agricultural University of Athens. These fungi have been isolated during the 2012 harvesting period (from August to September) and identified at species level by molecular techniques as reported in previous work (Kizis et al., 2014). The remaining 67 fungi were isolated during the 2014 harvesting period (in September) and identified at species level by PCR-RFLP of 5.8S-ITS gene region as detailed elsewhere (Bisbal et al., 2009; Kizis et al., 2014). Reference strains were obtained from the fungal collection of the LFMB for *A. carbonarius* (F26, F40), *A. niger* (F7, F88), and *A. tubingensis* (F31, F65).

6.2.2 DNA extraction and amplification of fungal isolates

6.2.2.1 Culture conditions and DNA extraction

Unidentified isolates from the fungal collection of the harvest year 2014 and reference strains were grown in Yeast Extract Sucrose broth (YES; yeast extract 20 g; sucrose 150 g; distilled water, *c.* 1000 mL) at 30 °C for 48 h. Then, mycelia were aseptically collected, washed thoroughly with ethanol (96%, *v/v*) and dried using Whatman No. 1 filter paper. Finally, 100–200 mg of each sample were lyophilized for 24 h and ground into a fine powder. DNA extractions were performed using the innuPREP Plant DNA kit (Analytik Jena, Germany) according to the manufacturer's instructions.

6.2.2.2 PCR amplification

The isolates were amplified using PCR primers ITS1 and ITS4. PCR reactions were performed in a 50 µl final volume, containing 1 × standard reaction buffer (Kapa Biosystems, Japan), 2.0 mM MgCl2, 300 Mm dNTPs (each), 300 nM primers (each), 100 ng DNA template and 1.25 U of Taq polymerase (Kapa Biosystems, Japan). The reaction mixtures for PCR primers ITS1/ITS4 were performed in a thermal cycler (Bio-Rad Laboratories, USA), starting with an initial denaturation step at 95 °C for 5 min, followed by 39 cycles consisting of 30 s at 95 °C, 30 s at 52 °C and 40 s at 72 °C, and a final extension step at 72 °C for 10 min. Then, PCR products were digested at 37 °C for 3 h with the restriction enzymes *HhaI*, *HinfI* and *RsaI* (New England Biolabs, UK). Digestions were performed in a 20 µl reaction volume containing 2 µl of 10 × reaction buffer, 10 µl of each amplicon and 1.5 U restriction endonuclease and 0.2 µl BSA (10 µg/l) for *HhaI* digestions at 37 °C for 3 h. All PCR amplicons and their restriction digestion fragments were separated by electrophoresis at 100 V, 1 × TAE buffer, in 1% and 3% agarose gels, respectively. Finally, gels were stained in ethidium bromide solution (0.5 mg/ml) and photographed under Ultra Violet (UV) light using a Gel Doc XR + system (Bio-Rad Laboratories, USA). Molecular sizes of the DNA were estimated by comparison with the DNA standard GeneRuler 100 and 50 bp DNA ladders (Thermo Scientific, USA).

6.2.3 FT-IR Analysis

6.2.3.1 Fungal culture and sample preparation

All fungal species were cultivated on Malt Extract Agar (MEA; malt extract, 20g; peptone, 1g; glucose, 20g; bacteriological agar, 20g; distilled water, *ca.* 1000 mL) medium into 9 cm Petri dishes for 7 days at 25 °C. Then, spores of each culture were extracted with the aid of a loop, transferred into a Petri dish containing 20 mL of YES broth medium (composition as mentioned previously) and incubated at 30 °C for 36 h. Mycelia, free of fungal spores, were collected, washed thoroughly with distilled water, dried using Whatman No. 1 filter paper and stored into eppendorf tubes. All tubes containing the mycelia were lyophilized for 24 h. After freeze-drying the final samples were transferred into a glass desiccator until FT-IR analysis.

6.2.3.2 Recording of DRIFT spectra

Samples were ground into a fine powder and approximately 2 mg of each sample were placed into a micro sampling cup (Spectra-Tech Inc., USA). Spectra of three replicates for each species were recorded using a Thermo Nicolet 6700 FT-IR spectrophotometer (Thermo Electron Corporation, Madison, WI, USA). Measurements were recorded in the range of 4000-400 cm^{-1} at 4 cm^{-1} spectral resolution and 100 scans per sample. The background spectra were collected using pure dried potassium bromide (KBr) to eliminate signals of the spectrometer and its environment from the sample spectrum (Zervakis et al., 2012). Spectral data were processed using the software of the spectrophotometer (OMNIC ver. 7.3 – Thermo Fisher Scientific Inc., Waltham, MA, USA). FT-IR spectra were smoothed using the 'automatic smooth' function of the software, which uses the Savitsky-Golay algorithm (5 point moving second degree polynomial). This function (default setting) is automatically smoothing the high-frequency component of the sample data, which improves the appearance of peaks obscured by noise. Afterwards, the baseline was corrected using the 'automatic baseline correct function' (default setting) of the software that automatically corrects the tilted baselines of the selected spectra with the baseline points selected by the software (Zervakis et al., 2012). Finally, the average spectrum of the three replicates was calculated for each sample and subjected to multivariate analysis.

6.2.3.3 Analysis of FT-IR spectra

Following smoothing and baseline correction, the obtained spectra were subjected to discriminant analysis using TQ Analyst software ver. 8.0.0.245 (Thermo Fisher Scientific Inc., Waltham, MA, USA). The spectral regions of: i) 3002-2801 cm^{-1}, ii) 1773-1550 cm^{-1}, and iii) 1286-952 cm^{-1} were used in the analysis. Three actual classes (*A. carbonarius* and *A. niger* aggregate) were defined. Spectral analysis was evaluated by computing the distance from each class center in Mahalanobis distance units. Mahalanobis distance is using an algorithm for calculating the distance of a sample from the mean of a set of standards using the following equation:

$$D^2 = (X - Xavg)T\ S^{-1}\ (X - Xavg)$$

Where, *D* equals the distance (as a scalar), *X* the data vector (n x 1), *Xavg* the mean data vector (n x 1), *S* the covariance matrix (n x n), $(X - Xavg)T$ denotes the transpose of $(X - Xavg)$, and *n* the number of data points in X.

Three mean spectra (one of each class) were computed by the software and a distribution model was generated by estimating the variance at each frequency in the analysis range. Afterwards, each sample was re-placed in each of the above three classes (calculated classes).

6.3 Results and discussion

6.3.1 Molecular Identification

Molecular characterization of 67 isolates and reference strains was primarily implemented by the use of PCR of the 5.8S-ITS region using ITS1-ITS4 primers, resulting to amplicon molecular sizes of approximately 600 bp for all samples (data not shown). All PCR products were digested with three endonucleases to identify *A. carbonarius*, *A. niger* and *A. tubingensis*. Restriction with enzymes *HhaI* and *HinfI* were able to differentiate *A. carbonarius* from *A. niger* and *A. tubingensis*. Moreover, restriction patterns of all *A. carbonarius* isolates were compared with those reported from the reference strains and identified correctly. *RsaI* digestion can distinguish further *A. niger* from *A. tubingensis* generating different restriction patterns for these two species. All results were also confirmed

by those of reference strains. Notwithstanding the above technique was precise in identifying *A.* section *Nigri* isolates.

6.3.2 FT-IR spectroscopy

In this work the potential of FT-IR spectroscopy to discriminate ochratoxigenic species of *A. carbonarius* from species of *A. niger* aggregate was evaluated. Fig. 6.1 illustrates the three typical mean spectra of the examined fungal species after pre-processing with automatic smoothing and baseline correction. Additionally, Fig. 2 shows the comparison between all recorded spectra (4000–400 cm^{-1}) of *A. carbonarius*, *A. tubingensis* and *A. niger*. Each mean spectrum is the average of three replicate spectra for each fungus. As shown in this figure, there are small variations in the spectra indicating that information derived from infrared spectroscopy require further analysis. Therefore, the use of multivariate analysis is needed to distinguish regions with biological meaning for the discrimination among *Aspergillus* species.

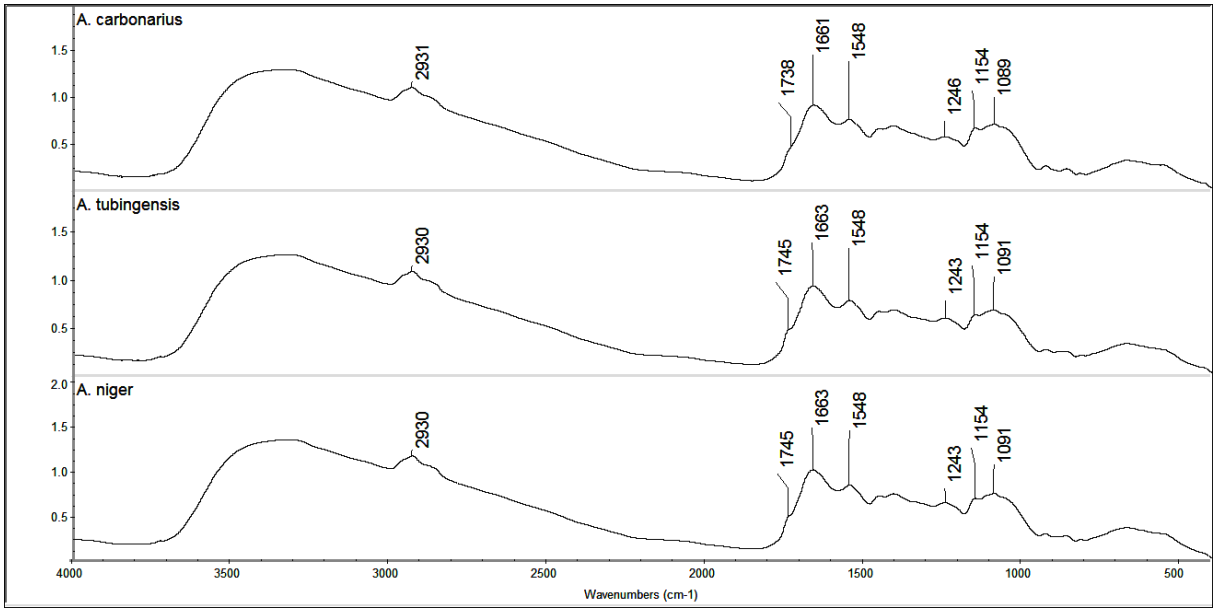

Figure 6.1: General view (4000–400 cm^{-1}) of absorption mean spectra after automatic smoothing for *A. carbonarius*, *A. tubingensis* and *A. niger* (wavenumbers indicate the most important functional groups within this spectral region).

Detailed analysis of the whole spectrum showed small differences and thus differentiation among the species was inadequate. Thus, spectral regions with different band patterns, known to be characteristic of certain chemical compounds, were screened for better results. Ranges presenting the highest variability among the species were selected for fungal discrimination. Specifically, the C-H stretching bonds which are related to absorption of lipids (i.e., fatty acids of cell membrane) at 3002-2801 cm^{-1} were primarily used (Naumann, 2000). Additionally, the regions between 1773-1550 cm^{-1} and 1286-952 cm^{-1} were evaluated. The broad peaks at 1773-1550 cm^{-1} and particularly absorption at 1738 cm^{-1} has been attributed mainly to the C=O stretching bonds, whereas the peaks at the fingerprint region between 1286-952 cm^{-1} to C-O-C and C=S stretching (Socrates, 2001; Tralamazza et al., 2013). The former peaks indicate the absorption of phospholipids and proteins, in particular amide I near 1650 cm^{-1} and amide II near 1540 cm^{-1}, whereas the latter peaks show the absorption of carbohydrates. Tralamazza et al. (2013) also used the regions of 1706 and 1758 cm^{-1} which are related to amide I absorption and referenced that bands designated as ‘‘amide I’’ are frequently cited as an important microbial marker in FT-IR spectroscopy. The bands at 1738 and 1745 cm^{-1} correspond to C=O bonds of phospholipids and were mainly accentuated in *A. niger* and *A. tubingensis* species. However, isolates of *A. carbonarius* did not show the same intensity in these wavelengths compared to the other two fungal species. Conclusively, the spectral profiles of *A. niger* and *A. tubingensis* were closer to each other than to *A. carbonarius* spectra. These two species are also consistent in macroscopic morphology and molecular phylogeny as well (Abarca et al., 2004; Samson et al., 2007). More specifically, Kusters-van Someren et al. (1991) were the first researchers who attempted to solve the classification of these important fungi based on RFLP analysis. They observed two different patterns of rDNA RFLP digestion with SmaI enzyme and proposed strains of group I as *A. niger* and group II as *A. tubingensis* strains. Mégnégneau et al. (1993) also confirmed these results by means of RFLP analysis of the total DNA digested with three restriction enzymes (*Sma*I, *Eco*RI and *Pst*I). The aforementioned researchers proved the high intraspecific variability among strains belonging to the *A. niger* aggregate by RAPD analysis. Visser et al. (1996) also confirmed the separation of the *A. niger* aggregate into the two species, *A. niger* and *A. tubingensis* by molecular means. Additionally, Parenicova et al. (2000) performed analysis of the sequences of the ITS1-5.8S rDNA-ITS2 region of some black aspergilli strains and observed that differences between the sequences of these two species were low with only 3 nucleotides separating the sequence of *A. niger* from *A. tubingensis*.

Moreover, a band at approximately 1150 cm^{-1} which has been correlated with the C-O stretching bonds of β-(1→4)-glucan was noticeable in all species (Kacurakova et al., 2000). Jäger (2003) investigated the production and mode of action of β–glucosidase isolated from *A. carbonarius* and *A. niger* and reported that both species were strong producers of β-glucosidase. Nevertheless, this observation was not considered as an effective criterion for the discrimination among the three species. The peaks at 1089-1091 cm^{-1} indicate the PO_2 symmetrical stretching attributed to absorption of DNA, RNA and phospholipids (Socrates, 2001).

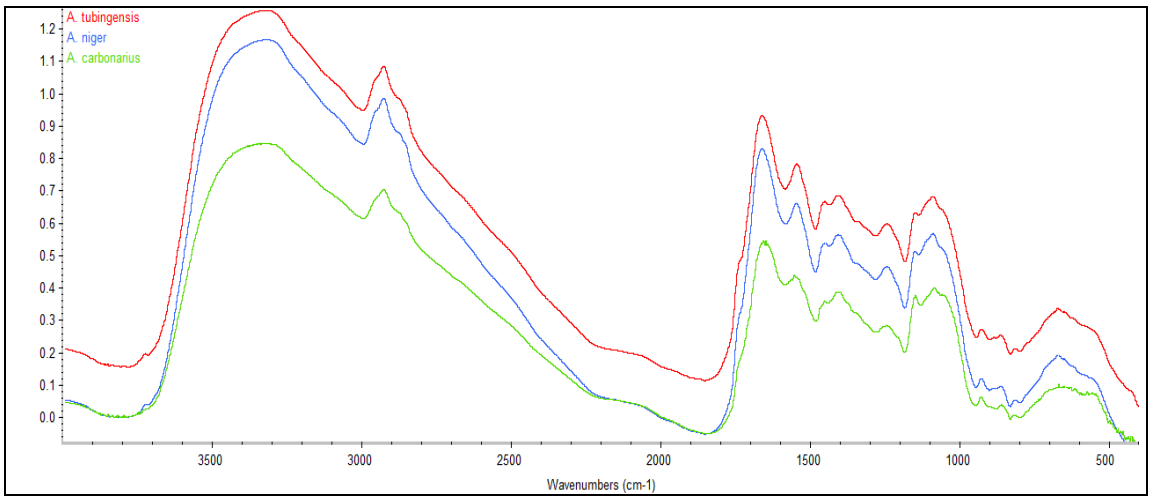

Figure 6.2: Comparison of DRIFT mean spectra (4000-400 cm^{-1}) of *A. tubingensis*, *A. niger* and *A. carbonarius*. Each spectrum is the average of the spectra recorded from three independent replicates for each fungus.

6.3.3 Discriminant analysis

The extracted spectral data were used as input in Mahalanobis discriminant classifier to separate the fungal species. The average spectra of 130 fungal isolates were used for model calibration based on Discriminant analysis performed by TQ Analyst software. The remaining 52 spectra were used for external model validation. The full spectrum as well as the combination of several spectral regions was investigated. Eventually, three wavelength ranges were chosen, specifically: i) 3002-2801 cm^{-1}, ii) 1773-1550 cm^{-1} and iii) 1286-952 cm^{-1}, and three actual classes (*A. carbonarius*, *A. niger* and *A. tubingensis*) were defined. The software used 19 principal components (factors) and described 100% variability of the selected spectral

regions (Fig. 6.3). This figure shows the principal components needed to calculate for all the spectral variation in the calibration standards. Each principal component describes a source of variation in the standards. Most of the variation should occur in the selected spectral regions (3002-2801 cm^{-1}, 1773-1550 cm^{-1} and 1286-952 cm^{-1}) because the full spectrum variation provides information about other sources which may not be relative to the analysis.

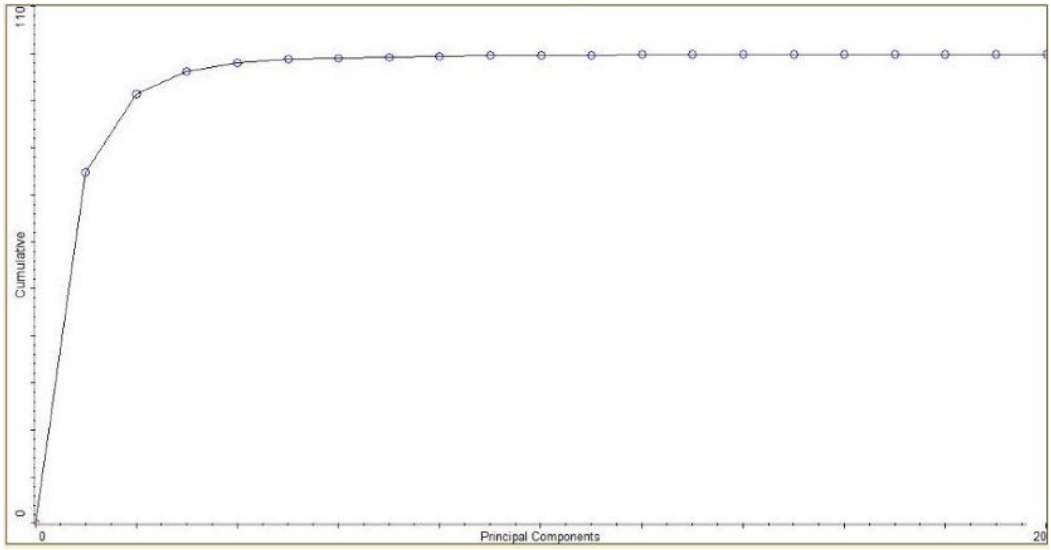

Figure 6.3: Cumulative Eigen values of 19 principal components calculated by the TQ Analyst software.

During model calibration, *A. carbonarius* showed classification accuracy of 95.3% (62 out of 65 spectra) when compared to the other two species (Table 6.1), whereas *A. niger* aggregate gave 100% (65 out of 65 spectra) per class accuracy presenting a higher performance in fungal discrimination. Only three isolates of *A. carbonarius* were assigned to the *A. niger* aggregate class in the calibration model. This is an important observation since all *A. niger* aggregate isolates were discriminated from *A. carbonarius,* which is the major OTA producer within the black aspergilli group, indicating the potential of FT-IR to discriminate these species. The external validation based on Discriminant analysiς performed by the TQ Analyst software showed 100% overall classification accuracy for both classes (Table 6.2). No fungal isolate was misclassified indicating high accuracy for the validation model. It can be concluded that working with the selected spectral regions higher percentage of variability could be explained with less number of principal components compared to full spectrum. Finally, the Mahalanobis distance of actual and calculated classes obtained by the

TQ Analyst software and the distance until the next class is shown in Supplementary Table 6.1. Moreover, a scatter plot of the Mahalanobis distances illustrated a clear differentiation between the two fungal classes (Fig. 6.4). Conclusively, from both calibration and validation models, it was observed that almost all predicted *A. niger* aggregate spectra were classified correctly, whereas eight isolates of *A. carbonarius* were misclassified only in the calibration model. To sum up, 179 isolates out of 182 were correctly classified and gave an average overall correct classification of 98.3% correct classification for all fungal species in both calibration and validation datasets.

Table 6.1: Percentage of classified spectra for model calibration based on Discriminant analysis performed by TQ Analyst software.

From / To	***A. carbonarius***	***A. niger* aggregate**	***per class accuracy (%)***
A. carbonarius (n=65)	62	3	95.3
A. niger aggregate (n=65)	0	65	100
overall correct classification (%)			**97.6**

Table 6.2: Percentage of classified spectra for model validation based on Discriminant analysis performed by TQ Analyst software.

From / To	***A. carbonarius***	***A. niger* aggregate**	***per class accuracy (%)***
A. carbonarius (n=26)	26	0	100
A. niger aggregate (n=26)	0	26	100
overall correct classification (%)			**100**

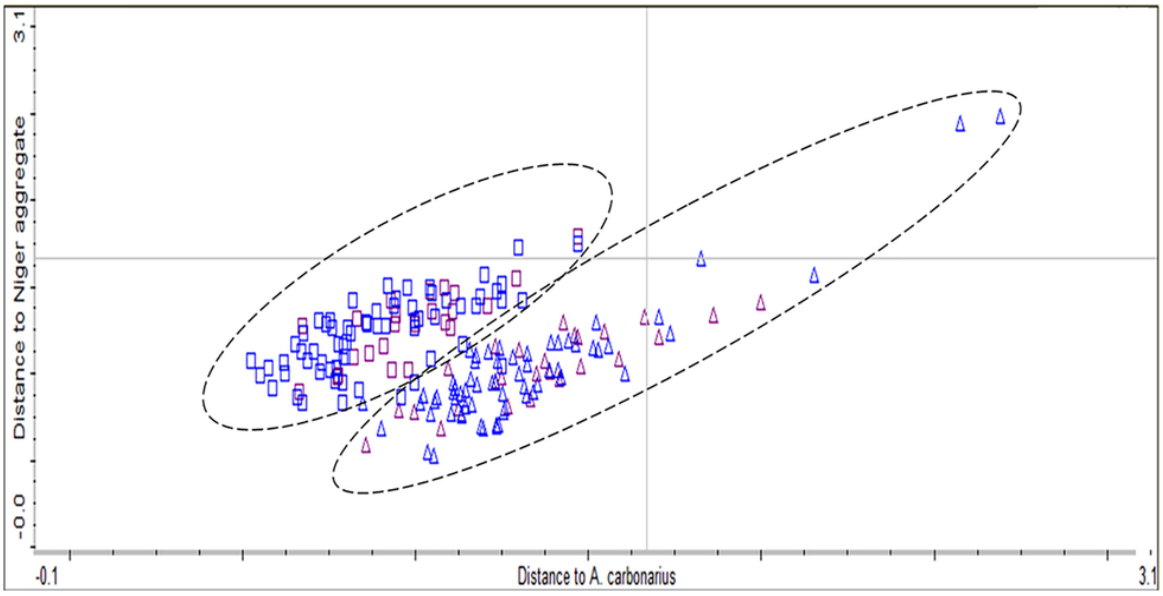

Figure 6.4: Scatter plot illustrating the discrimination between *A. carbonarius* (□) and *A.niger* aggregate (Δ) based on Mahalanobis distance (blue and red points indicate calibration and validation datasets, respectively). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

6.4 Conclusion

Fungal taxonomy is a difficult and complex process which requires robust identification keys for fungal discrimination and identification. In particular, *Aspergillus* section *Nigri* group is one of the most difficult groups to differentiate species, especially within the *A. niger* aggregate and therefore new techniques apart from morphological characteristics should be developed. In this study, discrimination among the black aspergilli species reached 98.3% overall correct classification for both model calibration and validation, based on well-characterized species, which have been previously identified by molecular techniques. Especially, *A. niger* aggregate showed classification accuracy of 100% in both model calibration and validation. Lower classification rates, were observed for *A. carbonarius* during calibration reaching 95.3%, whereas for validation the accuracy was 100%. One of the important outcomes of this study is the development of a well-characterized fungal database in FT-IR spectroscopy which is a helpful tool in identifying unknown fungal species using a less fastidious, low cost and easy-to-apply method. Due to these promising results it is necessary to extend the existing database with spectra from additional isolates of the fungi

under study and also include other species of the *Aspergillus* section *Nigri* group to infer on the applicability and limitations of FT-IR for rapid identification of filamentous fungi.

6.5 Supplementary File

Supplementary Table 6.1

The Mahalanobis distance of actual and calculated classes obtained by the TQ Analyst software and the distance to next classes. Bold letters indicate misclassified cases.

Actual Class	Calculated Class	Distance	Next Class	Next Distance
A. carbonarius	*A. carbonarius*	*1.9827*	*Niger aggregate*	*2.273*
A. carbonarius	*A. carbonarius*	*3.0468*	*Niger aggregate*	*3.0757*
A. carbonarius	*A. carbonarius*	*1.0418*	*Niger aggregate*	*1.4764*
A. carbonarius	*A. carbonarius*	*1.0825*	*Niger aggregate*	*1.494*
A. carbonarius	*A. carbonarius*	*0.8655*	*Niger aggregate*	*1.113*
A. carbonarius	*A. carbonarius*	*1.1084*	*Niger aggregate*	*1.3574*
A. carbonarius	*A. carbonarius*	*1.2907*	*Niger aggregate*	*1.546*
Niger aggregate	*Niger aggregate*	*0.7654*	*A. carbonarius*	*1.1052*
Niger aggregate	*Niger aggregate*	*1.143*	*A. carbonarius*	*1.5287*
A. carbonarius	*A. carbonarius*	*1.1118*	*Niger aggregate*	*1.4672*
Niger aggregate	*Niger aggregate*	*1.3345*	*A. carbonarius*	*1.8613*
Niger aggregate	*Niger aggregate*	*1.0784*	*A. carbonarius*	*1.3742*
A. carbonarius	*A. carbonarius*	*1.2073*	*Niger aggregate*	*1.3858*
Niger aggregate	*Niger aggregate*	*1.0077*	*A. carbonarius*	*1.3899*
Niger aggregate	*Niger aggregate*	*0.8515*	*A. carbonarius*	*1.3323*
A. carbonarius	*A. carbonarius*	*1.0851*	*Niger aggregate*	*1.3009*
A. carbonarius	*A. carbonarius*	*1.4693*	*Niger aggregate*	*1.7889*
A. carbonarius	*A. carbonarius*	*0.6731*	*Niger aggregate*	*1.2786*
A. carbonarius	*A. carbonarius*	*1.1005*	*Niger aggregate*	*1.265*
A. carbonarius	*A. carbonarius*	*1.048*	*Niger aggregate*	*1.364*
A. carbonarius	*A. carbonarius*	*0.7604*	*Niger aggregate*	*1.2652*
Niger aggregate	*Niger aggregate*	*1.2408*	*A. carbonarius*	*1.5457*
A. carbonarius	*A. carbonarius*	*0.9794*	*Niger aggregate*	*1.0214*
Niger aggregate	*Niger aggregate*	*1.1357*	*A. carbonarius*	*1.2998*

Niger aggregate	*Niger aggregate*	*0.9366*	*A. carbonarius*	*1.3537*
A. carbonarius	*A. carbonarius*	*0.7784*	*Niger aggregate*	*0.9883*
Niger aggregate	*Niger aggregate*	*1.5734*	*A. carbonarius*	*2.1525*
A. carbonarius	*A. carbonarius*	*0.9303*	*A. carbonarius*	*1.4232*
Niger aggregate	*Niger aggregate*	*0.9968*	*A. carbonarius*	*1.3507*
Niger aggregate	*Niger aggregate*	*1.4145*	*A. carbonarius*	*2.0001*
A. carbonarius	*A. carbonarius*	*0.8209*	*Niger aggregate*	*1.0962*
Niger aggregate	*Niger aggregate*	*1.1552*	*A. carbonarius*	*1.2323*
A. carbonarius	*A. carbonarius*	*0.9442*	*Niger aggregate*	*1.3336*
A. carbonarius	*A. carbonarius*	*0.9093*	*Niger aggregate*	*1.1548*
A. carbonarius	*A. carbonarius*	*1.0012*	*Niger aggregate*	*1.29*
A. carbonarius	*A. carbonarius*	*0.7737*	*Niger aggregate*	*0.9834*
A. carbonarius	*A. carbonarius*	*0.6654*	*Niger aggregate*	*0.8953*
Niger aggregate	*Niger aggregate*	*0.8149*	*A. carbonarius*	*1.2676*
Niger aggregate	*Niger aggregate*	*1.2219*	*A. carbonarius*	*1.4612*
Niger aggregate	*Niger aggregate*	*1.3255*	*A. carbonarius*	*1.6627*
Niger aggregate	*Niger aggregate*	*0.9554*	*A. carbonarius*	*1.2321*
Niger aggregate	*Niger aggregate*	*0.6886*	*A. carbonarius*	*1.0736*
Niger aggregate	*Niger aggregate*	*0.7861*	*A. carbonarius*	*0.9524*
Niger aggregate	*Niger aggregate*	*0.9651*	*A. carbonarius*	*1.4161*
Niger aggregate	*Niger aggregate*	*0.6897*	*A. carbonarius*	*0.9022*
Niger aggregate	*Niger aggregate*	*1.0438*	*A. carbonarius*	*1.4792*
Niger aggregate	*Niger aggregate*	*1.0321*	*A. carbonarius*	*1.0962*
Niger aggregate	*Niger aggregate*	*1.2136*	*A. carbonarius*	*1.7051*
Niger aggregate	*Niger aggregate*	*0.9747*	*A. carbonarius*	*1.2511*
Niger aggregate	*Niger aggregate*	*1.2914*	*A. carbonarius*	*1.4294*
A. carbonarius	*A. carbonarius*	*0.9397*	*Niger aggregate*	*1.2813*
Niger aggregate	*Niger aggregate*	*1.2151*	*A. carbonarius*	*1.4707*
Niger aggregate	*Niger aggregate*	*0.7765*	*A. carbonarius*	*0.9965*
Niger aggregate	*Niger aggregate*	*0.5887*	*A. carbonarius*	*0.8577*
Niger aggregate	*Niger aggregate*	*0.8008*	*A. carbonarius*	*1.1257*
Niger aggregate	*Niger aggregate*	*0.9073*	*A. carbonarius*	*1.1456*

Niger aggregate	*Niger aggregate*	*1.1853*	*A. carbonarius*	*1.4428*
Niger aggregate	*Niger aggregate*	*1.1641*	*A. carbonarius*	*1.4637*
A. carbonarius	*A. carbonarius*	*0.9329*	*Niger aggregate*	*1.0219*
Niger aggregate	*Niger aggregate*	*0.7819*	*A. carbonarius*	*1.2562*
Niger aggregate	*Niger aggregate*	*0.8692*	*A. carbonarius*	*1.1157*
Niger aggregate	*Niger aggregate*	*0.9115*	*A. carbonarius*	*1.1173*
Niger aggregate	*Niger aggregate*	*0.8436*	*A. carbonarius*	*1.0558*
A. carbonarius	*A. carbonarius*	*0.832*	*Niger aggregate*	*1.317*
Niger aggregate	*Niger aggregate*	*0.9*	*A. carbonarius*	*1.1103*
Niger aggregate	*Niger aggregate*	*0.9232*	*A. carbonarius*	*1.3157*
Niger aggregate	*Niger aggregate*	*1.0197*	*A. carbonarius*	*1.4134*
Niger aggregate	*Niger aggregate*	*1.1132*	*A. carbonarius*	*1.3232*
Niger aggregate	*Niger aggregate*	*0.8278*	*A. carbonarius*	*1.0147*
Niger aggregate	*Niger aggregate*	*1.1062*	*A. carbonarius*	*1.175*
Niger aggregate	*Niger aggregate*	*0.8041*	*A. carbonarius*	*1.2578*
Niger aggregate	*Niger aggregate*	*1.2303*	*A. carbonarius*	*1.7362*
A. carbonarius	*A. carbonarius*	*0.991*	*Niger aggregate*	*1.3754*
A. carbonarius	*A. carbonarius*	*1.0432*	*Niger aggregate*	*1.4959*
Niger aggregate	*Niger aggregate*	*0.9642*	*A. carbonarius*	*1.1597*
A. carbonarius	*A. carbonarius*	*1.2346*	*Niger aggregate*	*1.4797*
Niger aggregate	*Niger aggregate*	*0.8372*	*A. carbonarius*	*0.8476*
A. carbonarius	*A. carbonarius*	*1.2493*	*Niger aggregate*	*1.4271*
Niger aggregate	*Niger aggregate*	*0.8795*	*A. carbonarius*	*1.3214*
Niger aggregate	*Niger aggregate*	*1.0205*	*A. carbonarius*	*1.3855*
A. carbonarius	*A. carbonarius*	*0.8046*	*Niger aggregate*	*1.2678*
A. carbonarius	*A. carbonarius*	*0.7292*	*Niger aggregate*	*1.0634*
A. carbonarius	*A. carbonarius*	*0.9961*	*Niger aggregate*	*1.3065*
A. carbonarius	*A. carbonarius*	*0.9427*	*Niger aggregate*	*1.4378*
A. carbonarius	*A. carbonarius*	*0.5527*	*Niger aggregate*	*0.9942*
A. carbonarius	*A. carbonarius*	*0.6221*	*Niger aggregate*	*1.0056*
A. carbonarius	*A. carbonarius*	*0.7404*	*Niger aggregate*	*1.28*
A. carbonarius	*A. carbonarius*	*0.9135*	*Niger aggregate*	*1.2799*

A. carbonarius	*A. carbonarius*	*1.2961*	*Niger aggregate*	*1.7254*
A. carbonarius	*A. carbonarius*	*0.7523*	*Niger aggregate*	*1.0182*
A. carbonarius	*A. carbonarius*	*0.8124*	*Niger aggregate*	*1.2315*
A. carbonarius	*A. carbonarius*	*0.7071*	*Niger aggregate*	*1.1308*
A. carbonarius	*A. carbonarius*	*0.7701*	*Niger aggregate*	*1.0325*
A. carbonarius	*A. carbonarius*	*0.6872*	*Niger aggregate*	*1.0725*
A. carbonarius	*A. carbonarius*	*0.7533*	*Niger aggregate*	*1.3015*
A. carbonarius	*A. carbonarius*	*0.7935*	*Niger aggregate*	*1.0909*
A. carbonarius	*A. carbonarius*	*0.659*	*Niger aggregate*	*0.869*
A. carbonarius	*A. carbonarius*	*0.7674*	*Niger aggregate*	*0.9571*
A. carbonarius	*A. carbonarius*	*0.5235*	*Niger aggregate*	*1.0789*
A. carbonarius	*A. carbonarius*	*0.6514*	*Niger aggregate*	*1.1713*
A. carbonarius	*A. carbonarius*	*0.6192*	*Niger aggregate*	*1.0633*
A. carbonarius	*A. carbonarius*	*0.676*	*Niger aggregate*	*1.2277*
A. carbonarius	*A. carbonarius*	*0.9205*	*Niger aggregate*	*1.5011*
A. carbonarius	*A. carbonarius*	*0.7207*	*Niger aggregate*	*1.3012*
A. carbonarius	*A. carbonarius*	*1.0088*	*Niger aggregate*	*1.3217*
A. carbonarius	*A. carbonarius*	*0.8904*	*Niger aggregate*	*1.2711*
A. carbonarius	*A. carbonarius*	*0.8202*	*Niger aggregate*	*1.4219*
A. carbonarius	*A. carbonarius*	*0.5747*	*Niger aggregate*	*1.0373*
A. carbonarius	*A. carbonarius*	*1.1319*	*Niger aggregate*	*1.3921*
A. carbonarius	*A. carbonarius*	*1.3094*	*Niger aggregate*	*1.4212*
Niger aggregate	*Niger aggregate*	*1.0822*	*A. carbonarius*	*1.5895*
A. carbonarius	*A. carbonarius*	*1.045*	*Niger aggregate*	*1.086*
A. carbonarius	*A. carbonarius*	*0.6718*	*Niger aggregate*	*0.8358*
A. carbonarius	*A. carbonarius*	*0.7882*	*Niger aggregate*	*0.9453*
A. carbonarius	*A. carbonarius*	*1.1983*	*Niger aggregate*	*1.5727*
A. carbonarius	*A. carbonarius*	*0.9383*	*Niger aggregate*	*1.394*
Niger aggregate	*Niger aggregate*	*1.0796*	*A. carbonarius*	*1.1726*
A. carbonarius	*A. carbonarius*	*0.7769*	*Niger aggregate*	*1.1697*
A. carbonarius	*A. carbonarius*	*0.8566*	*Niger aggregate*	*1.2947*
A. carbonarius	*Niger aggregate*	*0.8605*	*A. carbonarius*	*0.9599*

A. carbonarius	*Niger aggregate*	*1.0433*	*A. carbonarius*	*1.2503*
A. carbonarius	*A. carbonarius*	*1.0477*	*Niger aggregate*	*1.4639*
Niger aggregate	*Niger aggregate*	*0.871*	*A. carbonarius*	*1.0253*
Niger aggregate	*Niger aggregate*	*1.1765*	*A. carbonarius*	*1.4127*
A. carbonarius	*Niger aggregate*	*0.9504*	*A. carbonarius*	*0.9968*
A. carbonarius	*A. carbonarius*	*0.9777*	*Niger aggregate*	*1.4939*
Niger aggregate	*Niger aggregate*	*1.6682*	*A. carbonarius*	*1.8254*
A. carbonarius	*A. carbonarius*	*0.7237*	*Niger aggregate*	*1.014*
A. carbonarius	*A. carbonarius*	*0.5879*	*Niger aggregate*	*0.9164*
Niger aggregate	*Niger aggregate*	*0.981*	*A. carbonarius*	*1.4233*
Niger aggregate	*Niger aggregate*	*1.1431*	*A. carbonarius*	*1.2429*
A. carbonarius	*A. carbonarius*	*0.9966*	*Niger aggregate*	*1.2642*
A. carbonarius	*A. carbonarius*	*1.1369*	*Niger aggregate*	*1.1696*
Niger aggregate	*Niger aggregate*	*1.3319*	*A. carbonarius*	*1.7039*
Niger aggregate	*Niger aggregate*	*0.9473*	*A. carbonarius*	*1.2319*
A. carbonarius	*A. carbonarius*	*0.7931*	*Niger aggregate*	*1.156*
Niger aggregate	*Niger aggregate*	*0.5532*	*A. carbonarius*	*1.0349*
Niger aggregate	*Niger aggregate*	*0.7663*	*A. carbonarius*	*1.1344*
Niger aggregate	*Niger aggregate*	*0.6814*	*A. carbonarius*	*1.1978*
Niger aggregate	*Niger aggregate*	*0.6814*	*A. carbonarius*	*1.1978*
Niger aggregate	*Niger aggregate*	*0.8922*	*A. carbonarius*	*1.3415*
Niger aggregate	*Niger aggregate*	*1.0035*	*A. carbonarius*	*1.2993*
Niger aggregate	*Niger aggregate*	*0.5281*	*A. carbonarius*	*1.054*
Niger aggregate	*Niger aggregate*	*1.0977*	*A. carbonarius*	*1.2816*
Niger aggregate	*Niger aggregate*	*1.1818*	*A. carbonarius*	*1.3927*
Niger aggregate	*Niger aggregate*	*0.8894*	*A. carbonarius*	*1.2524*
A. carbonarius	*A. carbonarius*	*1.1747*	*Niger aggregate*	*1.3881*
Niger aggregate	*Niger aggregate*	*0.7088*	*A. carbonarius*	*1.2336*
Niger aggregate	*Niger aggregate*	*1.144*	*A. carbonarius*	*1.5149*
Niger aggregate	*Niger aggregate*	*0.7015*	*A. carbonarius*	*1.2418*
Niger aggregate	*Niger aggregate*	*0.8112*	*A. carbonarius*	*1.1465*
Niger aggregate	*Niger aggregate*	*0.8613*	*A. carbonarius*	*1.0624*

Niger aggregate	*Niger aggregate*	*1.1231*	*A. carbonarius*	*1.2114*
A. carbonarius	*A. carbonarius*	*0.8884*	*Niger aggregate*	*1.3599*
A. carbonarius	*A. carbonarius*	*1.4714*	*Niger aggregate*	*1.7443*
A. carbonarius	*A. carbonarius*	*1.0892*	*Niger aggregate*	*1.4255*
A. carbonarius	*A. carbonarius*	*0.8591*	*Niger aggregate*	*1.2886*
Niger aggregate	*Niger aggregate*	*0.6958*	*A. carbonarius*	*1.2364*
A. carbonarius	*A. carbonarius*	*1.0554*	*Niger aggregate*	*1.3248*
A. carbonarius	*A. carbonarius*	*0.8381*	*Niger aggregate*	*0.905*
A. carbonarius	*A. carbonarius*	*0.7968*	*Niger aggregate*	*1.0952*
Niger aggregate	*Niger aggregate*	*1.1372*	*A. carbonarius*	*1.1587*
Niger aggregate	*Niger aggregate*	*1.2943*	*A. carbonarius*	*1.523*
Niger aggregate	*Niger aggregate*	*1.2943*	*A. carbonarius*	*1.523*
A. carbonarius	*A. carbonarius*	*1.2502*	*Niger aggregate*	*1.5192*
Niger aggregate	*Niger aggregate*	*0.7555*	*A. carbonarius*	*1.1345*
A. carbonarius	*A. carbonarius*	*0.7905*	*Niger aggregate*	*0.8312*
Niger aggregate	*Niger aggregate*	*0.946*	*A. carbonarius*	*1.224*
Niger aggregate	*Niger aggregate*	*1.0564*	*A. carbonarius*	*1.3234*
Niger aggregate	*Niger aggregate*	*1.0732*	*A. carbonarius*	*1.2383*
A. carbonarius	*A. carbonarius*	*0.8012*	*Niger aggregate*	*1.1811*
Niger aggregate	*Niger aggregate*	*1.004*	*A. carbonarius*	*1.6068*
Niger aggregate	*Niger aggregate*	*0.7695*	*A. carbonarius*	*1.045*
Niger aggregate	*Niger aggregate*	*1.1545*	*A. carbonarius*	*1.5597*
Niger aggregate	*Niger aggregate*	*0.8497*	*A. carbonarius*	*1.1389*
Niger aggregate	*Niger aggregate*	*0.8197*	*A. carbonarius*	*1.1635*
Niger aggregate	*Niger aggregate*	*0.6915*	*A. carbonarius*	*1.1914*
Niger aggregate	*Niger aggregate*	*0.9369*	*A. carbonarius*	*1.1781*
Niger aggregate	*Niger aggregate*	*0.9507*	*A. carbonarius*	*1.1108*
A. carbonarius	*A. carbonarius*	*0.6715*	*Niger aggregate*	*1.1319*
Niger aggregate	*Niger aggregate*	*0.8814*	*A. carbonarius*	*1.1297*
Niger aggregate	*Niger aggregate*	*2.4425*	*A. carbonarius*	*2.5762*
Niger aggregate	*Niger aggregate*	*2.4863*	*A. carbonarius*	*2.6926*

CHAPTER 7

Concluding remarks and future perspectives

Members of the *Aspergillus* section *Nigri* group are distributed worldwide and regarded as common food spoilage fungi that may produce Ochratoxin A (OTA), which is a dangerous mycotoxin often detected in various food products including grapes, grape juices and wines. Therefore, control of fungal growth and OTA presence is of primary importance in protecting consumer's health and, in this context, several approaches for inhibiting OTA production have been investigated lately. Among them, the use of biological control agents (BCA), such as non-ochratoxigenic species, or natural antifungal compounds, like pine-resin, seem to provide promising perspectives against OTA.

The main objective of this thesis was to provide an overview on the significance of black aspergilli focusing to modern control approaches as means for fungal growth and OTA production control, as well as to utilize predictive modelling in an attempt to accurately assess the effect of these approaches on fungal growth and OTA accumulation. Among the *Aspergillus* section *Nigri* contributed in this study, *A. carbonarius* was the dominant OTA producer where almost 100% of its isolates released OTA, followed by the *A. niger* aggregate species (*A. niger* and *A. tubingensis*). Fungal kinetics were determined and mathematical models for fungal growth and OTA prediction were developed combined with different ecological factors and antimicrobial compounds. Moreover, the taxonomy of this group is not clear and many attempts have been made in order to improve the existing taxonomic criteria. A new classification approach among species of *Aspergillus* section *Nigri* was obtained by the use of Diffuse Reflectance Infrared Fourier Transform Spectroscopy.

In Chapter 2, models were developed to quantify the effect of temperature, a_w and sodium metabisulphite (NaMBS) concentration, on the growth and OTA production of *A. carbonarius* on a Grape Juice based Medium (GJM). Colony diameter of the fungus was measured daily and fitted to the primary model of Baranyi and Roberts in order to estimate the maximum growth rates (μ_{max}) and lag phase duration (λ). Subsequently, the kinetic parameters derived from the Baranyi and Roberts model were expressed as a function of temperature, a_w and NaMBS concentration using the secondary cardinal model with inflection (CMI). In addition, OTA production was recorded and modelled as a function of the same parameters through a quadratic polynomial model. Results showed that NaMBS increased the lag phase of *A. carbonarius*, particularly at 38 °C/0.98 a_w and 38 °C/0.96 a_w, as well as at lower a_w levels regardless of temperature. At higher concentrations (100 and 150 mg L^{-1}) fungal growth was delayed and at concentrations of 200 mg L^{-1} no growth was observed irrespective of temperature and a_w levels. This study complemented the findings on the

ecophysiology of *A. carbonarius* using NaMBS as an inhibitory agent. Indeed, results were very comprehensive and models could describe effectively fungal behavior in different ecophysiological conditions in combination with the antimicrobial agent. Future studies with other natural antimicrobial compounds in diverse environmental conditions may be undertaken in order to develop models that provide additional information on fungal ecology and OTA minimization.

Additionally, the potential of natural compounds, namely natamycin and pine-resin, to suppress fungal growth and OTA production was demonstrated in Chapter 5. Natamycin increased the lag phase duration and reduced OTA production at intermediate temperature conditions (20 and 25 °C). On the other hand, pine-resin had no effect on the growth rate and increased OTA levels at the same conditions assayed. However, at high temperatures (30 °C) OTA was detected at lower levels after treatment with both antimicrobials. Moreover, both compounds completely inhibited fungal growth at lower temperature and water activity conditions (16 °C/0.94 a_w). Natamycin concentration up to 1 μg mL^{-1} was recommended for use as a possible fungicidal agent against *A. carbonarius*. Conclusively, more studies are needed in order to investigate the efficacy of pine-resin on *A. carbonarius* growth parameters and OTA production, since there is no literature available for this antimicrobial compound against this fungal species.

The complex fungal interactions determined by *in vitro* experiments resulted to the conclusion that fungal interspecific variability may act as a biological control factor, given that the presence of non-ochratoxigenic *Aspergillus* section *Nigri* species or other fungal grape-related species presented in Chapter 4, inhibited OTA accumulation by *A. carbonarius*. When *P. spinulosum*, *A. ibericus* and *B. cinerea* were co-cultured *in vitro* with *A. carbonarius*, OTA was remarkably reduced. Generally, for the target strains of *A. carbonarius*, even at conditions where the growth rate was stimulated by the fungal competitors, OTA concentration was decreased. Fungal interactions inhibited in most cases OTA production by the examined *A. carbonarius* strains. It is clear that the influence of changes in climatic conditions compared to fungal interactions might be a crucial parameter in mycotoxin control. Further studies must be conducted to examine the combined effect of different environmental factors and fungal interactions between toxigenic and atoxigenic species in order to elucidate the potential of some fungal species to eliminate OTA.

Another experiment with different mixed spore ratios of two non-toxigenic *Aspergillus* section *Nigri* species (*A. tubingensis* and *A. japonicus*) and three toxigenic *Aspergillus carbonarius* isolates was presented in Chapter 3. The *in vitro* potential of OTA production by *A. carbonarius* was measured and in some cases the toxin was inhibited whereas in others it was stimulated after fungal co-culture. Very few studies have focused on interactions between fungal strains or species under different ecological conditions and how these conditions can affect toxin concentration. The importance of this study concerns the understanding of interspecific interactions on OTA production by *A. carbonarius* in an attempt to find ways to ensure toxin elimination in grapes and their derivatives. Further studies are required to examine the efficacy of temperature, a_w, fungal interactions and competition on OTA minimization. Also, other non-toxigenic species of the *Aspergillus* section *Nigri* group or other fungi from the grape mycobiota may be used in mixed spore populations together with *A. carbonarius* isolates in order to have a better knowledge and understanding of their influence on OTA production.

In the last experiment presented in Chapter 6, the potential of FT-IR spectroscopy was evaluated to distinguish ochratoxigenic species of *A. carbonarius* from the species of *A. niger* aggregate. Even though *A. carbonarius* is the major OTA producer within the *Aspergillus* section *Nigri* group, it is very difficult to know the extent of its natural occurrence in foods because all black aspergilli are commonly regarded as *A. niger*. Species identification is the main goal in mycological taxonomy and requires a polyphasic approach to generate quality data that are both accurate and useful. FT-IR spectroscopy has demonstrated its potential as a new technique applied to the identification and characterization of several filamentous fungi and yeast strains. Results showed classification accuracy of 100% for *A. niger* aggregate species for both model calibration and validation. Lower classification rates were obtained for *A. carbonarius* reaching 95.3% and 100% per class accuracy during model calibration and validation, respectively. The advantages of this new approach are: (a) simple sample preparation procedure, (b) short time of analysis and (c) reliability of the data. One of the important outcomes of this study is the development of a well-characterized fungal database in FT-IR spectroscopy which is a helpful tool in identifying unknown fungal species using a less fastidious, low cost and easy-to-apply method.

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1. **Efstathia A. Kogkaki**, Pantelis I. Natskoulis, Naresh Magan, Efstathios Z. Panagou, 2015. Effect of interaction between *Aspergillus carbonarius* and non-ochratoxigenic grape-associated fungal isolates on growth and ochratoxin A production at different water activities and temperatures. *Food Microbiology* 46, 521-527.

2. **Kogkaki E.A.**, Natskoulis P.I., Nychas G.-J., Panagou E.Z., 2015. Effect of water activity, temperature and mixed fungal spore interactions on ochratoxin A production by *Aspergillus carbonarius*. *Journal of Food Protection* 78, 376-382.

3. Angelos-Gerasimos Ioannidis, **Efstathia A. Kogkaki**, Pantelis I. Natskoulis, George-John E. Nychas, Efstathios Z. Panagou, 2015. Modelling the influence of temperature, water activity and sodium metabisulphite on the growth and OTA production of *Aspergillus carbonarius* isolated from Greek wine grapes. *Food Microbiology* 49, 12-22.

4. **Efstathia A. Kogkaki**, Pantelis I. Natskoulis, Efstathios Z. Panagou, 2016. Modelling the effect of natamycin, pine-resin and environmental factors on the growth and OTA production by *Aspergillus carbonarius* using response surface methodology. *Food Research International* 79, 19-28.

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Conferences

1. **E. Kogkaki**, P.I. Natskoulis, D. Kizis, G.-J.E. Nychas, E.Z. Panagou, 2014. Probabilistic models for the effect of temperature, water activity and sodium metabisulphite concentration on the growth and OTA production boundaries of *Aspergillus carbonarius* isolated from Greek wine grapes. European Symposium of IAFP, 7-9 May, Budapest, Hungary.

2. **E. Kogkaki**, P.I. Natskoulis, G.-J.E. Nychas, E.Z. Panagou, 2014. Study of interactions on growth and OTA production between an *A. carbonarius* ochratoxigenic strain and non-ochratoxigenic grape-associated fungal strains at different water activities and temperatures. 24th International ICFMH Conference-FoodMicro, 1-4 September, Nantes, France.

3. **E. Kogkaki**, P.I. Natskoulis, G.-J.E. Nychas, E.Z. Panagou, 2015. Effect of natamycin and environmental factors on growth and OTA production by two *A. carbonarius* isolates using a central composite design experiment. IAFP's European Symposium on Food Safety, 20-22 April, Cardiff, Wales.

4. **E. Kogkaki**, P.I. Natskoulis, G.-J.E. Nychas, E.Z. Panagou, 2015. Modelling the effect of natamycin, resin and ecological factors on the growth, lag phase duration and OTA production by *A. carbonarius* using a central composite design. 9th International Conference on Predictive Modelling in Food-ICPMF, 8-12 September, Rio de Janeiro, Brazil.

5. **E. Kogkaki**, M. Sofoulis, G.-J.E. Nychas, E.Z. Panagou, 2016. Fourier transform infrared spectroscopy as a rapid and innovative technique for discrimination of three most important black aspergilli species isolated from grapes. The Food Factor I Barcelona Conference, 2-4 November, Barcelona, Spain.

6. **Kogkaki E.**, Striftou S., Nychas G.-J., Panagou E.Z., 2017. The effect of fungal quorum sensing molecules on growth and OTA production of two *Aspergillus carbonarius* isolates. 7th Symposium of the Scientific Society of Mikrobiokosmos, 7-9 April, Athens, Greece.