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INNOVATION, SAFETY AND MANAGEMENT"

(*XXXII CYCLE*)

Aspergillus from grapes: ochratoxin A risk in relation to climate change and new strategies for reducing contamination

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LIST OF ABBREVIATIONS

AFB_1, Aflatoxin B_1

AFB_2, Aflatoxin B_2

AFG_1, Aflatoxin G_1

AFG_2, Aflatoxin G_2

AFM_1, Aflatoxin M_1

AFs, Aflatoxins

ALT, Altenuene

ALTs, Altertoxins

AME, Alternariol Monomethyl Ether

AOH, Alternariol

a_w, Water Activity

BEN, Balkan Endemic Nephropathy

BM, Basal Medium

CAC, Codex Alimentarius Commission

CC, Climate Change

DON, Deoxynivalenol

EC, European Commission

EFSA, European Food Safety Authority

FAO, Food and Agricultural Organization

FB_1, Fumonisin B_1

FB_2, Fumonisin B_2

GAP, Good Agricultural Practices

GHG, Green Houses Gases

GJM, Grape Juice Medium

GMP, Good Manufacturing Practices

GSP, Good Storage Practices

HACCP, Hazard Analysis and Critical Control Points

HT-2, HT2 toxin

IARC, International Agency for Research on Cancer

IPCC, Intergovernmental Panel on Climate Change

NIH, National Institute of Health

OTA, Ochratoxin A

OTα, Ochratoxin alfa

OTβ, Ochratoxin beta

PAT, Patulin

RASFF, Rapid Alert System for Food and Feeds

SNM, Synthetic Nutrient Medium

T, Temperature

T-2, T2 toxin

TCIN, Tunisian Nephropathy

WHO, World Health Organization

ZEA, Zearalenone

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SUMMARY

Ochratoxin A (OTA) is one of the most-abundant food-contaminating mycotoxins produced by species belonging to *Aspergillus* and *Penicillium* genera. Wine is reported to be the second major source of OTA intake after cereals. OTA is a potent nephrotoxin, classified as a group 2B, possible human carcinogen. *Aspergillus carbonarius*, which belong to the *Aspergillus* section *Nigri,* has been identified as the major cause of OTA contamination in grape-berries. OTA contamination of grapes is strongly related to plant phenology, geographical area and meteorological conditions and varies from season to season. Indeed, climate represents the key-factor in the agro-ecosystem that influences fungal colonization and mycotoxin production in crops. Climate change (CC) is expected to have a profound effect on our landscape worldwide, and also to have an important impact on sustainable food production system. Based on existing information on climate change, it is estimated that the environment in which crops will be grown in the next 25-50 years may change markedly with atmospheric CO_2 concentration expected to double or triple (from 400 to 800-1200) and temperature to increase (+2-5°C). These projected changes in climate conditions are likely to have a detrimental effect on food safety, with mycotoxins representing one of the major hazards. In fact, CC effects are already evident for the widening of area exposed to aflatoxins risks, like south east of Europe.

In addition, it is reported that in high risk years OTA contamination could exceed the legislative limit (2 $\mu g \cdot L^{-1}$) in wine and other grape-derived products. In this respect, various strategies for decontamination of wine from OTA have been studied in the last decade but no reliable technique/system is available except the use of activated charcoal by reducing also wine quality.

A better solution, potentially safe and environmentally friendly, to decontaminate grape derived products by OTA could be biodegradation, using microorganisms or their enzymes. Nevertheless, this technology is not commonly used in food processing.

With the present PhD project, we studied the impact of combined CC factors (i.e. increasing temperature, water stress and elevated CO_2 concentrations) on the ecophysiology, expression of OTA related genes and phenotypic OTA production in *A. carbonarius* strains. Such activities were carried out *in vitro* by using a grape-based medium and simulating climate conditions typical of Apulia region, which is an area in South Italy susceptible to OTA contamination.

Moreover, it has been evaluated the possibility to develop a new strategy to reduce the risk of OTA contamination by exploiting the already known capability of *A. carbonarius* to degrade the

toxin. The ultimate aim was to identify and characterize proteases responsible of OTA degradation into the less toxic compound OTα.

Interesting results has been obtained evidencing as the expected raise of day/night temperature may not be associated to an increase of OTA risk in the area studied. On the other hand, experiments with 2.5x higher CO_2 concentrations showed that the probability of OTA contamination may enhance and may lead to redefine the distribution of the ochratoxigenic risk areas.

Reduced levels of OTA and the concomitant slight increase of OTα, confirmed the *A. carbonarius* ability to degrade OTA. Interestingly, increased levels of some proteases encoding genes, suggested that these might have played a key role in degrading OTA excluding that the reduction observed was due to the also known capability of the fungus to retain the toxin. An aspartyl protease (acid protease) was selected for further studies as a possible de-contamination application in grape by-products.

RIASSUNTO

L'ocratossina (OTA) è una delle più abbondanti micotossine contaminanti gli alimenti prodotta da specie fungine appartenenti ai generi *Aspergillus* e *Penicillium*. Il vino è considerato la seconda fonte di contaminazione da OTA dopo i cereali. OTA è una potente nefrotossina, classificata come possibile cancerogeno per l'uomo (Gruppo 2B). *Aspergillus carbonarius*, appartenente alla sezione *Nigri*, è considerato il maggiore responsabile della contaminazione da OTA nell'uva. A seconda dello stadio fenologico della pianta, della zona geografica, delle condizioni metereologiche, la contaminazione può variare da un anno all'altro. Infatti, la colonizzazione fungina e la produzione di micotossine negli ecosistemi agricoli è influenzata dalle condizioni climatiche. Sulla base delle informazioni esistenti relativamente al cambiamento climatico, si stima che le coltivazioni potrebbero essere soggette a variazioni climatiche caratterizzate dall' aumento della concentrazione di CO_2 (da 400 a 800-1200 ppm) e delle temperature (+2-5°C). Nello scenario di tali cambiamenti, la sicurezza alimentare potrebbe essere compromessa e le micotossine rappresentare un crescente pericolo per la salute umana. Gli effetti del cambiamento climatico (CC) sono già evidenti risultando, ad esempio, nell'ampliamento dell'area esposta al rischio di contaminazione da aflatossine nei paesi del sud Est Europeo. Inoltre, in stagioni favorenti la contaminazione di OTA il limite legislativo stabilito per la sua presenza in uva e prodotti da essa derivati (2 $\mu g \cdot L^{-1}$) potrebbe essere superato. Al riguardo, sono state proposte numerose strategie per la decontaminazione del vino da OTA sebbene l'uso di adsorbenti, tra cui il carbone attivo associato ad una diminuzione dei parametri organolettici e nutritivi del prodotto finale, ad oggi sia l'unico sistema impiegato nell'industria alimentare. Una soluzione migliore, potenzialmente sicura ed ecosostenibile, potrebbe essere rappresentata dalla biodegradazione attraverso microorganismi ed enzimi. Tuttavia, questa strategia non è comunemente usata.

Con il presente progetto di dottorato, abbiamo studiato l'effetto combinato di fattori del CC (ad es. l'aumento di temperatura, stress idrico e incremento della concentrazione di CO_2) sull'ecofisiologia, l'espressione di geni correlati alla biosintesi di OTA e la produzione fenotipica della stessa in ceppi di *A. carbonarius*. Per lo svolgimento delle seguenti attività è stato utilizzato un sistema *in vitro* preparando un substrato a base di succo d'uva e simulando condizioni climatiche tipiche della regione Puglia, un'area particolarmente suscettibile al problema della contaminazione da OTA.

Inoltre, la già nota capacità di *A. carbonarius* di degradare la tossina, è stata considerata come possibile strategia di riduzione di OTA. Lo scopo finale è stato quello di identificare e caratterizzare proteasi responsabili della degradazione dell'ocratossina in OTα, un suo derivato meno tossico.

Gli interessanti risultati ottenuti evidenziano che il preannunciato aumento delle temperature sia diurne che notturne potrebbe non determinare un aumento del rischio ocratossigeno nell'areale del Sud della Puglia. D'altro canto, i risultati sperimentali in riferimento all'incremento della CO_2, indicano un possibile aumento della contaminazione da OTA. Inoltre, a causa dei cambiamenti climatici molte specie fungine potrebbero risultare resilienti oppure trovare condizioni più idonee al loro sviluppo in zone diverse da quelle già note. Una rinnovata valutazione e definizione delle aree di rischio potrebbero risultare necessarie.

La riduzione dei livelli di OTA e il concomitante aumento di OTα, hanno confermato la capacità di *A. carbonarius* di degradare OTA. Infatti, l'aumento dell'espressione di alcuni geni esaminati codificanti per proteasi, ha suggerito che queste potrebbero svolgere un ruolo chiave nella degradazione della micotossina. Un'aspartil proteasi (proteasi acida) è stata selezionata per ulteriori studi e per il suo possibile impiego come decontaminante di prodotti derivanti dall'uva.

INTRODUCTION

1. INTRODUCTION TO MYCOTOXINS

Mycotoxins - from Greek *mykes*, fungus and *toxicon*, poison - are non-volatile, relatively low-molecular weight, toxic secondary metabolites produced by filamentous fungi (commonly known as moulds), naturally occurring in food and feed (Bräse et al., 2009). These compounds constitute a toxigenically and chemically heterogeneous group which are gathered together by the fact that they can exert a dangerous effect both on human beings and other vertebrates.

The term mycotoxin was coined in 1962 in the aftermath of an unusual veterinary crisis near London, England, during which approximately 100.000 poultry died (Blount, 1961; Forgacs, 1962). When this mysterious turkey X disease was discovered to be linked to a peanut meal contaminated with secondary metabolites (aflatoxins) from *Aspergillus flavus*, it began the so known mycotoxin gold rush during which scientists started to investigate on the possibility that other occult mould metabolites might be deadly (Maggon et al., 1977). Mycotoxins are not only hard to define, they are also challenging to classify, due to their diverse chemical structures and biosynthetic origins, their biological effects, the variety of fungal species that can produce them. Currently, almost 400 compounds are now recognized as mycotoxins, of which approximately a dozen groups regularly receive attention as a threat to human and animal health (Bennet and Klich, 2003).

1.1. Main mycotoxins

Mycotoxins are mainly produced by fungi belonging to *Aspergillus*, *Penicillium*, *Alternaria*, *Fusarium*, *Claviceps* genera. The most relevant mycotoxins are: aflatoxins, produced by *Aspergillus* species; ochratoxin A, produced by both *Aspergillus* and *Penicillium* species; trichothecenes (Type A: HT-2 and T-2 toxin, Type B: deoxynivalenol), zearalenone, fumonisins B_1 and B_2, and the emerging mycotoxins (fusaproliferin, moniliformin, beauvericin and enniatins) produced mainly by *Fusarium* species; ergot alkaloids produced by *Claviceps*; altenuene, alternariol, alternariol methyl ether, altertoxin, tenuazonic acid produced by *Alternaria* species (Barkai-Golan, 2008; Bottalico & Logrieco, 1998) (Table 1).

Table 1. Main mycotoxins and producing species

Mycotoxin	Acronym	Species producing
Aflatoxins B_1, B_2, G_1, G_2	AFB_1 AFB_2 AFG_1 AFG_2	*Aspergillus* section *Flavi* *A.* section *Ochraceorosei* *A.* section *Nidulantes*
Ochratoxin A	OTA	*Aspergillus* section *Circumdati* *A.* section *Nigri* *A.* section *Flavi* *(A. albertensis, A. alliaceus)* *Penicillium verrucosum* *Penicillium nordicum* *Penicillium thymicola*
Fumonisins B_1, B_2	FB_1, FB_2	*Fusarium* section *Liseola* *Aspergillus niger*
Patulin	PAT	*Penicillium expansum,* *Bysochlamis nivea,* *Aspergillus clavatus*
HT-2, T-2 toxins (Type A trichothecenes)	HT-2 T-2	*Fusarium acuminatum, F. poae* *F. sporotrichioides, F. langsethiae*
Deoxynivalenol (Type B trichothecenes)	DON	*Fusarium graminearum,* *F. culmorum, F. cerealis*
Zearalenone	ZEA	*Fusarium graminearum,* *F. culmorum, F. equiseti, F.cerealis,* *F. verticillioides,* *F. incarnatum*
Alternariol	AOH	*Alternaria alternata*
Alternariol monomethyl ether	AME	*Alternaria alternata, A. solani*
Tenuazoic acid	TeA	*Alternaria alternata*
Altertoxins	ALTs	*A. tenuissima*
Altenuene	ALT	*Alternaria alternata*

Based on Marin et al., 2013; Perrone & Gallo, 2017

1.2. Occurrence of toxigenic fungi throughout the food chain

Invasion by fungi and production of mycotoxins in commodities may occur at various points in the food chain: at pre-harvest, harvest and post-harvest. Poor agricultural and harvesting practices, improper drying, handling, packaging, storage and transport conditions promote fungal growth, increasing the risk of mycotoxin production. Fungi that are frequently found in the field include *Aspergillus flavus*, *Alternaria longipes*, *A. alternata*, *Claviceps purpurea*, *Fusarium verticillioides*, *F. graminearum* and a number of other *Fusarium spp*. Species most likely introduced at harvest, include *Fusarium sporotrichioides, Stachybotrys atra*, *Cladosporium spp*., *Myrothecium verrucaria*, *Trichothecium roseum*, as well as *A. alternata*. Most penicillia are storage fungi such as *Penicillium citrinum*, *P. cyclopium*, *P. citreoviride*, *P. islandicum*, *P. rubrum*, *P. viridicatum*, *P. verrucosum*, *P. expansum*, which are capable of producing mycotoxins *in grains* and foods. Other toxigenic storage fungi are: *A. parasiticus*, *A. flavus*, *A. versicolor*, *A. ochraceus*, *A. steynii*, *A. westerdjikiae, A. clavatus*, *A. fumigatus*, *A. rubrum*, *A. chevallieri*, *F. moniliforme*, *F. tricinctum*, *F. nivale* and several other *Fusarium spp*. Thus, most of the mycotoxin-producing fungi belong to three genera, namely *Aspergillus*, *Fusarium* and *Penicillium* (Hesseltine, 1976). However, not all species in these genera are toxigenic. Fungal colonization, growth and/or mycotoxin production are generally influenced by a variety of factors. In particular, weather conditions, plant characteristics (e.g. composition, pH, water activity, oxygen content..), competition with other fungi or microbes are key factors in determining fungal colonization in the field and on the crops/fruits (Chu, 2006).

During storage and transportation, water activity, temperature, crop damage plus a variety of chemical factors – such as aeration (O_2, CO_2 levels), pH, presence of inhibitors, chemical treatment – are important. In general, mould growth in the grains or foods is necessary before subsequent onset of toxin production and optimal conditions for toxin formation generally have a narrow window than those for mould growth. For example, the optimal temperatures and a_w for the growth of *A. flavus* and *A. parasiticus* are around 35-37°C (range 6-54°C) and 0.95 (range 0.78-1.0), respectively; for aflatoxin production they are 28-33°C and 0.90-0.95 (0.83-0.97), respectively (Lacey, 1989).

1.3. Mycotoxins as a public health concern

Exposure to mycotoxins occur primarily through ingestion of contaminated foods from vegetal and animal origin. An alternative way of exposure is the inhalation of contaminated dust or skin contact. The diseases caused by mycotoxins in human or animals are called "mycotoxicosis". While most animal mycotoxicoses have been experimentally confirmed, human mycotoxicoses are less well understood and not clearly defined (Bennett and Klich, 2003; Ruyck et al., 2015). Depending on dose and time of exposure, they can exert both acute and chronic toxicities. Acute toxicity generally has a rapid onset and an obvious toxic response, while chronic toxicity is characterized by low-dose exposure over a long time period, resulting in cancers and other generally irreversible effects (James, 1985). Some of the most frequently encountered mycotoxins, ochratoxin A (OTA) and deoxynivalenol (DON) are reported to interfere with mammalian cellular processes including DNA replication and protein synthesis (Benassi et al., 2009; Pfohl-Leszkowicz et al., 2012). Other mycotoxins, particularly aflatoxin B_1 (AFB_1) and its metabolic precursor sterigmatocystin, have been identified as carcinogenic by the World Health Organization's (WHO) International Agency for Research on Cancer (IARC) Monographs Program. IARC Monographs evaluated the toxicity of the most commonly occurring mycotoxins and proved the carcinogenic risk in human and animals of some of them (Table 2).

Furthermore, considering that one fungal species may produce different mycotoxins and the same mycotoxin may also be produced by several fungal species, there is a very high likelihood of multiple mycotoxins co-occurring in food and feed products (Mngadi et al., 2008). Consequently, in the richly varied modern Western diet, individuals undergo dietary exposure to a very wide variety of mycotoxins (Abia et al., 2013; Solfrizzo et al., 2014). Unfortunately, third-world nations with relatively consistent diet are no safer, being reliant on cereal crops grown and processed under laxer regulation and prone to significantly higher levels of contaminations (Kitya et al., 2010).

Table 2. Evaluation of carcinogenicity of some mycotoxins

Mycotoxin	Degree of carcinogenicity		Evaluation of carcinogenicity
	Human	*Animal*	
AFs occurring as mixture of	S	S	Group 1
AFB_1	S	S	Group 1
AFB_2		L	
AFG_1		S	
AFG_2		I	
AFM_1	I	S	Group 2B
OTA	I	S	Group 2B
Toxins from *F. graminearum*, *F. culmorum*	I		Group 3
Zearelenone		L	
Deoxynivalenol		I	
Nivalenol		I	
Toxins derived from *F. moniliforme*	I	S	Group 2B
Fumonisins occurring as mixture of	I		
Fumonisins B_1		S	Group 2B
Fumonisins B_2		I	
Fusarin C		L	
Toxins derived from *F. sporotrichioides*	I[a]		Group 3
T-2 toxin		L	

Degrees of evidence: S= sufficient; L= limited; I= inadeguate; N=negative.

Group 1: carcinogenic to humans, Group 2A: probably carcinogenic to humans, Group 2B: possibly carcinogenic to humans, Group 3: not classifiable as to its carcinogenicity to humans.

[a] No data available

International Agency for Research on Cancer (IARC), 1993 and 1999

1.4. Management of mycotoxigenic risk and food safety

In addition to the public health risks, mycotoxin contamination causes major economic losses at all levels of the food production chain. According to the Food and Agriculture Organization of the United Nations (FAO,1999), approximately 25% of the agricultural crops produced in the world are contaminated by mycotoxins above the EU and Codex limits. Recently, Eskola et al., 2019 pointed that this value may underestimate the real occurrence of mycotoxins worldwide above the limit of detection (up to 60-80%) due to obsolete analytical methods used at that time and the emerging issue of climate change.

To minimize the public health risk and reduce the economic losses, it would be ideally to eliminate them from the food chain. However, on a practical level this is not possible and the general recommendation is to reduce the mycotoxin levels in food and feed as low as technologically possible (Bennett and Klich, 2003).

The Codex Alimentarius (CAC, 2003) has set recommendations for prevention and reduction of mycotoxins contamination in several crops (cereals, nuts, fresh fruit) and raw materials. These recommendations consist in good agricultural practices (GAP) to be applied at the pre-harvest stage and include soil preparation, crop rotation, selection of resistant varieties, avoid plant stress (irrigation) and pest control (chemical pesticides and biological agents). Good manufacturing practices (GMP), during handling and good storage (GSP) and processing practices are required for the management of the mycotoxigenic risk at post-harvest stage. Control of storage/transport conditions (T, a_w, gaseous atmosphere..) to avoid fungal development and mycotoxin production is critical. Safe conditions could be determined through the development of predictive models specific of fungi-mycotoxins in foodstuffs. They can be used in the implementation of Hazard Analysis and Critical Control Point (HACCP) plans (Marin et al., 2008).

However, contamination of foods with mycotoxins cannot be completely avoided.

Therefore, pre-harvest and post-harvest preventive strategies have to be supplemented with decontamination/detoxification approaches.

Processes to reduce the presence of mycotoxins should be able to:

- destroy, inactivate or remove mycotoxins;
- not produce or leave toxic, carcinogenic or mutagenic residues in the final products;
- not adversely affect the desirable physical and sensory properties of the product;

- destroy fungal spores and the mycelium in order to avoid mycotoxin formation under favourable conditions;

These decontamination methods can be classified as physical (e.g. cleaning, mechanical sorting, filtration, heat treatment, ultrasonic treatment and irradiation), physicochemical (e.g. adsorbing agents), chemical (e.g. acids, bases, oxidizing agents, chlorinating or reducing agents, salts, formaldehyde) and biological (e.g. microbes and their enzymes).

Nevertheless, chemical decontamination and mycotoxin dilution by mixing batches of product are specifically banned by European Commission (EC, 2006). Besides, detoxification is less effective and sometimes restricted because of concerns of safety, possible losses in nutritional quality of the treated commodities and cost implications (Yang et al., 2014).

In addition, in order to protect consumers' health, many countries have also implemented legislation that imposes limits on the presence of major mycotoxins in commodities. The number of countries with specific regulation is increasing over the years. However, the limits can change according to countries as a result of their stage of development, different perceptions about the levels considered safe for health, or economic interests related to local cultures (van Egmond and Jonker, 2008).

The European Union (EU) has one of the highest food safety standards in the world, largely thanks to the solid set of EU legislation in place, which ensures that food is safe for consumers. The European Directive n° 1881/2006 is the landmark regulation in the field of food safety establishing the admissible limits for approximately 40 toxins/food commodities combination. With respect to feed, the only mycotoxin regulated to date is aflatoxin B_1, and its maximum value can be found in the Directive 2002/32/EC (EC, 2006b). The Commission Recommendation 2006/576/EC (EC, 2006c), established the limits for the other mycotoxins (DON, ZEA, OTA, T-2, HT-2, FBs) in feed.

A key tool to ensure the flow of information to enabling swift reaction when risks to public health are detected in the food chain is RASFF – the Rapid Alert System for Food and Feeds. Created in 1979, RASFF enables information to be shared efficiently between its members and provides a round-the-clock service to ensure that urgent notifications are sent, received and responded to collectively and efficiently.

RASFF notifications can be distinguished in:

- **Alerts:** foods/feeds present a serious health risk on the market and rapid actions are required;

- **Border rejections:** food/feeds are rejected at the external borders of the EU when a health risk has been found;
- **Information notification:** foods/feeds not highly risky which are i) on the market only in the notifying country ii) no more on the market iii) expired (***Information notification for attention***); foods/feeds products not highly risky that are on the market or may be introduced in another EU country (***Information notification for follow-up***).

Data retrieved from the RASFF web portal showed that a total of 3949 notifications related to both food/feed safety concerns were registered in the last year (2018-2019). Mycotoxins represented the second hazard category in number of total notifications received (566, 14.33%) after pathogenic microorganisms (903, 22.86%). Figure 1 shows the prevalence of notifications associated to the presence of mycotoxins both in food and feed products. Aflatoxins were the primary mycotoxins whose presence was notified mostly in nuts, nut products and seeds followed by ochratoxin A, mainly found in fruits and vegetables.

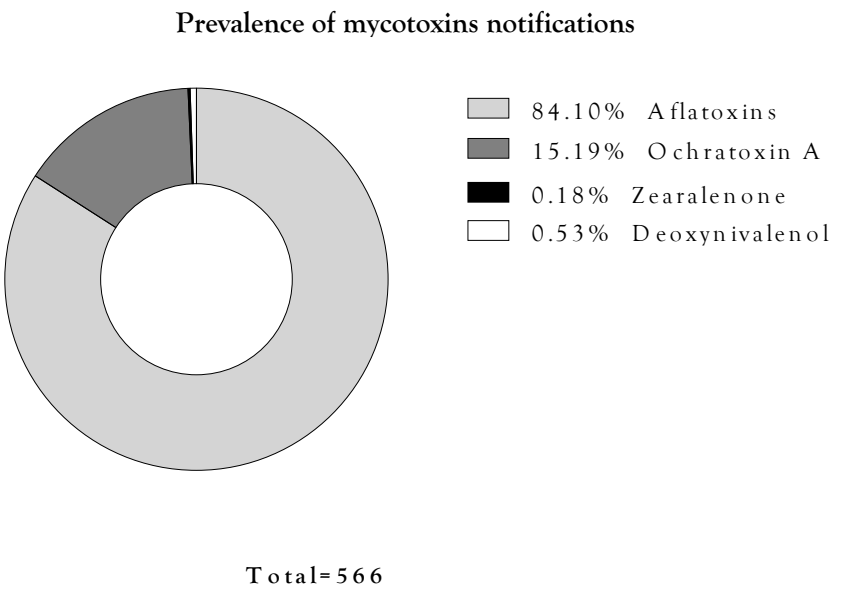
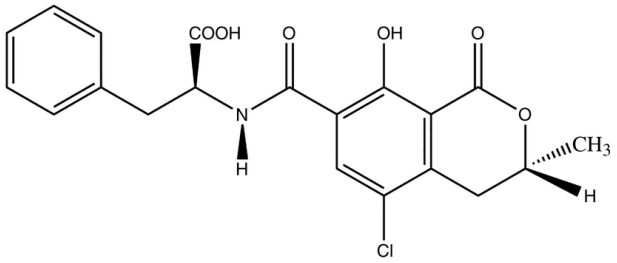


Figure 1. Prevalence of mycotoxin notifications during 2018-2019 (RASFF, 2019).

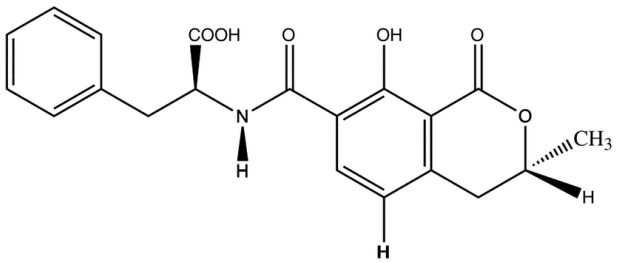
2. OCHRATOXIN A

2.1. Physicochemical properties

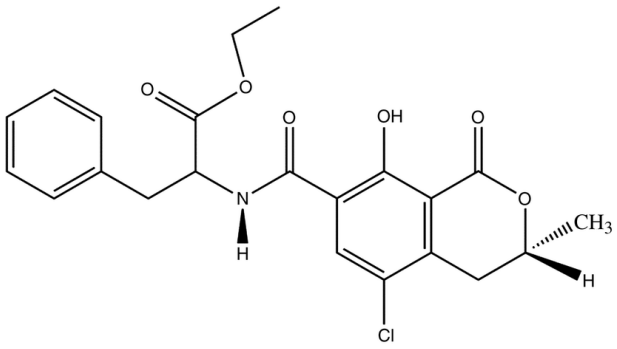
Ochratoxin A (OTA) is, among the small group of ochratoxins, the most important compound in terms of toxicity and economic losses determined by its presence in foodstuffs (Malir et al., 2016). Its chemical structure consists of a dihydroisocumarin moiety coupled to L-β-phenylalanine. Other metabolites related to OTA are the dechloro analogue ochratoxin B (OTB), its ethyl ester ochratoxin C (OTC), and the hydroxylated forms (Figure 2). OTα and OTβ are products of the hydrolysis of the peptide bond of OTA and OTB, respectively, and lack the phenylalanine moiety.



Ochratoxin A



Ochratoxin B



Ochratoxin C

Figure 2. Structure of main ochratoxins

The systematic chemical IUPAC name for OTA is (2S)-2-{[(3R)-5-chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydroisochromene-7-carbonyl] amino}-3-phenylpropanoic acid. OTA is a crystalline compound varying from colourless to white, intensely fluorescent in ultraviolet light, emitting green and blue fluorescence in acid and alkaline solutions, respectively (IARC, 1993; Budavari, 1989). It is moderately soluble in organic solvents (e.g. chloroform, ethanol, methanol, xylene) slightly soluble in water and insoluble in petroleum ethers and saturated hydrocarbons (WHO, 1990). OTA is unstable to air and light, though ethanol solutions are stable for longer than one year if refrigerated and in the dark. OTA is partially degraded under cooking conditions (Müller, 1982). Solutions of OTA are completely degraded by treatment with an excess of sodium hypochlorite solution (Castegnaro et al., 1991).

2.2. Ochratoxigenic fungi and OTA occurrence in foodstuffs

OTA was first isolated as a secondary metabolite from *Aspergillus ochraceus* in 1965 (Van der Merwe et al., 1965). Since then, several OTA producing species of *Aspergillus* and *Penicillium* genera have been discovered to differently contaminate a variety of food commodities depending on climatic conditions. Indeed, *Aspergillus* species predominate in warm and temperate regions while *Penicillium* isolates are frequent in colder areas (Malir et al., 2016).

The genus *Aspergillus* includes the major number of OTA producing species (currently 27); in *Penicillium* three species are confirmed as OTA producers, namely: *P. nordicum*, *P. verrucosum* and the recently described *P. thymicola* (Visagie et al., 2014; Varga et al., 2011; Nguyen et al., 2016).

OTA-producing *Aspergillus* species belong to the sections *Circumdati* (also called *Aspergillus ochraceus* group) and *Nigri*, with only two minor producing species in Sec. *Flavi*.

In Sec. *Circumdati*, eleven species produce large amounts of OTA with the most important being *A. ochraceus*, *A. westerdijkiae* and *A. steynii*, mainly responsible for OTA contamination of coffee, rice, cocoa, beverages and other foodstuffs (Visagie et al., 2014).

OTA producing fungi belonging to the section *Nigri*, also named "black Aspergilli", have a significant impact on modern society as they cause food spoilage, but they are also used in biotechnology for the production of (extracellular) enzymes, organic acids, vitamins and antibiotics applied in food fermentations (Perrone & Gallo, 2017). Taxonomy and classification in the *Nigri* section is challenging and has changed over the last decades, but a polyphasic approach suggested by Samson et al., 2014 based on the combination of morphology, electrolytes and phylogenetic analyses has currently resulted in 28 species with the recently discovered *A.*

labruscus within the *A. homomorphus* clade and *A. serratalhadensis* in the *A. aculeatus clade* (Fungaro et al., 2017; Crous et al., 2018).

A. carbonarius is the main responsible for OTA contamination of wine, grape derived products and raisins; followed by *A. niger* and its cryptic sister species *A. welwitschiae* (Perrone et al., 2007). Among the two OTA producing species of the *Flavi* group, only *A. alliaceus* has been rarely isolated in figs and tree nuts in California (Bayman et al., 2002).

Within the *Penicillium* genus, *P. verrucosum* and *P. nordicum* are the two most OTA producing species, with the former predominantly contaminating grains while the latter has been mainly found in cheese and fermented meats (Visagie et al., 2014; Varga et al., 2011). Recently, *P. thymicola* has been identified as a new specie evolutionary related to *P. nordicum* and *P. verrucosum*, isolated from thyme, soil and sorghum grain, while the specific strain studied for OTA production has been isolated from cheddar cheese (Nguyen et al., 2016). In 2017, the production of OTA was claimed from new species of *Penicillium* (*P. commune* and *P. rubens*), *Aspergillus* (*A. aculeatus*) and *Talaromyces* (*T. rugulosus*) (Zhang et al., 2016) however these data were commented and needed further confirmation that has not yet been published (Perrone et al., 2017; Zhang et al., 2017).

Based on the EU Rapid Alert System for Food and Feed (RASFF) web portal, in the last year (October 2018 - October 2019), the presence of OTA in foodstuff has received 86 notifications of which 61.62% border rejections, 26.74% alerts and 10.46% information (6.97% information for attention and 3.48% information for follow-up).

Table 3 shows the relation between the occurrence of OTA in food products and the type of notifications received. Based on these data, fruits and vegetables were the food product category with the highest number of notifications (44: border rejections, 10: alerts, 4: information).

Table 3. OTA notifications by food products

	Alert	Border Rejection	Information for attention	Information for follow-up
Fruits and vegetables	10	44	3	1
Cereals and bakery products	4		1	2
Herbs and spices	5	1	2	
Nuts, nuts products, seeds	2	7		
Other*	3	1		
	24	**53**	**6**	**3**

*Other=dietetic foods, food supplements, fortified foods and other food products/mixed

Based on RASFF, 2018-2019

2.3. OTA toxicity

Nephropathy is the major toxic effect of OTA. Several epidemiological studies on animals showed that OTA plays a key role in the aetiology of porcine and poultry nephropathy. In addition, this mycotoxin was also associated with human nephropathy and it is suspected to be the cause of the human fatal disease known as Balkan Endemic Nephropathy (BEN), a chronic disease affecting the south-eastern population of Europe (Pfohl-Leszkowicz et al., 1998; Fuchs et al., 2005). It is also considered to be the major cause of the Tunisian Nephropathy (TCIN) (Maaroufi et al., 1995; Grosso et al., 2003).

Based on a number of carcinogenicity studies, some of which were life-long studies, supported by the International Agency for Research on Cancer (IARC, 1993) and the National Institute of Health (NIH) within the framework of the National Tumor Programs (Boorman, 1989), ochratoxin A has been classified as a group 2B carcinogen. This means that the substance is carcinogenic to animals and possible carcinogen to humans. At present, new information regarding genotoxicity of OTA (formation of OTA-DNA adducts), its role in oxidative stress, the identification of epigenetic factors involved in OTA carcinogenesis, the synergistic action of diverse mycotoxins (e.g. OTA and citrinin, reported by Kanisawa,1984; Sandor et al.,1991), the immunosuppressor effect (Creppy et al., 1991) could lead to a reclassification of OTA in the direction of group 2A (probably carcinogenic to humans) (IARC,2006) or even to group 1 (carcinogenic to humans).

2.4. Biosynthetic pathway and regulatory mechanisms

Unlike to other important mycotoxins, the biosynthetic pathway of OTA has not been completely elucidated yet. However, it is known that the pathway involves some crucial steps, such as the biosynthesis of the isocoumarin group through the catalysing action of a polyketide synthase (PKS), its ligation with the amino acid phenylalanine in a reaction catalysed by a peptide synthase and the chlorination step. In this regard, several schemes have been proposed. Initially, Huff and Hamilton (1979) suggested that the mellein, catalysed by a polyketide synthase, was oxidized to OTβ and then transformed to the chlorinated analogue OTα by a chloroperoxidase. Subsequently, OTα is converted to OTC by a peptide synthase that links the phenylalanine ethyl ester to OTα. Finally, a de-esterification reaction transforms OTC in OTA. In this scheme, the putatively ubiquitous intermediate OTB its not considered.

Harris and Mantle (2001), proposed a different pathway which involves the passage from OTβ to OTα and then to OTA, with a chlorinating step prior to the ligation of the polyketide (OTα) to phenylalanine. Moreover, they proposed an alternative pathway in which the formation of OTA goes through the synthesis from OTβ to OTB, but in this case, a biosynthetic role for OTα could not be explained. Availability of genome sequence of *A. carbonarius* allowed the identification of key OTA biosynthetic genes. Gallo et al., 2012, proposed that ochratoxin β and L-phenylalanine were converted to OTB by a non-ribosomal peptide synthetase (NRPS) and that the OTB was transformed to OTA through chlorination in *Aspergillus carbonarius*. This study provided the first evidence that in *A. carbonarius*, the addition of phenylalanine to polyketide dihydroisocoumarin precedes the chlorination step to form OTA and that ochratoxin α is a product of hydrolysis of OTA. Subsequently, a gene encoding for a polyketide synthase (PKS) within the putative OTA cluster of *A. carbonarius* has been identified (Gallo et al., 2014). Sequencing of the genomic region of *A. carbonarius* carrying the *pks* and *nrps* genes involved in OTA biosynthesis allowed the identification of a gene encoding a putative flavin-dependent halogenase, which is involved in the final stage of the biosynthetic pathway with a chlorination step which convert OTB to OTA (Ferrara et al, 2016). These studies importantly contributed to elucidate the order of the enzymatic steps of OTA biosynthesis in *A. carbonarius* (Figure 3).

Other studies reported the identification of OTA biosynthetic genes in other ochratoxigenic species. Wang et al., 2015 characterized two polyketide synthases (PKSs) involved in OTA biosynthesis in *A. ochraceus.* Farber and Geisen (2004) demonstrated the potential role of a putative halogenase through the analysis of differentially expressed OTA biosynthesis genes in

Penicillium nordicum. Recently, Wang et al. (2018) demonstrated the involvement of P450 monooxygenase and bZIP transcription factor identified in the OTA cluster by gene inactivation giving evidence of the presence of five genes in all the sequenced genomes of OTA producing species.

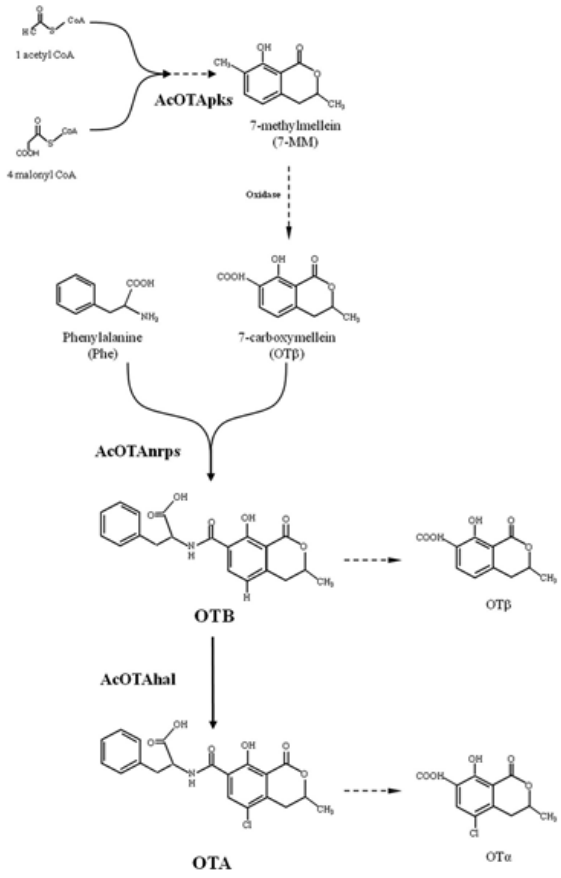


Figure 3. Schematic representation of the order of OTA biosynthetic pathway and the enzymes involved in *A. carbonarius* (Ferrara et al., 2016).

Several studies reported the existence of one or more regulatory specific genes playing a key role in controlling the expression of aflatoxins (Payne et al., 1993; Fernandes et al., 1998), fumonisins (Brown et al., 2007), zearalenone (Park et al., 2015) and tricothecenes (Merhej et al., 2011; Peplow et al., 2003; Tag et al., 2001; Proctor et al., 1995; Tannous et al., 2014) biosynthetic pathways. Very few evidences are reported about OTA biosynthetic pathway-specific regulators (Bacha et al., 2012; Wang et al., 2018, Gallo & Perrone, 2017).

In addition to specific regulatory mechanisms, biosynthesis of secondary metabolites is also responsive to environmental factors that could be under the control of general regulatory genes located outside the cluster. In *A. carbonarius*, OTA production is influenced by carbon and

nitrogen sources (Abbas et al., 2009; Ferreira and Pitout, 1969; Medina et al., 2008) and pH signaling by PacC (Tilburn et al., 1995). Recently, the heterotrimeric velvet complex VelB/VeA/LaeA that couples in *A. nidulans* secondary metabolism with fungal development, including asexual and sexual growth in response to light, was identified (Bayram et al., 2008) (Figure 4). VeA is a global regulator that, under dark conditions, is transported from cytoplasm to nucleus where it reacts with LaeA, regulating production of secondary metabolites and with VelB, inducing sexual development (Palmer et al. 2013). In *A. carbonarius*, Crespo-Sempere et al. (2013) found that the inactivation of VeA and LaeA caused a reduction of both OTA and conidial production showing their important contribute to the regulation of important biological processes in this fungus. Furthermore, in wild-type strain of *A. carbonarius*, they revealed that OTA production was promoted by darkness while conidiation was enhanced by light conditions.

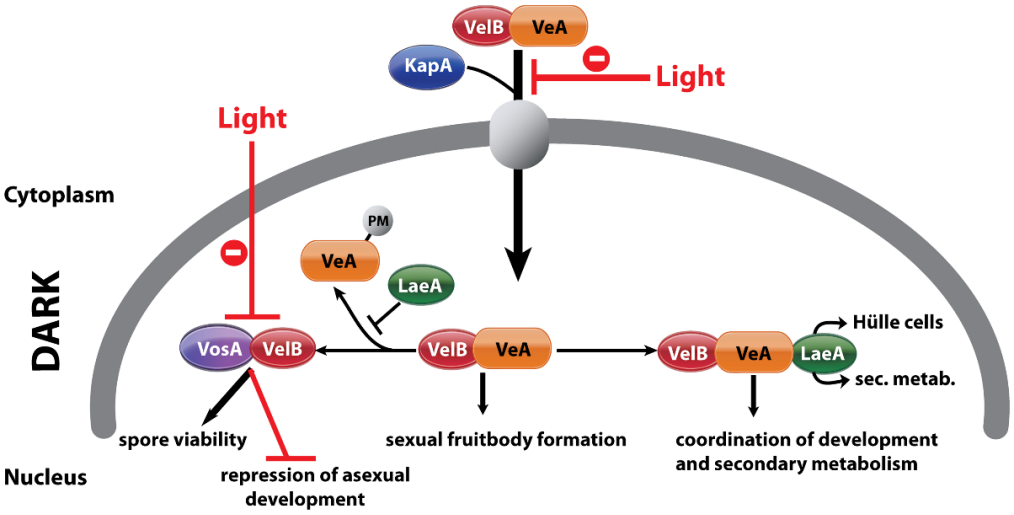


Figure 4. Representation of velvet complex proteins (Bayram et al., 2010).

2.5. Control strategies and regulations

As already described in the general discussion on the mycotoxigenic risk, OTA controlling strategies are primarily based on preventive measures (e.g. GAP, fungal resistant crop varieties, correct application of fungicides, proper storage of commodities) that could, ideally, be part of a Hazard Analysis Critical Control Point (HACCP) scheme. However, fully implemented HACCP schemes are rare and when the individual measures fail or are not in place, OTA remains in food and feed products. Decontamination/detoxification procedures can be used to remove or to reduce OTA levels. These measures, which are technologically diverse, are usually classified into physical (e.g. segregation, sorting, cleaning, peeling and shelling processes, adsorbents), chemical (e.g. ammoniation, nixtamalization, ozonation) and biological (e.g. microorganisms, enzymes) (Amezqueta et al., 2009). Chemical methods are reported generally as effective in the elimination

of OTA and other mycotoxins (Riley et al., 1999) although the toxicological safety of the final products is not always guaranteed since some chemical residues may remain in products and the toxicity of the reaction products formed is not usually studied. Biological methods are the technologies of choice for decontamination purposes because they present several advantages from being mediated by enzymatic reactions. For example, they are very specific, efficient, environmentally friendly and they preserve the nutritive quality of food products. To assess the non-pathogenicity of the microorganism and the non-toxicity of the reaction products formed is essential (Karlovsky, 1999). Numerous microorganisms and/or their enzymes are capable of degrading, adsorbing and detoxifying OTA (Chen et al., 2018). One of the most common strategy to biodegrade OTA consists in the application of proteolytic enzymes. This process assumes two possible mechanisms. First, OTA can be biodegraded through the hydrolysis of the amide bond that links the L-β-phenylalanine molecule to the OTα moiety (Figure 5a). Since OTα and L-β-phenylalanine are not toxic, this mechanism can be considered as a possible detoxification pathway. Second, a more hypothetical process involves OTA being degraded via the hydrolysis of the lactone ring (Figure 5b). In this case, the final degradation product is an opened lactone form of OTA, which is of similar toxicity to OTA when administered to rats (Xiao et al., 1996; Li et al., 1997).

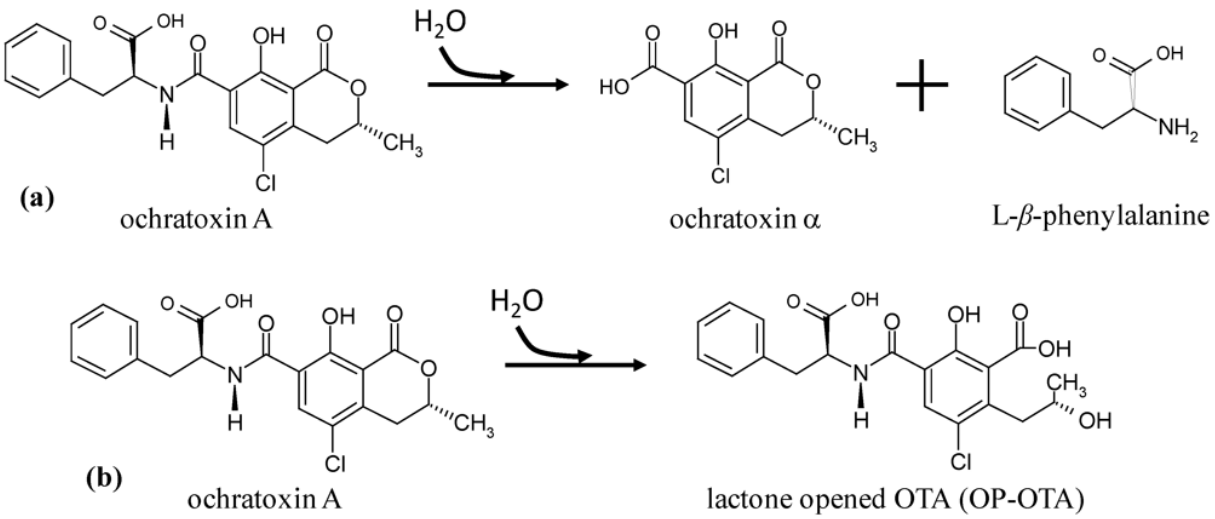


Figure 5. Ochratoxin A biodegradation pathways. **a)** Amide bond hydrolysis of OTA; **b)** Lactone ring hydrolysis of OTA (Abrunhosa et al., 2010).

The toxicity of OTA became more evident by the end of the 1970s although a real debate on whether OTA in food and feed shall be regulated on a national and international level does not seem to predate the 1990s. Worldwide surveys reported that the number of countries adopting

regulations for OTA levels increased from 11 in 1991 (Van Egmond et al., 1991) to 37 in 2003 (FAO,2003). No such large-scale survey has been reported ever since (Duarte et al.,2010). At the European level, limits on OTA in food were first established by the Commission Regulation (EC) n° 472/2002 amending Regulation (EC) n°466/2001 which was then replaced by the Commission Regulation (EC) n° 1881/2006, which is still in force today. The Regulation n°1881/2006 sets maximum limits on OTA in a variety of foods (Table 4). These limits are legally binding on all the EU Member States, which are obliged to completely apply these rules. Moreover, the Commission Recommendation 2006/576/EC (EC, 2006c) sets non-binding limits for the presence of OTA, deoxynivalenol, zearalenone, T-2 and HT-2, fumonisins in products intended for animal feeding.

Table 4. Maximum limits of OTA in foodstuffs in the EU

Foodstuff	**Maximum level ($\mu g \cdot kg^{-1}$)**
Dietary foods for special medical purposes intended specifically for infants	0.5
Green coffee, dried fruit other than dried vine fruit, beer, cocoa and cocoa products, liqueur wines, meat products, spices and liquorice	0.5
Wine	2
Processed cereal-based foods and baby foods for infants and young children	2
All products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption	3
Unprocessed cereals	5
Roasted coffee beans and ground roasted coffee, excluding soluble coffee	5
Wheat gluten not solid directly to the consumer	8
Dried vine fruits (currants, raisins and sultanas)	10
Soluble coffee (instant coffee)	10
Various species of spices	15

Commission Regulation (EC) n° 1881/2006

3. OTA RISK IN THE GRAPE-WINE FOOD CHAIN

3.1. Black Aspergilli and OTA occurrence in vineyards

Since the discovery of Ochratoxin A in wines in 1996, its presence in imported and locally produced wines has been reported from a number of European and other countries (see Table 5). Recently and for the first time, high levels of OTA (>2 μg·L^{-1}) have been also detected in USA wines (De Jesus et al., 2018).

Table 5. Summary of highly OTA contaminated wines (>2 μg·L^{-1}).

Region	#wines (%) >2 μg·L^{-1}	References
North America		
USA	2/41 (4.9%)	De Jesus et al.,2018
Europe		
Italy	6/55 (10.9%)	Visconti et al., 1999
	9/112 (8.0%)	Perrone et al., 2007
	22/783 (2.8%)	Brera et al., 2008
	29/1206 (2.4%)	Spadaro et al., 2010
Greece	1/105 (<1%)	Labrinea et al., 2011
	3/35 (8.6%)	Soufleros et al., 2003
Spain	18/188 (9.6%)	Burdaspal et al., 2007
Turkey	1/47 (2.1%)	Anli et al., 2005
Portugal	1/60 (1.7%)	Pena et al., 2010
South America		
Argentina	3/47 (6.4%)	Ponsone et al., 2010
Africa		
Morocco	1/30 (3.3%)	Filali et al., 2001

Based on De Jesus et al., 2018

It is widely known that OTA contamination in wine is closely related to fungal colonization in the vineyards.

Aspergillus section *Nigri*, also called black Aspergilli, are the primary aetiological agent of the black rot of grapes. The disease appears on the berries as a black rot due to fungal sporulation which consumes the berries making them completely shrunken and dry (Figure 6). Colonies of these fungi are present from fruit setting and increase in amount from early veraison to harvest. However, the incidence of colonised berries is highly related to climatic conditions during the ripening stage and to the geographical area (Cozzi et al., 2007; Visconti et al., 2008). The principal pathway of infection for black Aspergilli is damage to berry skins, caused by many factors including fungal diseases (downy mildew, powdery mildew), pests (grape berry moth *Lobesia*

Botrana, bunch mites) and environmental factors (wind, hail, rain or sunburn injury, berry splitting). This disease has received more attention since it has been associated with contamination of grapes and grape derived products by OTA. *A. carbonarius,* is the major ochratoxigenic fungal species, indeed almost 100% of the strains of this specie have been shown to produce OTA (Somma et al., 2012). Other Black Aspergilli occurring on grapes are *A. japonicus*, *A. uvarum*, *A. aculeatus* belonging to the *A. aculeatus* clade (uniseriate group). However, none of them has been reported as OTA producer. In addition, within the *A. niger* "aggregate" clade (biseriate), *A. niger*, *A. welwitschiae*, *A. tubingensis,* have been frequently found on grapes with a low percentage of OTA-producing strains. To a lesser extent, *A. brasiliensis*, *A. luchuensis (A. foetidus=A. acidus)* have been also reported to be grape colonizers although they are never reported as OTA producing species.

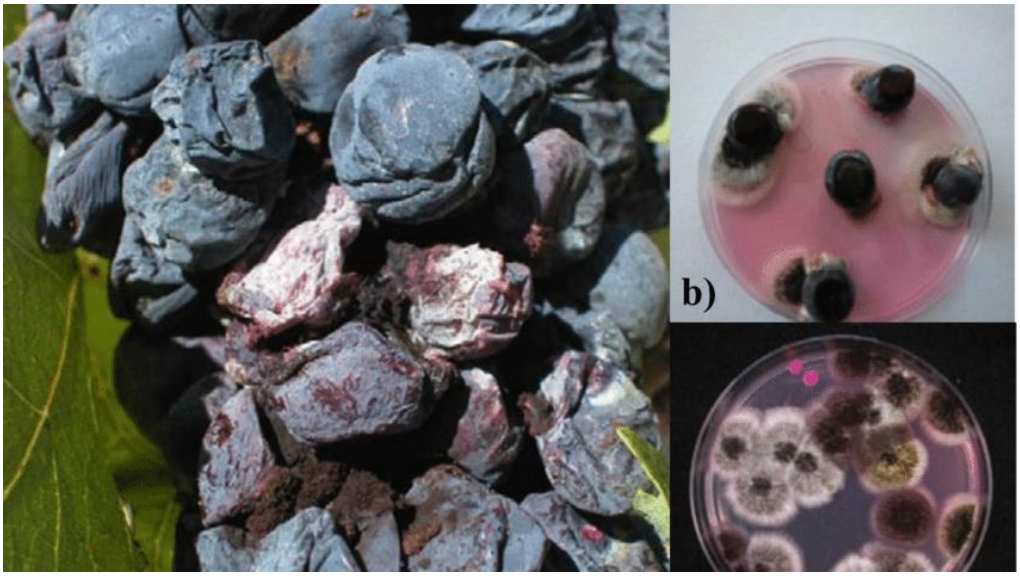


Figure 6. Black aspergilli on grapes: A) black rot of berries caused by black aspergilli; B) direct plating of berries on DRBC agar plates; C) different black *Aspergilli* colonies from berries' homogenate diluted and plated on DRBC (Somma et al., 2012).

3.2. Management of ochratoxin A risk in grapes

Since OTA represents the major mycotoxin risk in the grape production chain, several systems could be applied to reduce its presence at different levels in the grape-wine production chain.

- **In the vineyard**

In the vineyards the best way to reduce OTA production is to control the presence of ochratoxigenic fungi. The appliance of good agricultural practices (GAP), such as keeping constant the moisture content of the soil and limit/avoid tillage, in particular from veraison to harvest (Leong et al., 2006b) is always recommended. It is also useful to avoid excess vigour and vegetative growth and to promote the aeration of bunches. Black aspergilli colonization can be controlled by applying 1-2 chemical treatments. The mixture cyprodinil/fludioxonil is the most effective combination of antifungal compounds (Tjamos et al., 2004; Belli et al., 2007) especially when applied at 21 days before harvesting in high risk conditions. This treatment, in the same dosage and timing, is effectively used against the grey mould caused by *Botrytis cinerea*. Moreover, insecticide treatment against *L. botrana* in combination with the fungicide contributes significantly to the reduction of OTA level in the field, especially in crop years of high contamination risk (Cozzi et al., 2007). Promising results were also obtained using biological control strategies, such as yeasts (Dimakopoulou et al., 2005; Bleve et al., 2006). However, in high conducive years (high rains prior harvest and high temperature) and in high risk area (i.e. South Apulia) it is difficult to counteract the risk of contamination by OTA in the field.

- **Post-harvest**

Regarding wine-making, preventive actions are to harvest early in high OTA risk areas when favourable conditions occur, segregate rotted bunches at harvest and minimize/avoid storage time before processing the grapes for wine making. In the case of table grapes, which could be subjected to fungal infection during storage, it could be useful to reduce the storage time and to discard visibly rotted bunches. In addition, it has been reported that the incidence of black aspergilli in post-harvest could be reduced with sulphur dioxide in cold storage (0°C). For dried vine fruit production, a rapid drying at greater than 30°C is recommended (Hocking et al., 2007).

- **During wine-making**

Several fining agents have been tested for their ability to remove OTA from contaminated must/wines (Castellari et al., 2001; Leong et al., 2006a), with oenological charcoal showing the highest adsorption capacity for ochratoxin A (Visconti et al., 2008). Charcoal for oenological use

has only recently been accepted as a corrective action, by using the lowest possible and most effective doses (CAC, 2007). However, the efficacy of these fining agents, charcoal in particular, is directly related to reductions in quality parameters of treated wines such the polyphenol content. In addition, effective OTA absorption was reported by using some lactic acid bacteria (Del Prete et al., 2007) during wine fermentation and several studies showed that OTA removal was also possible by using yeast strains, both dead or alive (Scott, 2008; Ciconova et al., 2010; Ponsone et al., 2011; Var et al., 2011). However, the efficacy of yeasts for OTA reduction at the industrial level has not been shown and it has been reported that they could be involved in the loss of colour of wines (Visconti et al., 2008). In addition, an innovative and environmentally friendly corrective measure to reduce ochratoxin A through repassage of contaminated musts or wines over grape pomaces in red wines has been proposed. The experiment was also tested at an industrial scale and resulted in a useful technique especially for wineries located in high risk regions for OTA contamination (Solfrizzo et al., 2009); these results lead to the registering of a Patent (Solfrizzo et al, 2019).

4. CLIMATE CHANGE: A CHALLENGE FOR THE MYCOTOXIGENIC RISK IN FOOD

The last report of the Intergovermental Panel on Climate Change (IPCC, 2014) revealed that warming of the climate system is unequivocal. Since the 1950s many of the observed changes are unprecedented over the last decades. The atmosphere and ocean have warmed, the amounts of snow and ice have diminished, the sea level has risen. Anthropogenic greenhouse gas (GHG) emissions have increased since the pre-industrial era driven largely by economic and population growth and are now higher than ever. This led to atmospheric concentrations of carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) that are unprecedented in at least the last 800.000 years.

Based on present available data, atmospheric concentrations of CO_2 are expected to double or triple (from 350-400 to 800-1200 ppm) in the next 25-50 years. Thus, different regions in Europe especially those facing the Mediterranean basin, will be impacted by the increase of temperature of 2-5°C coupled with elevated CO_2 (800-1200 ppm) and drought episodes (Bebber et al., 2013, 2014). Similar impacts have been predicted in other areas of the world, especially parts of Asia, Central and South America which are important producers of wheat, maize and soya beans for food and feed uses on global basis (IPCC, 2014).

Agriculture is highly dependent on climate variability and such changes are expected to have a profound effect on the productivity and quality of crop and livestock production systems, raising uncertainties about both food security and food safety.

Food security is determined by sufficient food availability, access to this food and quality and utilisation of the food in terms of both nutritional and cultural perspectives (FAO, 1996). Food safety is the absence, or safe, acceptable levels, of hazards in food that may harm the health of consumers. With the predicted increase of population on global scale and in the context of such climate change scenario, to produce and delivery enough and safe staple foods represent a big challenge (Battilani et al., 2012, 2016).

With respect to food safety, one of the most important hazards likely affected by climate change will be the contamination by mycotoxins. Almost all mycotoxins present in food and feeds are produced in field. Thus, their presence is strongly influenced by climatic factors.

The environmental changes occurring now are slowly shaping the relationship between plant growth and the associated fungal diseases. Indeed, the traditional and classic balanced triangle between pathogen/pest, host plant and environment (Garrett,2008; Grulke, 2011) is changing

and becoming skewed because of the relative importance and pressure of the environmental component which may shape both plant agronomy and ultimately yield of key staple foods (Pautasso et al., 2012, Figure 7). This shift in these interactions could be in a state of flux depending on the push and pull of the interacting factors and have significant impacts on the food supply chains in terms of food quality and quantity necessary to satisfy a rapidly expanding world population.

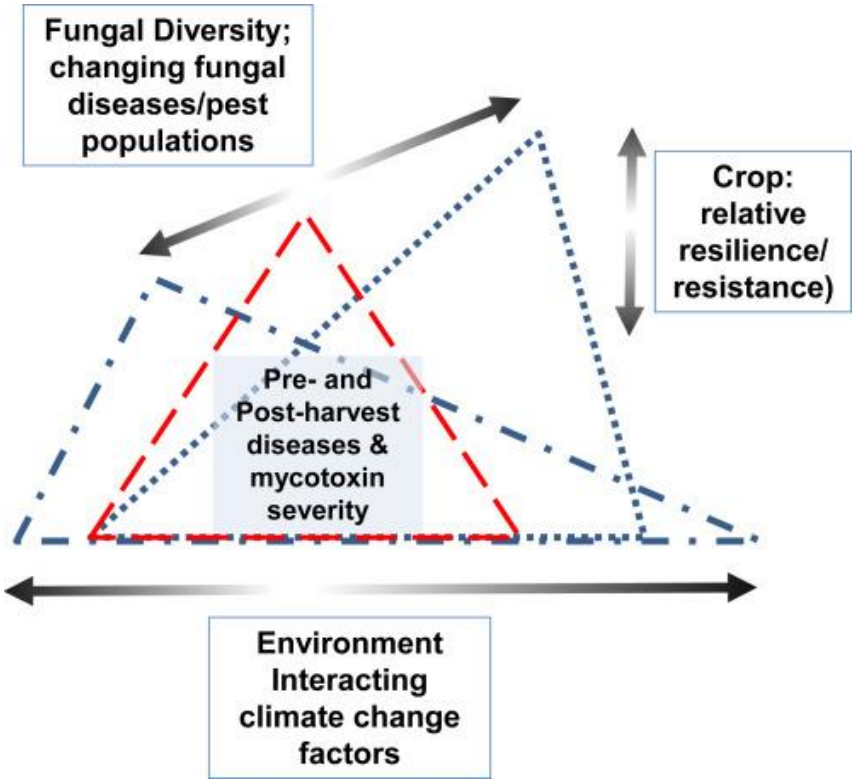


Figure 7. Climate change factors as drivers in change of the classical crop/fungal disease/environment triangle which may occur due to the pressure of climate change scenario (Medina et al., 2017).

In the context of mycotoxigenic fungal pathogens this could result in a switch from the so-called mycotoxin suppressive to conducive conditions. In addition, fungal plant pathogens are predicted to move globally and change the diversity of diseases and pests invading staple crops with both economic and social costs (Bebber & Gurr, 2015; Medina et al., 2015). Recent predictions suggest that on global scale, pests and diseases are migrating to the poles at the rate of 3-5 km/year and the diversity of pest populations will also change and have profound economic impacts on staple food production systems (Bebber et al., 2013; Bebber et al., 2014; Crespo-Perez et al., 2015).

These evidences had lay the foundations for the beginning of a new area of investigation dealing with the understanding of the impact of climate change (CC) scenarios on the most

occurring mycotoxins in the food chain. In this regard, predictive models by using historical or current climatic conditions datasets have been performed in order to estimate, in a more realistic way, the impact of CC factors in fungal colonization and mycotoxin contamination (Battilani et al., 2012, 2016; Van Der Fels-Klerx et al., 2012, 2016). With a different research approach, the effect of three-way interactions between environmental factors (temperature x water availability x CO_2) on the ecophysiology of mycotoxigenic fungi and mycotoxin accumulation, have been recently investigated by several authors (Medina et al., 2015; Vaughan et al., 2014; Marin et al., 2010; Akbar et al., 2016; Verheecke-Vaessen et al., 2019).

Considering the available information, several questions remain unanswered and research efforts are needed to improve current knowledge. Key questions include whether under CC scenarios will mycotoxin production patterns change? What is going to happen with masked mycotoxins? Are the current control/mitigation strategies going to be effective in the future? Will interactions between mycotoxigenic fungi and other microbiota in the phyllosphere and in the rhizosphere ecological niches change resulting in different community structures and dominance of different species? Will the agricultural practices, including Good Agricultural Practices (GAP) and Hazard Analysis Critical Control Point (HACCP), change in order to minimize the mycotoxin contamination when environmental shifts and climate change fluxes become the norm? (Medina et al., 2017). More research is urgently required to address these key questions to effectively predict the level of risk of different mycotoxins in economically important staple food crops and to understand whether they are resilient enough to tolerate the expected CC conditions (Medina et al., 2015).

5. REFERENCES

Abbas, A., Valez, H., Dobson, A.D.W., 2009. Analysis of the effect of nutritional factors on OTA and OTB biosynthesis and polyketide synthase gene expression in *Aspergillus ochraceus*. Int. J. Food Microbiol. 135, 22–27. https://doi.org/10.1016/j.ijfoodmicro.2009.07.014

Abia, W.A., Warth, B., Sulyok, M., Krska, R., Tchana, A., Njobeh, P.B., 2013. Bio-monitoring of mycotoxin exposure in Cameroon using a urinary multi-biomarker approach. Food Chem. Toxicol. 62, 927–34. https://doi.org/10.1016/j.fct.2013.10.003

Abrunhosa, L., Paterson, R. R., Venâncio, A., 2010. Biodegradation of ochratoxin a for food and feed decontamination. Toxins, 2(5), 1078–1099. https://doi.org/10.3390/toxins2051078

Akbar, A., Medina, A., Magan, N., 2016. Impact of climate change factors on growth and ochratoxin A production by *Aspergillus* sections *Circumdati* and *Nigri* species on coffee. World Mycotoxin J. 9, 863-874. http://dx.doi.org/10.3920/WMJ2016.2041

Amezqueta, S., Gonzalez-Penas, E., Murillo-Arbizu, M., De Cerain, A.L.,2009. Ochratoxin A decontamination: A review. Food Control. 20, 326–333. http://dx.doi.org/10.1016/j.foodcont.2008.05.017

Anli, E., Cabuk, B., Vural, N., Baspinar, E., 2005. Ochratoxin in Turkish wines. J. Food Biochem. 29, 611–623. https://doi.org/10.1111/j.1745-4514.2005.00043.x

Bacha, N., Mathieu, F., Liboz, T., Lebrihi, A., Bacha, N., Mathieu, F., Liboz, T., Lebrihi, A., 2012. Polyketide synthase gene *aolc35-12* controls the differential expression of ochratoxin a gene *aoks1* in *Aspergillus westerdijkiae*. World Mycotoxin J. 2012, 5, 177–186. https://doi.org/10.3920/WMJ2011.1374

Barkai-Golan, R., Paster, N., 2008. Mouldy fruits and vegetables as a source of mycotoxins: Part 1. World Mycotoxin J. 1, 147–159. https://doi.org/10.3920/WMJ2008.x018.

Battilani, P., Rossi, V., Giorni, P., Pietri, A., Gualla, A., Van der Fels-Klerx, H.J., Booij, C.J.H., Moretti, A., Logrieco, A., Toscano, P., Miraglia, M., De Santis, B., Brera, C., 2012. Modelling, predicting and mapping the emergence of afla- toxins in cereals in the EU due to climate change. Scientific/- Technical Report submitted to EFSA. Available at: http: //www.efsa.europa.eu/en/supporting/pub/223e.htm.

Battilani, P., Toscano, P., Van der Fels-Klerx, H.J., Moretti, A., Camardo Leggieri, M., Brera, C., Rortais, A., Goumperis, T., Robinson, T., 2016. Aflatoxin B1 contamination in maize in Europe increases due to climate change. Sci. Rep. 6, 24328. http://dx.doi.org/10.1038/srep24328

Bayman, P., Baker, J.L., Doster, M.A., Michailides, T.J., Mahobey, N.E., 2002. Ochratoxin production by the *Asergillus ochraceus* group and *Aspergillus alliaceus*. Appl. Environ. Microbiol. 68, 2326-9. https://doi.org/10.1128/aem.68.5.2326-2329.2002

Bayram, Ö., Krappmann, S., Ni, M., Bok, J., Helmstaedt, K., Valerius, O., Braus-Stromeyer, S., Kwon, N.J., Keller, N., Yu, J.H., Braus, G., 2008. VelB/VeA/LaeA Complex Coordinates Light Signal with Fungal Development and Secondary Metabolism. Science. 320, 1504-6. https://doi.org/10.1126/science.1155888

Bebber, D.P., Gurr, S.J., 2015. Crop-destroying fungal and oomycete pathogens challenge food security. Fungal Genet. Biol. 74, 62-64. https://doi.org/10.1016/j.fgb.2014.10.012

Bebber, D.P., Holmes, T., Gurr, S.J., 2014. The global spread of crop pests and pathogens. Glob. Ecol. Biogeogr. 23, 1398e1407 https://doi.org/10.1111/geb.12214

Bebber, D.P., Ramotowski, M.A.T., Gurr, S.J., 2013. Crop pests and pathogens move polewards in a warming world. Nat. Clim. Change 3, 985e988. https://doi.org/10.1038/nclimate1990

Bellí, N., Marín, S., Argiles, E., Ramos, A.J., Sanchis, V.. 2007. Effect of chemical treatments on ochratoxigenic fungi and common mycobiota of grapes (Vitis vinifera). J. Food Prot. 70, 157–163.https://doi.org/10.4315/0362-028x-70.1.157

Benassi, F., El Golli-Bennour, E., Abid-Essefi, S., Bouaziz, C., Hajlaoui, M.R., Bacha, H., 2009. Pathway of deoxynivalenol-induced apoptosis in human colon carcinoma cells. Toxicology. 264(1-2),104-9. https://doi.org/10.1016/j.tox.2009.07.020

Bennett J.W., Klich, M., 2003. Mycotoxins. Clin Microbiol Rev.16(3), 497–516. https://doi.org/10.1128/cmr.16.3.497-516.2003

Bleve, G., Grieco, F., Cozzi, G., Logrieco, A., A., Visconti, 2006. Isolation of epiphytic yeasts with potential for biocontrol of *Aspergillus carbonarius* and *A. niger* on grape. Int. J. Food Microbiol. 108, 204–209. https://doi.org/10.1016/j.ijfoodmicro.2005.12.004

Blount, W. P., 1961. Turkey 'X' Disease, Turkeys, 9 (7), 52-61

Boorman, G., Ed.; NTP Technical Report on the Toxicology and Carcinogenesis Studies of Ochratoxin A (CAS No. 30-47-9) in F344/N Rats (Gavage Studies), NIH Publication No. 89-2813; U.S. Department of Health and Human Services, National Institutes of Health: Research Triangle Park, NC, USA, 1989

Bottalico, A., Logrieco, A., 1998. Toxigenic *Alternaria* species of economic importance. In: Sinha KK and D Bhatnagar (eds) Mycotoxins in Agriculture and Food Safety (pp 65–108) Marcel Dekker, Inc, New York

Bräse, S., Encinas, A., Keck, J., Nising, C.F., 2009. Chemistry and biology of mycotoxins and relaed fungal metabolites. Chem. Rev. 109, 3903 – 3990. https://doi.org/10.1021/cr050001f

Brera, C., Debegnach, F., Minardi, V., Prantera, E., Pannunzi, E., Faleo, S., de Santis, B., Miraglia, M., 2008. Ochratoxin A contamination in Italian wine samples and evaluation of the exposure in the Italian population. J. Agric. Food Chem. 56, 10611–10618. https://doi.org/10.1021/jf8016282

Brown, D., Butchko, R.M., Proctor, R., 2007. The *Fusarium verticillioides* FUM gene cluster encodes a Zn(II)2Cys6 protein that affects FUM gene expression and fumonisin production. Eukaryot. Cell. 6, 1210–1218. https://doi.org/10.1128/EC.00400-06

Budavari, S., The Merck Index, 11th ed.; Merck & Co.: Rahway, NJ, USA, 1989; p. 1068

Burdaspal, P., Legarda, T., 2007. Occurrence of ochratoxin A in sweet wine produced in Spain and other countries. Food Addit. Contam. 24, 976–986. https://doi.org/10.1080/02652030701311155

Castegnaro, M., Canadas, D., Vrabcheva, T., Petkova-Bocharova, T., Chernozemsky, I.N., Pfhol-Leszkowicz, A., 2006. Balkan endemic nephropathy: role of ochratoxins A through biomarkers. Mol. Nutr. Food Res. 50, 519-529. https://doi.org/10.1002/mnfr.200500182

Castellari, M., Versari, A., Fabiani, A., Parpinello, G.P., Galassi, S., 2001. Removal of ochratoxin A in red wines by means of adsorption treatments with commercial fining agents. J. Agric. Food Chem. 49, 3917–3921. https://doi.org/10.1021/jf010137o

Chen, W., Li, C., Zhang, B., Zhou, Z., Shen, Y., Liao, X., Yang, J., Wang, Y., Li, X., Li, Y., Shen, X.L. 2018. Advances in Biodetoxification of Ochratoxin A-A Review of the Past Five Decades. Front. Microbiol. 9,1386. https://doi.org/ 10.3389/fmicb.2018.01386

Chu, F. S., Mycotoxins and alimentary mycotoxicoses. Claridge, M. F., Dawah, H. A., and Wilson M. R. (Eds.) (1997). Species: The Units of Biodiversity. Chapman and Hall, London

Cicoňova, P., Laciakova, A., Mate, D., 2010. Prevention of ochratoxin a contamination of food and ochratoxin a de- toxification by microorganisms – a review. Czech J. Food Sci. 28, 465–474 http://dx.doi.org/10.17221/307/2009-CJFS

Codex Alimentarius Commission (CAC), 2007. Code of Practice for the Prevention and reduction of ochratoxin a Contamination in Wine. Codex Committee on Contaminants in Foods. CAC/RCP 63-2007. FAO/WHO Joint Publications, January 2007. Available at: http://www.codexalimenta- rius.net/download/standards/10750/CXP_063e.pdf

Codex Alimentarius Commission (CAC), 2003. Code of practice for the prevention and reduction of mycotoxin contamination in cereals, including annexes on ochratoxin A, zearalenone, fumonisins and tricothecenes.

Cozzi, G., Perrone, G., Epifani, F., Pascale, M., Visconti, A.. 2007. Epidemiology of ochratoxin A producing fungi in Apulian vineyards. Poster 1422r presented at XII International IUPAC Symposium on Mycotoxins and Phycotoxins, May 21–25; Istanbul

Creppy, E.E., Betbeder, A.M. Gharbi, A., Counord, J., Castegnaro, M., Bartsch, H., Moncharmont, P., Fouillet, B., Chambon, P., Dirheimer, G., 1991. Human Ochratoxicosis in France; IARC Sci. Publ. Lyon, France,115,131–134.

Crespo-Perez, V., Regniere, J., Chuine, I., Rebaudo, F., Dangles, O., 2015. Changes in the distribution of multispecies pest asemblages affect levels of crop damage in warming tropical Andes. Glob. Change Biol. 21, 82-96. https://doi.org/10.1111/gcb.12656

Crespo-Sempere, A., Marín, S., Sanchis,V., Ramos, A.J., 2013. VeA and LaeA transcriptional factors regulate ochratoxin A biosynthesis in *Aspergillus carbonarius*. Int. J. Food Microbiol. 166,479-486. https://doi.org/10.1016/j.ijfoodmicro.2013.07.027.

Crous, P., Wingfield, M.J., Burgess, T., Hardy, G., Gené, J., Guarro, J., Baseia, I., García, D., Gusmao, L., Souza-Motta, C., Thangavel, R., Adamčík, S., Barili, A., Barnes, C., Bezerra, J., Bordallo, J.J., Cano, J., Oliveira, R., Ercole, E., Groenewald, J.Z. 2018. Fungal Planet description sheets: 716–784. Persoonia - Molecular Phylogeny and Evolution of Fungi. 40. https://doi.org/10.3767/persoonia.2018.40.10

De Jesus, C. L., Bartley, A., Welch, A. Z., Berry, J. P., 2017. High Incidence and Levels of Ochratoxin A in Wines Sourced from the United States. Toxins, 10, 1. https://doi.org/10.3390/toxins10010001

Del Prete, V., Rodriguez, H., Carrascosa, A.V., de las Rivas, B., Garcia-Moruno, E., Muñoz, R., 2007. *In vitro* removal of ochratoxin A by wine lactic acid bacteria. J. Food Prot. 70, 2155–2160

Dimakopoulou, M., Tjamos, S.E., Tjamos, E.C., Antoniou, P.P., 2005. Chemical and biological control of sour rot caused by black Aspergilli in the grapevine variety agiorgitico of Korinth region. Poster presented at the International Workshop on Ochratoxin A in Grapes and Wine: Prevention and Control; Marsala (TP) Italy, 2005 October 20–21. p 77

Eskola, M., Kos, G., Elliott, C.T., Hajšlová, J., Mayar, S., Krska R., 2019. Worldwide contamination of food-crops with mycotoxins: Validity of the widely cited 'FAO estimate' of 25%, Crit. Rev. Food Sci. Nutr. 3, 1-17. https://doi.org/ 10.1080/10408398.2019.1658570

European Commission, 2006a. Commission Regulation (EC) No 1881/2006 of 19 December 2006 on setting the maximum levels for certain contaminants in foodstuffs. Off. J. Eur. Union. L364,5–24.

European Commission, 2006b. Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. Off. J. Eur. Union. L140, 10–21

European Commission, 2006c. Commission recommendation 2006/576/EC of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding. Off. J. Eur. Union. L229,7–9

European Commission, 2001. Commission Regulation (EC) No 466/2001 of 8 March 2001 setting maximum levels for certain contaminants in foodstuffs. Off. J. Eur. Union. L77, 1–13

European Commission, 2002. Commission Regulation (EC) No 472/2002 of 12 March 2002 setting maximum levels for certain contaminants in foodstuffs. Off. J. Eur. Union, L75, 18–20

Färber, P., Geisen, R., 2004. Analysis of differentially-expressed Ochratoxin A biosynthesis genes of *Penicillium Nordicum*. Eur. J. Plant Pathol. 110, 661-669. https://doi.org/10.1023/B:EJPP.0000032405.21833.89

Fernandes, M., Keller, N.P., Adams, T.H., 1998. Sequence-specific binding by *Aspergillus nidulans* AflR, a C_6 zinc cluster protein regulating mycotoxin biosynthesis. Mol. Microbiol. 28, 1355-1365. https://doi.org/10.1046/j.1365-2958.1998.00907.x

Ferrara, M., Perrone, G., Gambacorta, L., Epifani, F., Solfrizzo, M., & Gallo, A., 2016. Identification of a Halogenase Involved in the Biosynthesis of Ochratoxin A in *Aspergillus carbonarius*. Appl. Environ. Microbiol. 82, 5631–5641. https://doi.org/10.1128/AEM.01209-16

Ferreira, N.P., Pitout, M.J., 1969. The biogenesis of ochratoxin. J. S. Afr. Chem. Inst. 22, S1–S8

Filali, A., Ouammi, L., Betbeder, A.M., Baudrimont, I., Soulaymani, R., Benayada, A., Creppy, E.E., 2001. Ochratoxin A in beverages from Morocco: A preliminary survey. Food Addit. Contam. 18, 565–568. https://doi.org/10.1080/02652030117365

Food and Agriculture Organization (FAO), 1996. Rome Declaration on Food Security and World Food Summit Plan of Action. FAO, Rome Italy.

Food and Agriculture Organization, 1993, Sampling plans for aflatoxin analysis in peanuts and com.FAO Food and Nutrition Paper, 55, Rome, Italy.

Forgacs, J.,1962. Mycotoxicoses—the neglected diseases. Feedstuffs, 34, 124-134.

Fuchs, R., Peraica, M., 2005. Ochratoxin A in human kidney diseases. Food Addit. Contam. 22 Suppl 1, 53-57, https://doi.org/10.1080/02652030500309368

Fungaro, M.H.P., Ferranti, L.S., Massi, F.P., da Silva, J.J., Sartori, D., Taniwaki, H.M., Frisvad, J.C., Iamanaka, B.T., 2017. *Aspergillus labruscus* sp. nov., a new species of *Aspergillus* section Nigri discovered in Brazil. 7, 6203. https://doi.org/10.1038/s41598-017-06589-y.

Gallo, A., Bruno, K.S., Solfrizzo, M., Perrone, G., Mulè, G., Visconti, A., Baker, S.E. 2012.New insight into the ochratoxin A biosynthetic pathway through deletion of a nonribosomal peptide synthetase gene in *Aspergillus carbonarius*. Appl. Environ. Microbiol. 78, 8208-8218. https://doi.org//10.1128/AEM.02508-12

Gallo, A., Knox, B.P., Bruno, K.B., Solfrizzo, M., Baker, S.E., Perrone, G., 2014. Identification and characterization of the polyketide synthase involved in ochratoxin A biosynthesis in *Aspergillus carbonarius*. Int. J. Food Microbiol. 179,10–11. https://doi.org/10.1016/j.ijfoodmicro.2014.03.013

Garrett, K.A., 2008. Climate change and plant disease risk. In: Relman, D.A., Hamburg, M.A., Choffnes, E.R., Mack, A. (Eds.), Global climate change and extreme weather events: understanding the contributions to infectious disease emergence. National Academies Press, Washington, DC, pp. 143-155

Grosso, F., Saïd, S., Mabrouk, I., Fremy, J.M., Castegnaro, M., Jemmali, M., Dragacci, S., 2003. New data on the occurrence of ochratoxin A in human sera from patients affected or not by renal diseases in Tunisia. Food Chem. Toxicol. 41, 1133-1140. https://doi.org/10.1016/S0278-6915(03)00067-X.

Grulke, N.E., 2011. The nexus of host and pathogen phenology: understanding the disease triangle with climate change. New Phytol. 189, 8-11 http://dx.doi.org/10.2307/40960871

Harris, J.P., Mantle, P.G., 2001. Biosynthesis of ochratoxins by *Aspergillus ochraceus*. Phytochemistry, 58, 709-716. https://doi.org/10.1016/S0031-9422(01)00316-8

Hesseltine, C. W., Shotwell, O. L., Kwolek, W. F., Lillehoj, E. B., Jackson, W. K., Bothast. R. J., 1976. Aflatoxin occurrence in 1973 corn at harvest. II. Mycological studies. Mycologia 68, 341-353.

Hocking, A.D., Leong, S.L., Kazi, B.A., Emmett, R.W., Scott, E.S., 2007. Fungi and mycotoxins in vineyards and grape products. Int. J. Food Microbiol. 119, 84–88. https://doi.org/10.1016/j.ijfoodmicro.2007.07.031

Huff, W., Hamilton, P.,1979. Mycotoxins-their biosynthesis in fungi: Ochratoxins-metabolites of combined pathways. J. Food Prot. 42, 815–820. https://doi.org/10.4315/0362-028X-42.10.815

International Agency for Research on Cancer (IARC), 1993. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. Monographs on the evaluation of carcinogenic risks to humans. Vol. 56, Lyon, France.

International Agency for Research on Cancer (IARC), 1998. Monographs on the evaluation of carcinogenic risks to humans: some traditional herbal medicines, some mycotoxins, naphthalene and styrene.

International Panel Climate Change (IPCC), 2014: synthesis report. Contribution of working group I, II, III to the fifth Assessement Report of the Intergovernmental Panel on Climate change. Geneva, Switzerland: Intergovernmental Panel on Climate Change (IPCC): 2014

James, R. C., 1985. General principles of toxicology, p.7-26. *In* P. L. Williams and J. L. Burson (ed.), Industrial toxicology. Van Nostrand Reinhold, New York, N.Y.

Kanisawa, M., 1984. Synergistic effect of citrinin on hepatorenal carcinogenesis of Ochratoxin A in mice. Dev. Food Sci. 7, 245–254

Karlovsky, P., 1999. Biological detoxification of fungal toxins and its use in plant breeding, feed and food production. Nat. Toxins. 7, 1–23

Kitya, D., Bbosa, G.S., Mulogo, E., 2010. Aflatoxin levels in common foods of South Western Uganda: A risk factor to hepatocellular carcinoma. Eur. J. Cancer Care. 19. 516-21. https://doi.org/10.1111/j.1365-2354.2009.01087.x.

Labrinea, E.P., Natskoulis, P.I., Spiropoulos, A.E., Magan, N., Tassou, C.C. 2011. A survey of ochratoxin A occurrence in Greek wines. Food Addit. Contam. Part B Surveill. 4, 61–66. https://doi.org/ 10.1080/19393210.2010.539707

Lacey, J., 1989. Pre- and Post-harvest ecology of fungi causing spoilage of foods and other stored product. J. Appl. Bacteriol. Symposium Suppl., 11-26

Leong SL, Hocking AD, Pitt JI, Kazi BA, Emmett RW, Scott ES. 2006a. Australian research on ochratoxigenic fungi and ochratoxin A. Int. J. Food Microbiol. http://dx.doi.org/10.1016/j.ijfoodmicro.2006.02.005

Leong, S.L., Hocking, A.D., Scott, E.S., 2006b. The effect of juice clarification, static or rotary fermentation and fining on ochratoxin A in wine. Aust. J. Grape Wine R.12, 245–251. http://dx.doi.org/10.1111/j.1755-0238.2006.tb00064.x

Li, S., Marquardt, R.R., Frohlich, A.A., Vitti, T.G., Crow, G., 1997. Pharmacokinetics of ochratoxin A and its metabolites in rats. Toxicol. Appl. Pharmacol. 145, 82–90.

Maaroufi, K., Achour, A., Hammami, M., El May, M., Betbeder, A., Ellouz, F., Creppy, E.E., Bacha, H., 1995. Ochratoxin A in human blood in relation to nephropathy in Tunisia. Hum. Exp. Toxicol. 14, 609–614. https://doi.org/10.1177/096032719501400710

Maggon, K.K., Gupta, S.K., Venkitasubramanian T.A., 1977. Biosynthesis of aflatoxins. Bacteriol Rev. 41(4), 822–855.

Malir, F., Ostry, V., Pfohl-Leszkowicz, A., Malir, J., Toman, J., 2016. Ochratoxin A: 50 Years of Research. 8(7). Toxins. https://doi: 10.3390/toxins8070191

Marín, P., Magan, N., Vázquez, C., González-Jaén, M.T., 2010. Differential effect of environmental conditions on growth and regulation of the fumonisin biosynthetic gene FUM1 in the maize pathogens and fumonisin-producers *F. verticillioides* and *F. proliferatum*. FEMS Microbiol. Ecol. 73, 303-311. https://doi.org/10.1111/j.1574-6941.2010.00894.x

Marin, S., Hodžić, I., Ramos, A.J., Sanchis, V., 2008. Predicting the growth/no-growth boundary and ochratoxin A production by *Aspergillus carbonarius* in pistachio nuts. Food Microbiol. 25, 683-689. https://doi.org/10.1016/j.fm.2008.03.006

Marin, S., Ramos, A.J., Cano-Sancho, G., Sanchis, V., 2013. Mycotoxins: occurrence, toxicology and exposure assessment. Food Chem. Toxicol. 60, 218-237. https://doi.org/10.1016/j.fct.2013.07.047.

Medina, A., Akbar, A., Baazeem, A., Rodríguez, A., Magan, N., 2017. Climate change, food security and mycotoxins: Do we know enough?. Fungal Biol. Rev. https://doi.org/10.1016/j.fbr.2017.04.002.

Medina, A., Mateo, E.M., Valle-Algarra, F.M., Mateo, F., Mateo, R.; Jimenez, M., 2008. Influence of nitrogen and carbon sources on the production of ochratoxin A by ochratoxigenic strains of *Aspergillus* spp. isolated from grapes. Int. J. Food Microbiol. 122, 93–99 https://doi.org/10.1016/j.ijfoodmicro.2007.11.055

Medina, A., Rodríguez, A., Sultan, Y., Magan, N., 2015. Climate change factors and A. flavus: effects on gene expression, growth and aflatoxin production. World Mycotoxin J. 8, 171-179 http://dx.doi.org/10.3920/WMJ2014.1726

Merhej, J., Richard-Forget, F., Barreau, C., 2011. Regulation of trichothecene biosynthesis in *Fusarium*: recent advances and new insights. Appl. Microbiol. Biotechnol.91, 519-528. https://doi.org/10.1007/s00253-011-3397-x

Mngadi, P., Govinden, R., Odhav, B., 2008. Co-occuring mycotoxins in animal feeds. Afr. J. Biotechnol. 7. 2239-2243

Müller, H.M., 1982. Decontamination of mycotoxins. I. Physical process (Ger.). Überischt. Tierenähr., 10, 95-122.

Nawaz, M. A., Rauf, S., Hayat, A., Catanante, G., Raza, R., Marty, J., 2017. Determination of Mycotoxins in Food. In Analysis of Food Toxins and Toxicants (eds. Y. Wong and R.J. Lewis). https://doi.org/10.1002/9781118992685.ch5

Nguyen, D., Rieu, I., Mariani, C., van Dam, N.M., 2016. How plants handle multiple stresses: hormonal interactions underlying responses to abiotic stress and insect herbivory. Plant Mol. Biol. 91, 727-40. https://doi.org/10.1007/s11103-016-0481-8.

Palmer, J.M., Theisen, J.M., Duran, R.M., Grayburn, W.S., Calvo, A.M., Keller, N.P., 2013. Secondary Metabolism and Development Is Mediated by LlmF Control of VeA Subcellular Localization in *Aspergillus nidulans*. PLoS Genet. 9(1): e1003193. https://doi.org/10.1371/journal.pgen.1003193

Park, A. R., Son, H., Min, K., Park, J., Goo, J.H., Rhee, S., Chae, S., Lee, Y., 2015. Regulatory mechanism of zearalenone biosynthesis. Mol. Microbiol. 97, 942-956. https://doi.org/10.1111/mmi.13078

Park, D. L., H. Njapau, and E. Boutrif. 1999. Minimizing risks posed by mycotoxins utilizing the HACCP concept. FAO Food, Nutrition and Agriculture Journal. 23:49–56.

Pautasso, M., Döring, T.F., Garbelotto, M., Pellis, L., Jeger, M.J., 2012. Impacts of climate change on plant diseases-opinions and trends. Eur. J. Plant Pathol. 133, 295-313. https://doi.org/10.1007/s10658-012-9936-1

Payne, G. A., Nystrom, G. J., Bhatnagar, D., Cleveland, T. E., Woloshuk, C. P., 1993. Cloning of the afl-2 gene involved in aflatoxin biosynthesis from *Aspergillus flavus*. App. Environ. Microbiol. 59, 156–162

Pena, A., Cerejo, F., Silva, L.J., Lino, C.M., 2010. Ochratoxin A survey in Portugese wine by LC-FD with direct injection. Talanta. 82, 1556–1561. https://doi.org/10.1016/j.talanta.2010.07.041

Peplow, A.W., Tag, A.G., Garifullina, G.F., Beremand, M.N.,2003. Identification of New Genes Positively Regulated by *Tri10* and a Regulatory Network for Trichothecene Mycotoxin Production. Appl. Environ. Microbiol. 69, 2731-2736. https://doi.org/10.1128/aem.69.5.2731-2736.2003

Perrone G., Gallo A., 2017. *Aspergillus* Species and Their Associated Mycotoxins. In: Moretti A., Susca A. (Eds.), Mycotoxigenic Fungi. Methods in Molecular Biology, Humana Press, New York, pp.33-49. https://doi.org/10.1007/978-1-4939-6707-0_3

Perrone G., Nicoletti, I., Pascale, M., De Rossi, A., De Girolamo, A., Visconti, A., 2007. Positive correlation between high levels of ochratoxin A and resveratrol-related compounds in red wines. J. Agric. Food Chem. 55, 6807–6812. https://doi.org/10.1021/jf070993l

Perrone, G., Gallo, A., 2017. *Aspergillus* Species and Their Associated Mycotoxins. Methods Mol Biol. 1542, 33-49. https://doi.org/10.1007/978-1-4939-6707-0_3

Perrone, G., Logrieco, A.F., Frisvad, J.C., 2017. Comments on "Screening and Identification of Novel Ochratoxin A-Producing Fungi from Grapes. Toxins 2016, 8, 333"-In Reporting Ochratoxin A Production from Strains of *Aspergillus*, *Penicillium* and *Talaromyces*. Toxins. 9(2) https://doi.org./10.3390/toxins9020065

Perrone, G., Susca, A., Cozzi, G., Ehrlich, K., Varga, J., Frisvad, J., Meijer, M., Noonim, P., Mahakarnchanakul, W., Samson, R., 2007. Biodiversity of *Aspergillus* species in some important agricultural products. Stud. Mycol. 59, 53-66. https://doi.org/ 10.3114/sim.2007.59.07

Pfohl-Leszkowicz, A., Manderville, R., 2007. Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans. Mol. Nutr. Food Res. 51, 61–99. https://doi.org/10.1002/mnfr.200600137

Pfohl-Leszkowicz, A., Manderville, R.A., 2012. An update on direct genotoxicity as a molecular mechanism of ochratoxin a carcinogenicity. Chem. Res. Toxicol. 25, 252–262 https://doi.org/10.1021/tx200430f

Ponsone, M.L., Chiotta, M.L., Combina, M., Dalcero, A., Chulze, S., 2011. Biocontrol as a strategy to reduce the impact of ochratoxin A and *Aspergillus* section *Nigri* in grapes. Int. J. Food Microbiol. 151, 70–77. https://doi.org/10.1016/j.ijfoodmicro.2011.08.005

Ponsone, M.L., Chiotta, M.L., Combina, M., Torres, A., Knass, P., Dalcero, A., Chulze, S., 2010. Natural occurrence of ochratoxin A in musts, wines and grape vine fruits from grapes harvested in Argentina. Toxins. 2, 1984–1996. https://doi.org/10.3390/toxins2081984

Proctor, R.H., Hohn, T.M., McCormick, S.P., Desjardins, A.E., 1995. Tri6 encodes an unusual zinc finger protein involved in regulation of trichothecene biosynthesis in *Fusarium* sporotrichioides. Appl. Environ. Microbiol. 61, 1923–1930.

Rapid Alert System for Food and Feed (RASFF), Consulted in October 2019. Available at: https://webgate.ec.europa.eu/rasff-window/portal/?event=searchForm&cleanSearch=1#

Riley, R.T., Norred, W.P.,1999. Mycotoxin prevention and decontamination—A case study on maize. Food Nutr. Agric. 23, 25–32.

Ruyck, K., De Boevre, M., De Huybrechts, I., De Saeger, S., 2015. Dietary mycotoxins, co-exposure and carcinogenesis in humans : Short review. Mutat. Res. Rev. Mutat. Res. 766, 32–41. https://doi.org/10.1016/j.mrrev.2015.07.003

Samson, R.A., Visagie, C.M., Houbraken, J., Hong, S.B., Hubka, V., Klaassen, C.H., Perrone, G., Seifert, K.A., Susca, A., Tanney, J.B., Varga, J., Kocsubé, S., Szigeti, G., Yaguchi, T., Frisvad, J.C., 2014. Phylogeny, identification and nomenclature of the genus *Aspergillus*. Stud. Mycol. 78,141-73. https://doi.org/10.1016/j.simyco.2014.07.004

Sarikaya Bayram, Ö., Bayram, Ö., Valerius, O., Park, H.S., Irniger, S., Gerke, J., Ni, M., Han, K.H., Yu, J.H., Braus, G.H., 2010. LaeA Control of Velvet Family Regulatory Proteins for Light-Dependent Development and Fungal Cell-Type Specificity. PLoS Genet. 6(12): e1001226. https://doi.org/10.1371/journal.pgen.1001226

Scott, P.M., 2008. Mycotoxins in alcoholic beverages and fruit juices: occurrence and analysis. Food Contaminants, ACS Symposium Series 1001, 170–191

Solfrizzo, M., Avantaggiato, G., Panzarini, G., Visconti, A., 2009. Removal of Ochratoxin A from Contaminated Red Wines by Repassage over Grape Pomaces. J. Agric. Food Chem. 58, 317-23. https://doi.org/10.1021/jf9030585

Solfrizzo, M., Gambacorta, L., Visconti, A., 2014. Assessment of Multi-Mycotoxin Exposure in Southern Italy by Urinary Multi-Biomarker Determination. Toxins. 6. 523-38. https://doi-org/10.3390/toxins6020523.

Solfrizzo, M.,Perrone, G., Fracchiolla, R., Leo, R., Piemontese, L., Zivoli, R., Ferara, M., Gambacorta, L., Magistà, D., 2019. Processo e impianto per il trattamento di mosti e vini. Patent 102016000083162

Somma, S., Perrone, G., Logrieco, A.F., 2012. Diversity of black Aspergilli and mycotoxin risks in grape, wine and dried vine fruits. Phytopathol. Mediterr. 51, 131-147. https://doi.org/10.14601/Phytopathol_Mediterr-9888

Soufleros, E.H., Tricard, C., Bouloumpasi, E., 2003. Occurrence of ochratoxin A in Greek wines. J. Sci. Food Agric. 83, 173–179. https://doi.org/10.1002/jsfa.1300

Tag, A.G., Garifullina, G.F., Peplow, A.W., Ake, C.Jr., Phillips, T.D., Hohn, T.M., Beremand, M.N.,2001. A Novel Regulatory Gene, *Tri10*, Controls Trichothecene Toxin Production and Gene Expression. Appl. Environ. Microbiol. 67, 5294 5302. https://doi.org/10.1128/AEM.67.11.5294-5302.2001

Tannous, J., Khoury, R.E., Snini, S.P., Lippi, Y., Khoury, A.E., Atoui, A., Lteif, R., Oswald, I.P., Puel, O., 2014. Sequencing, physical organization and kinetic expression of the patulin biosynthetic gene cluster from *Penicillium expansum*. Int. J. Food Microbiol. 189, 51–60. https://doi.org/10.1016/j.ijfoodmicro.2014.07.028.

Tilburn, J., Sarkar, S., Widdick, D.A., Espeso, E.A., Orejas, M., Mungroo, J., Peñalva, M.A., Arst, H.N.,1995. The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. EMBO J. 14, 779–790

Tjamos, S.E., Antoniou, P.P., Kazantzidou, A., Antonopoulos, D.F., Papageorgiou, I., Tjamos, E.C., 2004. *Aspergillus niger* and *Aspergillus carbonarius* in Corinth raisin and wine-producing vineyards in Greece: population composition, ochratoxin A production and chemical control. J. Phytopathol. 152,250–255. http://dx.doi.org/10.1111/j.1439-0434.2004.00838.x

Van der Fels-Klerx, H., Goedhart, P., Elen, O., Börjesson , T., Hietaniemi, V., Booij, C., 2012. Modelling deoxynivalenol contamination of wheat in northwestern Europe for climate change assessments. J. Food Prot. 75, 1099-1106. https://doi.org/10.4315/0362-028X.JFP-11-435

Van der Fels-Klerx, H.J., Liu, C., Battilani, P., 2016. Modelling climate change impacts on mycotoxin contamination. World Mycotoxin J. 9, 717-726. https://doi.org/10.3920/WMJ2016.2066

van der Merwe, K.J., Steyn, P.S., Fourie, L., Scott,D.B., Theron, J.J., 1965. Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus*. Nature. 205(976),1112-3. https://doi.org/10.1038/2051112a0

van Egmond, H.P., 1991. Regulatory aspects of mycotoxins in Asia and Africa. In:Fungi and Mycotoxins in Stored Products: proceedings of an international conference, Bangkok, Thailand, 23–26 April 1991. B.R. Champ, E. Highley, A.D. Hocking and J.J. Pitt, eds. ACIAR Proceedings no. 36, 198–204

van Egmond, H.P., Jonker M.A., 2008. Worldwide Regulations on Aflatoxins—The Situation in 2002. J. Toxicol. Toxin Rev. 23, 2-3. https://doi.org/10.1081/TXR-200027844

Var, I., Erginkaya, Z., Kabak, B., 2011. Inhibition of ochratoxin A production of *Aspergillus carbonarius* by yeast species. Czech J. Food Sci. 29, 291–297

Varga, J., Frisvad, J.C., Kocsubé, S., Brankovics, B., Tóth, B., Szigeti, G., Samson R.A., 2011. New and revisited species in *Aspergillus* section *Nigri*. Stud. Mycol. 69,1-17. https://doi.org/10.3114/sim.2011.69.01

Vaughan, M.M., Huffaker, A., Schmelz, E.A., Dafoe, N.J., Christensen, S., Sims, J., 2014. Effects of elevated CO2 on maize defence against mycotoxigenic *Fusarium verticillioides*. Plant Cell. Environ. 37, 2691-2706. https://doi.org/10.1111/pce.12337

Verheecke-Vaessen, C., Diez, L., Renaud, J., Sumarah, M., Medina, A., Magan, N., 2019. Interacting climate change environmental factors effects on *Fusarium langsethiae* growth, expression of Tri genes and T-2/HT-2 mycotoxin production on oat-based media and in stored oats. Fungal Biol. 123, 618-624. https://doi.org/10.1016/j.funbio.2019.04.008

Visagie, C.M., Houbraken, J., Frisvad, J., Hong, S.B., Klaassen, C.H.W., Perrone, G., Seifert, K.A., Varga, J., Yaguchi, T., Samson, R.A., 2014. Identification and nomenclature of the genus *Penicillium*. Stud. Mycol. 78, 343-371. https://doi.org/10.1016/j.simyco.2014.09.001.

Visconti, A., Pascale, M., Centonze, G., 1999. Determination of ochratoxin A in wine by means of immunoaffinity column clean-up and high-performance liquid chromatography. J. Chromatogr. A. 864,89–101. https://doi.org/10.1016/S0021-9673(99)00996-6

Visconti, A., Perrone, G., Cozzi, G., Solfrizzo, M., 2008. Managing ochratoxin A risk in the grape-wine food chain, Food Addit. Contam. Part A Chem. Anal. Control. Expo. Risk Assess. 25,193-202. https://doi.org/10.1080/02652030701744546

Wang, L., Wang, Y., Wang, Q., Liu, F., Selvaraj, J.N., Liu, L., Xing, F., Zhao, Y., Zhou, L., Liu, Y., 2015. Functional Characterization of New Polyketide Synthase Genes Involved in Ochratoxin A Biosynthesis in *Aspergillus Ochraceus*. Toxins. 7, 2723-2738. https://doi.org/10.3390/toxins7082723

Wang, Y., Wang, L., Wu, F., Liu, F., Wang, Q., Zhang, X., Selvaraj, J.N., Zhao, Y., Xing, F., Yin, W.B., Liu, Y., 2018. A Consensus Ochratoxin A Biosynthetic Pathway: Insights from the Genome Sequence of *Aspergillus ochraceus* and a Comparative Genomic Analysis. Appl. Environ. Microbiol. 84(19). https://doi.org/10.1128/AEM.01009-18

World Health Organization (WHO), 1990. Selected mycotoxins: ochratoxins, trichothecenes, Ergot (Environmental Health Criteria 105), Geneva

Xiao, H., Marquardt, R.R., Abramson, D., Frohlich, A.A., 1996. Metabolites of ochratoxins in rat urine and in a culture of *Aspergillus ochraceus*. Appl. Environ. Microbiol. 62, 648–655.

Yang, J., Li, J., Jiang, Y., Duan, X., Qu, H., Yang, B., Chen, F., Sivakumar, D., 2014. Natural occurrence, analysis, and prevention of mycotoxins in fruits and their processed products. Crit. Rev. Food Sci. Nutr. 54, 64-83. https://doi.org/10.1080/10408398.2011.569860

Zhang, X., Li, Y., Wang, H., Gu, X., Zheng, X., Wang, Y., Diao, J., Peng, Y., Zhang, H., 2016. Screening and Identification of Novel Ochratoxin A-Producing Fungi from Grapes. Toxins. 8(11), 333. https://doi.org/10.3390/toxins8110333

Zhang, X., Li, Y., Wang, H., Gu, X., Zheng, X., Wang, Y., Diao, J., Peng, Y., Zhang, H., 2017. Reply to Comments on "Screening and Identification of Novel Ochratoxin A-Producing Fungi from Grapes". Toxins 2016, 8, 333"-in Reporting Ochratoxin A Production from Strains of *Aspergillus*, *Penicillium* and *Talaromyces*. Toxins. 9(2). https://doi.org/10.3390/toxins9020066

OBJECTIVES AND WORK PLAN

In order to deal with the realization of the present PhD project, activities were divided as follows.

- **OTA RISK IN RELATION TO CLIMATE CHANGE**

Apulia region, in South Italy, is a very susceptible area to OTA contamination in grape and grape by-products, mainly (red) wines. OTA contamination is strongly influenced by meteorological conditions during the year of cultivation. Indeed, it is reported that rainy seasons, especially prior to harvest, favour black Aspergilli colonization causing the so called "Black rot of grapes" which lead to OTA production. In the context of the general increasing interest about the effect of climate change on the mycotoxigenic risk, we aimed to evaluate the impact of possible climatic variations through the grape wine food chain in the Apulia region. Several reports evidenced that OTA contamination follows an increasing gradient from Northern to Southern countries in Europe and also in Italy. In the present study, we extrapolated this information and defined two areas of interests - the North and the South areas of Apulia region, traditionally known to be less and more susceptible to OTA contamination, respectively - to understand whether variations in climatic conditions may exacerbate or not the ochratoxigenic risk. For each area two different day/night temperatures with associated photoperiod, representative of the month prior to harvest, were selected in order to simulate current and expected climate change (Table 6).

Table 6. Temperature cycles used in this study

	South Apulia	**North Apulia**
Current	18/31°C	15/28°C
Climate Change	20/37°C	18/34°C
Photoperiod	10h/14h dark/light	11.5h/12.5h dark/light

In the first part of the project, i) the combined effect of temperature cycles and water stress was investigated on the ecophysiology, toxin gene expression and OTA phenotypic production by *A. carbonarius* strains. Data about the South Apulia conditions were the object of a publication **(STUDY I)** while results about the effect of North Apulia conditions gave us the hints for further investigation. In fact, ii) temperature cycles representative of this area were chosen to study their combined effect with CO_2 variations on the ecophysiology, toxin gene expression and OTA production by *A. carbonarius* strains.

This activity was carried out at Cranfield University and was object of an article **(STUDY II)**.

- **NEW STRATEGIES FOR REDUCING OTA CONTAMINATION**

In high risk seasons, OTA levels in grape derived products may exceed the legislative limit (2 μg·L^{-1}) resulting in both increasing risk for humans and economic losses. Biodegradation, through the use of microorganisms or enzymes, could be a valid solution for OTA decontamination. The most acknowledged pathway of OTA degradation involves the hydrolysis of the amide bond of the L-β-phenylalanine to the OTα moiety. The degradation product OTα seems to not exert cytotoxic effects on cellular metabolism and therefore, this mechanism can be considered a detoxification strategy. A previous work reported that a mutant strain of *A. carbonarius* deleted for key genes of OTA pathway and thus unable to produce OTA retained the ability to degrade the toxin. In relation to this evidence, three mutant strains of *A. carbonarius* were tested for their ability to degrade the mycotoxin when exogenous OTA was added to the medium. In parallel, the expression levels of previously identified proteases encoding genes was analysed for their possible involvement in the degradation process.

Some of these proteases, among which an aspartyl protease, were further investigated to characterize the enzymatic activity with the final aim to identify some proteolytic enzymes able to reduce the OTA content in grape by-products, such as wine **(STUDY III).**

CLIMATE CHANGE *in vitro*

Ecophysiology | Toxin gene expression | OTA production

Aspergillus carbonarius

i) **Temperature x water activity**

SOUTH APULIA

18/31°C + 0.99/0.93a_w
20/37°C + 0.99/0.93a_w

10h/14h dark/light

STUDY I

NORTH APULIA

15/28°C + 0.99/0.93a_w
18/34°C + 0.99/0.93a_w

11.5h/12.5h dark/light

From these results we proceed in the next point

ii) **Temperature x CO_2**

15/28°C + 400/1000ppm
18/34°C + 400/1000ppm

11.5h/12.5h dark/light

STUDY II

NEW REDUCING STRATEGIES

Aspergillus carbonarius
mutant strains

Incubation in BM

OTA addition after 3d

Collection after
12 and 24h

Mycelium
Gene expression of proteases encoding

Liquid cultures
OTA and OTα analysis

Evaluation of *Aspergillus carbonarius* OTA degrading activity

STUDY III

RESULTS

STUDY I

Effects of temperature and water activity change on ecophysiology of *Aspergillus carbonarius* in field-simulating conditions

ABSTRACT

Ochratoxin A (OTA) is the primary mycotoxin threat in wine and dried vine fruits. Its presence in grape and wine is strongly related to climatic conditions and the expected climate change could represent a risk of increasing fungal colonization and OTA contamination in grapes. In this regard, the interacting effect of i) different conditions of water availability (0.93 and 0.99a_w) and ii) different 10h/14h dark/light alternating temperature conditions simulating a nowadays (18/31°C) and climate change scenario (20/37°C) in high OTA risk areas of Apulia region, were studied. Lag phases prior to growth, mycelial growth rate, the expression of biosynthesis, transcription factors and regulatory genes of OTA cluster and OTA production were analyzed in *Aspergillus carbonarius* ITEM 5010 under the combined effect of different climatic factors. At 18/31 °C and under water stress conditions (0.93 a_w) the growth rate was slower than at 0.99 a_w; on the contrary, at 20/37°C a higher growth rate was observed at 0.93 a_w. An over-expression of OTA genes and genes belonging to the global regulator Velvet complex was observed at 18/31°C and 0.99 a_w, with the specific OTA pathway transcription factor *bZIP* showing the highest expression level. The up-regulated transcription profile of the genes positively correlated with OTA production higher at 18/31°C than at 20/37°C and 0.99 a_w; while no OTA production was detected at 0.93 a_w at each of the temperature conditions tested. These findings provide preliminary evidence that the possible increase of the temperature, likely to happen in some areas of the Apulia region, may results in a reduction of both *A. carbonarius* spoilage and OTA production in grapes.

In *International Journal of Food Microbiology*

1. INTRODUCTION

Ochratoxin A (OTA) is one of the most abundant food contaminating mycotoxins produced by species belonging to *Aspergillus* and *Penicillium* genera; it is a potent nephrotoxin, classified as a possible human carcinogen (Group 2B) (IARC, 1993). To date, the European Union has established maximum OTA levels for different food products and the limit for grapes and wine was set at 2 μg/kg (EC, 2006 a). Studies performed over the last decade have provided evidence that all fungi responsible for OTA production in grapes belong to *Aspergillus* section *Nigri*, the so called "Black Aspergilli". In particular, *A. carbonarius* has been identified as the major cause of contamination in berries (Cabañes and Bragulat 2018; Perrone et al., 2008). This contamination is strongly related to various factors such as climatic conditions, geographical regions, grape varieties, damage by insects, growing season, with fluctuations in contamination rate occurring from one year to another (Somma et al., 2012; Visconti et al., 2008). A number of ecophysiological studies simulating field conditions revealed that *A. carbonarius* growth and OTA production were determined by environmental factors such as temperature, water availability and photoperiod (Bellí et al., 2006; Oueslati et al., 2010). In this respect, climate has always been one of the key-factors in the agro-ecosystem that influences fungal colonization and mycotoxin production in crops (Bellí et al., 2005a; Magan et al., 2003). Climate change is thus expected to have a profound effect both on our landscape worldwide and on sustainable food production system. It is estimated that the environment in which crops will be grown in the next 10-25 years may change markedly with atmospheric CO_2 concentration expected to double or triple, accompanied by episodes of heavy rainfall or periods of extreme drought. Because of the increase of greenhouse gases, the global temperature could rise between +2 and +5 °C (Botana et al., 2015; Medina et al., 2015). With respect to food safety, one of the most important hazards likely affected by climate change will be the contamination by mycotoxins of economically important commodities (Miraglia et al., 2009).

Mycotoxigenic fungi have their own specific temperature and humidity range for crop infection, mycotoxin production and survival, which reflects their geographical distribution and determines a gradient of mycotoxin contamination worldwide. Some species might shift their geographical distribution in response to global warming, leading to changes in the pattern of mycotoxin occurrence (Battilani et al., 2016). Recent studies have reported diverse observations on the effect of climate change on mycotoxins production. Under elevated CO_2 concentration, *Fusarium verticillioides*, a well-known pathogen of maize, showed an increased virulence, although

fumonisins production was relatively unchanged compared to control conditions (Vaughan et al., 2014). The interaction of environmental factors (water activity x temperature x CO_2) was reported to have a little effect on growth of *A. flavus* on maize kernels, while a significant increase in the production of the mycotoxin Aflatoxin B_1 was detected (Medina et al., 2014). Differential effects of interacting climate change factors on growth and OTA production in most species of *Aspergillus* on coffee were observed by Akbar et al. (2016). In particular, within section *Circumdati*, *A. westerdijkiae* strains were the ones showing a significant stimulation of OTA production at 0.90 a_w under elevated CO_2 concentrations. Moreover, a preliminary study on *A. ochraceus* and *A. carbonarius* evidenced a reduction of growth and OTA production when temperature increased (García-Cela et al., 2012). There is limited research investigating the toxigenic risk in grapes and wines towards a climate change scenario. In the present work, we evaluated the interacting effect of 0.99 and 0.93 a_w, reported to be representative of more and less conducive conditions, respectively, for the colonization and OTA contamination by *A. carbonarius* (Bellí et al., 2004, 2005a) and different alternating temperature conditions, 18/31°C and 20/37°C (10h/14h dark/light), simulating a nowadays and climate change scenario, respectively, in high OTA risk areas of Apulia region during the month preceding harvest. In fact, from early veraison to harvest, the incidence of *A. carbonarius* black rot in grape berries increases with a peak at ripening, due to the change in composition of berries that become mature and soft during this period (Leong et al., 2006a).

In this work, we studied the behavior of *A. carbonarius* ITEM 5010 grown on grape medium under different conditions related to a possible increase of temperatures and drought stress, by analyzing lag phase prior to growth, mycelial growth rate, expression levels of OTA biosynthetic genes and genes belonging to the regulatory Velvet complex in relation to OTA production.

2. MATERIALS AND METHODS

2.1. Fungal strain and growth conditions

The *A. carbonarius* strain ITEM 5010 isolated from Italian wine grapes and held in the Agro-Food Microbial Culture Collection – ITEM of the Institute of Sciences of Food Production, CNR, Bari, Italy (www.ispa.cnr.it/Collection), was used for this study.

To test the influence of temperature, water activity (a_w) and dark/light variation, a Grape Juice Medium (GJM) was prepared by mixing 50% (v/v) commercial pasteurized grape juice (composition per 100 mL: fat, 0.02 g; proteins, 0.3 g; carbohydrates, 16.10 g) and 1.2% agar in distilled water (Ioannidis et al., 2015). The a_w of GJM, measured by an AquaLab 4TE a_w meter at 25°C, was adjusted to 0.99 and 0.93 by adding the required grams of glucose. The pH of the medium was adjusted to 3.5 using 1 M KOH. Fungal inocula were prepared from cultures grown on Potato Dextrose Agar (PDA) plates for seven days at 25°C.

Different alternating conditions simulating a nowadays and climate change scenario of high OTA risk areas of Apulia region (18/31°C and 20/37°C, 10h/14h dark/light) were tested using a dynamic climate chamber (WTC Binder, Labortechnik GmbH, Tuttingen Germany).

Grape Juice Medium plates overlaid with sterile cellophane membranes were uniformly spread with 100 µl of a conidial suspension (10^5 conidia·mL $^{-1}$) of *A. carbonarius* strain ITEM 5010. Plates cultures with the same a_w were incubated into a transparent plastic box containing water or a water/glucose solution to minimize moisture loss and maintain constant the a_w value during incubation at different conditions. Fungal mycelium was harvested at 4 days and 7 days post inoculation (dpi) and stored at -20°C for OTA content analysis. Fungal mycelium was harvested at 4 days dpi and stored at -80°C for RNA extraction.

2.2. Growth measurements

Fungal growth was assessed daily for seven days and expressed as the average measure (mm) of two orthogonal diameters of centrally inoculated colonies (3 mm drop). The colony diameters (mm) were plotted against time (days) and the linear regression model, already described by (Medina et al., 2014), was used for calculating the growth rate expressed as the slope of the line. The lag phase (hours) was calculated by equaling the linear regression formula to the diameter of the inoculated drop. The square of the linear correlation coefficient was ≥ 0.98. Experiments were carried out with three replicates per treatment.

2.3. RNA extraction, cDNA synthesis and qRT-PCR

Total RNA was extracted from frozen mycelium ground in liquid nitrogen using the RNeasy Plant Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. RNA concentration was determined by using Nanodrop and its integrity was evaluated by doing a 1% agarose gel. First strand cDNA was synthesized using about 1 µg of total RNA in a reaction mix containing oligo $(dT)_{18}$ primer, random examers and SuperScript III Reverse Transcriptase (Invitrogen, San Diego, CA) according to the manufacturer's protocol.

The expression levels of OTA biosynthetic genes (*AcOTApks*, *AcOTAnrps*, *AcOTAhal*, *AcOTAp450*, *AcOTAbZip*) and heterotrimeric velvet complex genes (*VelB*,*VeA*,*Lae*A) of *A. carbonarius* were analyzed by using real-time quantitative reverse transcription-PCR (qRT-PCR); *β-tubulin* was used as internal reference gene. Nucleotide sequences of primers used in the qPCR assays are shown in Table 7.

Table 7. Primers for qRT-PCR used in this study.

Primer	Concentration	Sequence (5'-3')	Reference
RT_OTApks_Ac_FOR	200 nM	CGTGTCCGATACTGTCTGTGA	Gallo et al. 2014
RT_OTApks_Ac_REV	200 nM	GCATGGAGTCCTCAAGAACC	
RT_nrps_Ac_FOR	200 nM	ACGGGTCGCTGCTCTATATC	Ferrara et al. 2016
RT_nrps_Ac_REV	200 nM	ACTCACCACATCAACCACGA	
RT_hal_Ac_FOR	200 nM	GAACGCCAGTAGAGGGACAG	Ferrara et al. 2016
RT_hal_Ac_REV	200 nM	ATGGAGGTGGTGTTGTTGTG	
RT_AcOTAp450_F	200 nM	GTGGTTATCCCGCCCAATAC	Ferrara et al. 2016
RT_AcOTAp450_R	200 nM	TGCCAGATTCATCCCGATAC	
RT_Ac_OTAbZIP_for	250 nM	AATGGAACCAGCATTGATCTC	Ferrara et al. 2016
RT_Ac_OTAbZIP_rev	250 nM	GACCCAAGCATTCGCTCTA	
RT_AClaeA-1F	150 nM	AATGGGACCGCAATGAGTC	This study
RT_AClaeA-2R	150 nM	TCCTGCTCCTGTTCGTCAC	
RT_Ac_veA_FOR	150 nM	GGTGAATGAGACCGAGCA	This study
RT_Ac_veA_REV	150 nM	GCATTGTAGGCGAAGGTGA	
RT_Ac_VelB_For	150 nM	AGTGCGTTCCGACTGACTG	This study
RT_Ac_VelB_Rev	150 nM	TGGACTGATTACCGACATTTACA	
RT3 BT Ac_F	200 nM	CAAACCGGCCAGTGTGGTA	Ferrara et al. 2016
RT3 BT Ac_R	200 nM	CGGAGGTGCCATTGTAAACA	

Real time PCR were performed using the Viia 7 Real Time PCR system programmed to hold at 50°C for 2 min and at 95°C for 2 min and to complete 40 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s. Specificity of the PCR amplifications was confirmed by dissociation curve analysis. Real time PCR experiments were conducted in 96-well plates by using SYBR Select

MasterMix and different concentrations of primers pairs for each gene in a final volume of 10 µl. Data analysis was assessed by QuantStudio™ RT-PCR Software. The relative quantification of gene expression was established using the comparative $2^{-\Delta\Delta CT}$ method. PCR efficiency of each oligonucleotide pair was calculated from each linear regression of standard curves. Relative gene expression analysis was performed on three biological replicates for each of which three technical replicates were assayed.

2.4. Determination of ochratoxin A

For the determination of OTA in fungal mycelium, HPLC-FLD analysis was performed on three biological replicates

An aliquot of lyophilized mycelium (100 mg) was extracted with 2 mL of a mixture of methanol:acetonitrile:water (30:30:40 v/v/v) by shaking for 2 h at room temperature. After extraction, samples were centrifuged for 15 min at 3901 g and 600 µl of supernatant were diluted with 400 µl of acetonitrile:water:acetic acid (35.0:62.5:2.5 v/v/v) and vortexed for 30 s. This solution was filtered (0.22 µm) and 100 µl were injected into the HPLC-FLD apparatus. The mobile phase was an isocratic mixture of acetonitrile:water (45:55 v/v) containing 1% acetic acid. The limit of detection (LOD) of this method for OTA was 6.78 $ng \cdot g^{-1}$.

The HPLC apparatus was an Agilent 1260 Infinity system equipped with a G1312 binary pump, a G1367E autosampler with a 100 µl loop, a G1316C column thermostat set at 30°C, and a fluorescence detector (G1321B) (excitation wavelength, 333 nm; emission wavelength, 460 nm) from Agilent Technologies (Waldbronn, Germany). The column used was a 150 x 4.6 mm i.d., 5 µm, Zorbax C18, (Phenomenex, Torrance, CA, USA) with a 3 mm i.d. and a 0.45 µm pore size guard filter (Rheodyne, Cotati, CA, USA). The flow rate of the mobile phase was 1 $mL \cdot min^{-1}$. The standard solutions of OTA in acetonitrile were purchased from Romer Labs Diagnostic GmbH (Tulln, Austria). Calibration solutions of OTA were prepared in HPLC mobile phase, and calibration curves were prepared in the range of 0.1 to 100 $ng \cdot mL^{-1}$.

2.5. Data analysis

Statistical analysis was performed by using GraphPad Software (version 7.0, Prism, La Jolla California, USA). After checking the normal distribution of lag phases, growth rates and OTA production datasets with D'Agostino-Pearson test, the analysis of variance (ANOVA) was performed in order to test the effect of each single variable and their interaction on the results

obtained. Furthermore, significant differences among the means were established by using Tukey HSD *post hoc* test.

3. RESULTS

3.1. Lag phase, growth rate and sporulation

As shown in Figure 8A, at the two alternating temperatures 18/31°C, the drought stress (0.93 a_w) caused a lag phase considerably longer (40 h) than at 0.99 a_w (26 h), while at 20/37°C, the lag phase exhibited no significant differences when a_w of the substrate was changed. As regards growth rate, at 18/31°C, it was faster at 0.99 a_w (19 mm·day^{-1}) than at 0.93 a_w (10 mm·day^{-1}); on the contrary, at 20/37 °C, the fungus showed a faster growth at 0.93 a_w (11 mm·day^{-1}) than at 0.99 a_w (6 mm·day^{-1}) (Fig. 8B). The different ecophysiological behavior of *A. carbonarius*, both in terms of growth and sporulation, was macroscopically evident comparing mycelial growth on plates at 7 days after inoculation, as illustrated in Figure 9. In particular, at 20/37°C a less sporulated mycelium was observed at 0.93 a_w than at 0.99 a_w, although the growth rate resulted to be faster in water stress condition, as previously described. In order to confirm this new evidence, the experiment was repeated two times also on different strains.

ANOVA analysis showed that both lag phase and growth rate were significantly influenced ($p<0.001$) by temperature conditions, a_w, and their interaction (data not shown).

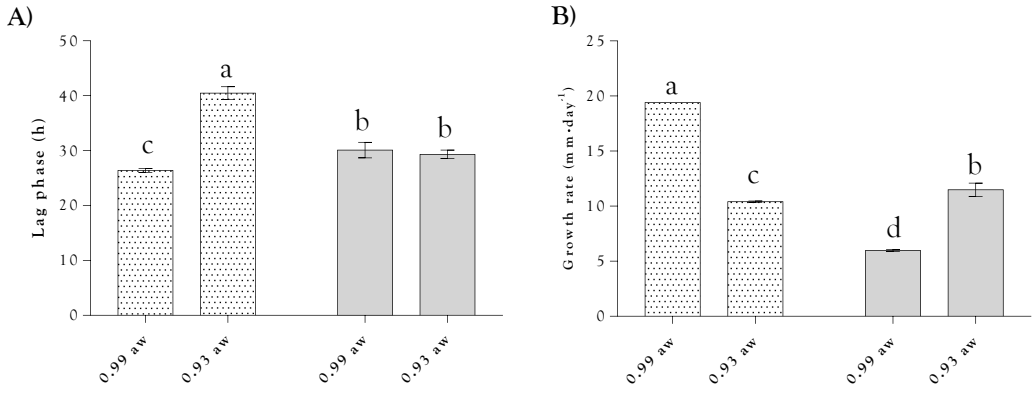


Figure 8. A) Lag phases (h) of *A. carbonarius* ITEM 5010 grown on GJM at 0.99 and 0.93 a_w and incubated at 18/31°C (white bars with dots) and 20/37°C (grey bars), 10h/14h dark/light photoperiod; **B)** Growth rate (mm·day^{-1}) of *A. carbonarius* ITEM 5010 grown on GJM at 0.99 and 0.93 a_w and incubated at 18/31°C (white bars with dots) and 20/37°C (grey bars), 10h/14h dark/light photoperiod. Letters indicate significant differences (Tukey HSD, $p<0.05$).

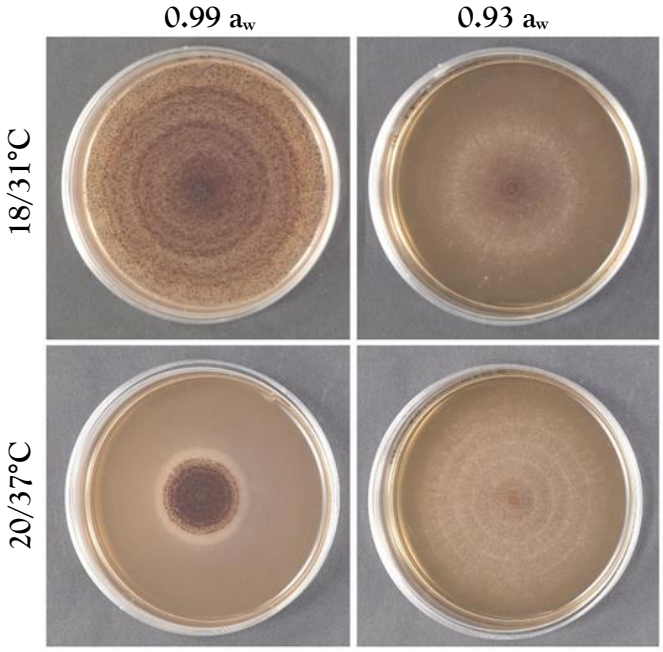


Figure 9. Pictures of *A. carbonarius* ITEM 5010 colonies centrally inoculated taken at 7 dpi at 18/31°C and 20/37°C 10h/14h dark/light, 0.99 and 0.93 a_w.

3.2. Gene expression and OTA production

The effect of the two alternating day/night temperature on the expression of both biosynthetic and regulatory genes in *A. carbonarius* was analysed after 4 days of growth at 0.99 a_w, which seems to be the most reliable time to detect the activation of the genes involved in OTA biosynthesis, preceding the later increase of OTA production. As shown in Figure 10, a general up-regulation of structural OTA biosynthesis genes (*AcOTApks, AcOTAnrps, AcOTAhal* and *AcOTAp450*) was observed at 18/31°C compared to 20/37°C. In particular, a minimum of about 2 fold change in the expression level was detected for the genes located in the OTA cluster with a peak of about 12 fold change of the gene coding for the cytochrome *p450* oxidase protein, expected to be involved in the oxidase step for the formation of the precursor metabolite OTβ in the biosynthesis pathway (Ferrara et al., 2016). Moreover, at 18/31°C, the gene coding for a *bZIP* transcription factor protein, likely acting as regulator of OTA gene expression, exhibited a 34-fold higher transcription level (Fig. 10A). The genes *lae*A, *ve*A, and *velB*, belonging to the global transcriptional complex regulating fungal secondary metabolism known as Velvet complex, showed a higher level of expression between 2 and 5-fold at 18/31°C temperature cycle as well (Fig. 10B).

Gene expression analysis performed after 4 days of incubation, was supported by the results of OTA production. At 0.99 a_w, the highest OTA production was observed in *A. carbonarius* grown at 18/31°C. In particular, at these temperatures OTA content was slightly higher at 4 days (554

ng·g^{-1}) than at 7 day (512 ng·g^{-1}) after inoculation. At 20/37°C, OTA accumulation was lower at 4 days (185 ng·g^{-1}) than at 7 days (226 ng·g^{-1}) (Fig. 11). The longer production timing at 20/37 °C is likely due to the slower growth rate observed at these higher temperature conditions. Under strong water stress conditions, *A. carbonarius* appeared unable to produce OTA, in fact no OTA was detected at 0.93 a_w, under the limit of detection, in any of the temperature conditions tested.

ANOVA analysis evidenced that OTA production was significantly affected by the different temperature conditions (Fig. 11).

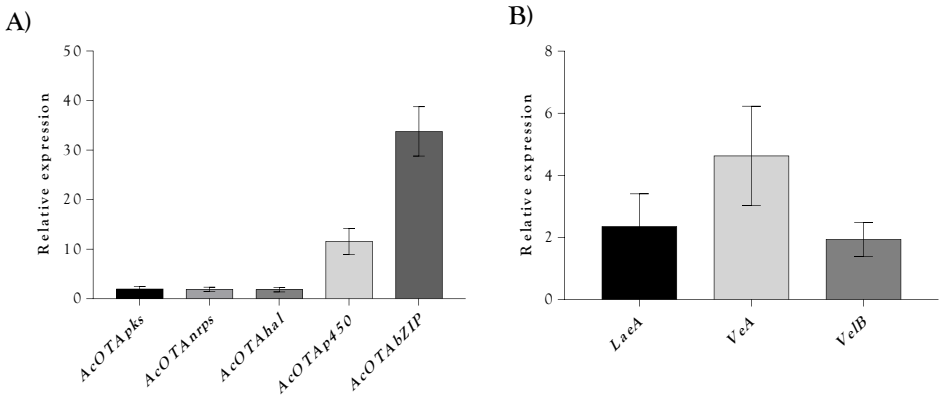


Figure 10. **A)** Relative expression of *A. carbonarius* OTA biosynthesis genes (*AcOTApks, AcOTAnrps, AcOTAhal, AcOTAp450, AcOTAbZIP)* at 18/31°C; **B)** Relative expression of the regulatory Velvet complex genes (*LaeA, VeA, VelB*) of *A. carbonarius* at 18/31°C. For each gene the expression is relative (fold change) to the expression at 20/37°C, considered as the control, at day 4 after inoculation.

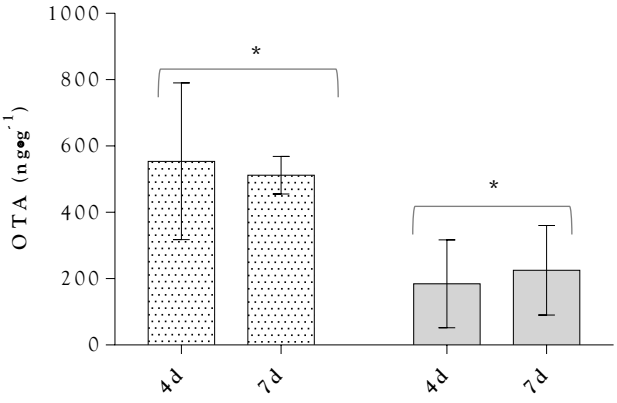


Figure 11. Ochratoxin A (OTA) production (ng·g^{-1}) by *A. carbonarius* ITEM 5010 at 18/31°C (white bars with dots) and 20/37°C (grey bars), 10h/14h dark/light at 0.99 a_w, after 4 and 7 days. Asterisks indicate significant differences between temperature conditions ($p<0.006$).

4. DISCUSSION

The studies of European Commission (2018) on adapting to climate change in Europe suggest that, in Southern Europe, changes may equate to a temperature increase of 4–5°C coupled with longer drought periods. The climate change scenario hypothesized in our study takes EU suggestions into account and is derived from climatic data series related to the high OTA risk areas of Apulia region as retrieved from "ilmeteo.it" website (https://www.ilmeteo.it/, 2017).

The combined effect of a_w and temperatures on the ecophysiology of *A. carbonarius* grown on substrates that simulate grape composition has been largely investigated (Astoreca et al., 2007; Bellí et al., 2005b; Chiotta et al., 2015), while only few studies have considered day/night alternating temperatures associated with the photoperiod (García-Cela et al., 2012; Oueslati et al., 2010). Performing *in vitro* studies which consider interacting climate change factors, may provide ecophysiological data quite similar to a real field situation. In our study, when the alternating cycle at 18°C for 10 h darkness and 31°C for 14 h light, corresponding to the nowadays climate condition in Apulia, was applied, we observed a higher growth rate and a shorter lag phase at high-water availability (0.99 a_w), compared to water stress (0.93 a_w). Our results are consistent with previous studies, reporting that the optimum for the growth of *A. carbonarius* varied from 25 to 30°C at 0.96-0.99 a_w and that water stress caused a general reduction of growth rate and extension of lag time (Astoreca et al., 2010). Moreover, Oueslati et al. (2010) studied the combined effect of three alternating temperatures (20/30, 20/37 and 25/42°C) and 11h/13h light/darkness photoperiod of *A. carbonarius* strains isolated from Tunisian grapes and grown on a Synthetic Nutrient Medium (SNM) at 0.99 a_w. They found that mycelia growth rate was significantly enhanced at 20/30°C than at 20/37°C and it was even slower at 25/42°C. This evidence supports our results in which, under water availability condition (0.99 a_w), *A. carbonarius* grew faster at 18/31°C than at 20/37°C.

In fact, at 20/37°C, simulating a climate change scenario, we observed a reduction of *A. carbonarius* growth rate at 0.99 a_w, which was in turn lower than that observed at 0.93 a_w at the same temperature, even though the mycelium appeared less sporulated and no differences were observed in terms of lag phase. These findings diverge from previous studies on *A. carbonarius*, in which it was reported that higher a_w determined a faster growth rate compared to lower values, regardless of temperature (Bellí et al., 2005b). However, other works underline how significant is the impact of the combination of a_w and temperature on fungal growth and mycotoxin production (Selouane et al., 2009).

In our study we considered the sporulation behavior of *A. carbonarius* only from the morphological point of view which revealed that a water stress condition (0.93 a_w) determined a visible reduction of sporulation compared to high water availability (0.99 a_w). In addition, a small but more sporulated colony was observed at the increased day/night temperature (20/37°C). No work has been carried out on the sporulation rate of *A. carbonarius* while few evidences exist on other mycotoxigenic fungi. Such studies suggested that middle water stress conditions (0.95-0.97 a_w) combined with constant high temperature (30-35°C) are generally suitable for spore production (Abdel-Hadi & Magan, 2009; Gervais and Molin, 2003; Giorni et al., 2008; Parra et al., 2004).

Ample evidence exists to suggest that environmental factors, like temperature and light and humidity, affect mycotoxin production by activating biosynthesis genes through a complex regulatory mechanism that occurs at several levels from cluster-specific regulators to global transcriptional complex (Keller, 2019).

Recent studies have made it possible to identify the main structural genes in the OTA biosynthesis cluster of *A. carbonarius*. The genes *AcOTApks*, *AcOTAnrps*, *AcOTAhal*, and *AcOTAp450* encoding for a polyketide synthase, a non ribosomal-peptide synthase, an halogenase and a cytochrome p450 monooxygenase, respectively, contribute to determine the molecular structure of OTA (Perrone and Gallo, 2017). In the cluster, the *AcOTAbZIP* gene, encoding for a bZIP (basic leucine zipper) transcription factor, is also present as a pathway specific regulator of OTA cluster as suggested by its transcription profile highly correlated to OTA production, observed in a previous study (Ferrara et al., 2016). An homologous *bZIP* gene has identified in the OTA cluster of *A. ochraceus* and its role as regulator of OTA biosynthesis has been corroborated by gene inactivation (Wang et al., 2018).

At 18/31°C, all the OTA genes analysed in this work showed expression levels higher than at 20/37°C. These results were positively correlated with OTA production which was higher and significantly different at 18/31°C than at 20/37°C (Fig. 4). Different studies reported that optimum conditions for the production of OTA by *A. carbonarius* are high a_w (0.95-0.99) at temperature ranging from 20°C to 30 °C (Astoreca et al., 2010; Bellí et al., 2005b; Mitchell et al., 2004; Selouane et al., 2009) however, this variation is also depending on the origin (Lasram et al., 2010) and intraspecific variability (Garcia et al., 2011) of the strains. It is generally agreed that increasing temperatures and decreasing a_w cause a reduction of OTA production by *A. carbonarius* (Bellí et al., 2005b; Marin et al., 2006). Indeed, no OTA production was observed under the limit

of detection, when we analysed *A. carbonarius* grown at 0.93 a_w at both the two temperature conditions tested and after extension of incubation time at 10 and 14 days (data not shown). The lack of OTA production at 0.93 a_w may be due to the glucose added in the medium to lower the a_w value; in fact, it was reported that increasing glucose in the medium causes a decreasing of OTA production in *A. carbonarius* (Stoll et al. 2013).

Our findings are also concordant with a research reporting that the increasing of night temperature from 20°C to 25 °C and diurnal temperature from 30°C to 42°C induced a drastic reduction in OTA accumulation (Oueslati et al., 2010). In our case, the major diurnal temperature increase (+6°C) rather than the slight nocturnal one (+2°C) could have negatively affected OTA production.

To date, very little has been reported about the molecular mechanisms by which environmental factors regulate OTA cluster activation. In a recent study on the production of OTA under the influence of temperature and a_w in *A. carbonarius*, the authors have observed that while a_w was the key factor affecting OTA production, only temperature acted as the key factor influencing transcript levels of some biosynthesis structural genes; however, no transcriptional factor internal to the cluster was investigated (Lappa et al., 2017). Generally, temperature and other abiotic factors affect mycotoxin production by acting on the transcription levels of biosynthesis genes, with the increasing production coupled to the rising expression of the structural genes. Instead, not conclusive or contradictory results about the expression levels of specific transcription factors in the cluster have been reported to date. In most of the studies on aflatoxin biosynthesis, for example, the transcript levels of the two regulatory genes, *AflR* and *AflS*, did not change significantly in comparison to the levels of structural genes, when conducive or repressive temperature conditions for mycotoxin production were applied (Gallo et al., 2016; O'Brian et al., 2007). On the contrary, we found that the highly increased expression levels of the putative transcription factor of OTA cluster (*AcOTAbZIP*) mirrored the increased expression of OTA structural genes and then the increased OTA production. Not all the genes including in the cluster of OTA biosynthesis and their corresponding functions have been identified. From the analysis of genomic sequences of ochratoxigenic *Aspergillus* species, a likely second transcription factor, a zinc finger DNA-binding protein, is present in the cluster and it could also have a role in the regulation of OTA production (Wang et al., 2018). So, the regulation of OTA biosynthesis is far to be completely clarified. Furthermore, the regulatory mechanism of secondary metabolites such as mycotoxins, is composed of overlapping and interconnected pathways, which

comprise global regulators and multiprotein complexes that respond to environmental clues. One of most important is the heterotrimeric Velvet complex (*VelB/VeA/LaeA*) that links sexual development with secondary metabolism in fungi in response to light, through a regulated spatial compartmentalization of the three proteins (Bayram et al., 2008). In details, under dark conditions, *VeA* is transported from cytoplasm to the nucleus where it reacts with *LaeA* and *VelB*, regulating production of secondary metabolites, and with *VelB*, controlling fungal sexual development (Palmer et al., 2013). In *A. carbonarius,* Crespo-Sempere et al. (2013), found that *VeA* and *LaeA* have an important role in regulating conidiation and OTA production in response to light, since the loss of *VeA* and *LaeA* led to a reduction of OTA and conidial production in mutant strains. In wild-type strain, they observed an increasing of OTA production under dark conditions and an activation of conidiation by light; however, the transcription of the *VeA* and *LaeA* genes appeared relatively constant under both light and dark conditions, suggesting that in the regulatory mechanism the subcellular localization of the protein is more important than the transcriptional level of the corresponding genes. Interestingly, in our study on *A. carbonarius* exposed to a light/dark cycle, the expression of the three genes of the global regulator complex appeared from 2 to 5 fold higher at the temperature conditions favorable to OTA production, 18/31°C, than at 20/37°C, in correlation with the transcription profile of biosynthesis genes. Actually, the role of *VeA* and more generally of the Velvet complex in the regulation of certain secondary metabolite clusters was suggested to be temperature-dependent as observed in a recent study on *A. fumigatus* under different temperature conditions (Lind et al., 2016). Therefore, the Velvet complex appears to be involved in both temperature and light based regulation of secondary metabolism in *Aspergillus*, providing support to the fact that regulation of mycotoxin production occurs in response to multiple environmental cues. The localization of VeA in the nucleus under dark conditions seems to be crucial for the Velvet complex in controlling the dark responsive secondary metabolism in fungi, while the precise mechanisms by which this complex and the interaction of its components regulate temperature influence on the secondary metabolism is still to be elucidated. A complex regulatory network involving multiple interconnecting proteins may be responsible to the response to various environmental stimuli.

With the present *in vitro* study, we investigated for the first time the behavior of *A. carbonarius* under the combined effect of two environmental factors in simulating field conditions of an area at high risk of OTA contamination, according to the expected values in a climate change scenario. On the basis of our results, we found that the possible increase of the temperature from

18/31 °C to 20/37°C 10h/14h dark/light, likely to happen in some areas of the Apulia region, may results in a reduction of both *A. carbonarius* spoilage and OTA production in grapes. Furthermore, molecular measurements gave us useful information not only as a more sensitive support to the chemical analysis, but also highlighting the complexity of the OTA biosynthesis by the involvement of some regulatory genes not necessarily located in the cluster.

5. REFERENCES

Abdel-Hadi, A., Magan, N., 2009. Influence of physiological factors on growth, sporulation and ochratoxin A/B production of the new *Aspergillus ochraceus* grouping. World Mycotoxin J. 2(4), 429-434. https://doi.org/10.3920/WMJ2009.1156

Akbar, A., Medina, A., Magan, N., 2016. Impact of interacting climate change factors on growth and ochratoxin A production by *Aspergillus* section *Circumdati* and *Nigri* species on coffee. World Mycotoxin J. 9, 863–874. https://doi.org/10.3920/WMJ2016.2041

Astoreca, A., Magnoli, C., Combina, M., Dalcero, A., 2007. Water activity and temperature effects on growth of *Aspergillus niger* , *A. awamori* and *A. carbonarius* isolated from different substrates in Argentina. Int. J. Food Microbiol. 119, 314–318. https://doi.org/10.1016/j.ijfoodmicro.2007.08.027

Astoreca, A.L., Magnoli, C.E., Dalcero, A.M., 2010. Ecophysiology of *Aspergillus* Section *Nigri* Species Potential Ochratoxin A Producers. Toxins (Basel). 2, 2593–2605. https://doi.org/10.3390/toxins2112593

Battilani, P., Toscano, P., Van Der Fels-Klerx, H.J., Moretti, A., Camardo Leggieri, M., Brera, C., Rortais, A., Goumperis, T., Robinson, T., 2016. Aflatoxin B 1 contamination in maize in Europe increases due to climate change. Sci. Rep. 6, 1–7. https://doi.org/10.1038/srep24328

Bayram, O., Krappmann, S., Ni, M., Kwon, N.-J., Braus-Stromeyer, S., Bok, J.W., Keller, N.P., Yu, J.-H., Braus, G.H., Helmstaedt, K., Valerius, O., 2008. VelB/VeA/LaeA Complex Coordinates Light Signal with Fungal Development and Secondary Metabolism. Science. 320, 1504–1506. https://doi.org/10.1126/science.1155888

Bellí, N., Ramos, A., Marín, S., 2004. Incubation time and water activity effects on ochratoxin A production by *Aspergillus* section *Nigri* strains isolated from grapes. Lett. Appl. Microbiol. 38, 72-77. http://dx.doi.org/10.1046/j.1472-765X.2003.01445.x.

Bellí, N., Mitchell, D., Marı, S., Alegre, I., Ramos, A.J., Magan, N., 2005a. Ochratoxin A-producing fungi in Spanish wine grapes and their relationship with meteorological conditions. Eur. J. Plant Pathol.133, 233–239. https://doi.org/10.1007/s10658-005-5547-4

Bellí, N., Ramos, A.J., Coronas, I., Sanchis, V., Marín, S., 2005b. *Aspergillus carbonarius* growth and ochratoxin A production on a synthetic grape medium in relation to environmental factors. J.

Appl. Microbiol. 98, 839–844. https://doi.org/10.1111/j.1365-2672.2004.02469.x

Bellí, N., Ramos, A.J., Sanchis, V., Marín, S., 2006. Effect of photoperiod and day-night temperatures simulating field conditions on growth and ochratoxin A production of Aspergillus carbonarius strains isolated from grapes. Food Microbiol. 23, 622–627. https://doi.org/10.1016/j.fm.2005.12.007

Botana, L.M., Sainz, M.J., 2015. Climate change and mycotoxins; Walter de Gruyter GmbH: Berlin, Germany

Cabañes, F.J., Bragulat, M.R., 2018. Black aspergilli and ochratoxin A-producing species in foods. Curr. Opin. Food Sci. 23, 1–10. https://doi.org/10.1016/j.cofs.2018.01.006

Chiotta, M.L., Sosa, D.M., Ponsone, M.L., Chulze, S.N., 2015. Effect of water activity and temperature on growth of *Aspergillus carbonarius* and *Aspergillus tubingensis* and their interactions on ochratoxin A production. World Mycotoxin J. 8, 99–105. https://doi.org/10.3920/WMJ2013.1692

Crespo-Sempere, A., Marín, S., Sanchis, V., Ramos, A.J., 2013. VeA and LaeA transcriptional factors regulate ochratoxin A biosynthesis in *Aspergillus carbonarius*. Int. J. Food Microbiol. 166, 479–486. https://doi.org/10.1016/j.ijfoodmicro.2013.07.027

European Commission, 2006. Commission Regulation (EC) No 1881/2006 of 19 December 2006 on setting the maximum levels for certain contaminants in foodstuffs. Off. J. Eur. Union, L364, 5–24.

EU Science Hub, 2018. Worrying effects of accelerating climate change on the Mediterranean Basin. Available at: https://ec.europa.eu/jrc/en/science-update/worrying-effects-accelerating-climate-change-mediterranean-basin

Ferrara, M., Perrone, G., Gambacorta, L., Epifani, F., Solfrizzo, M., 2016. Identification of a Halogenase Involved in the Biosynthesis of Ochratoxin A in *Aspergillus carbonarius*. Appl. Environ. Microbiol. 82, 5631–5641. https://doi.org/10.1128/AEM.01209-16

Gallo, A., Knox, B.P., Bruno, K.S., Solfrizzo, M., Baker, S.E., Perrone, G., 2014. Identification and characterization of the polyketide synthase involved in ochratoxin A biosynthesis in *Aspergillus carbonarius*. Int. J. Food Microbiol. 179, 10-17. https://doi.org/10.1016/j.ijfoodmicro.2014.03.013.

Gallo, A., Solfrizzo, M., Epifani, F., Panzarini, G., Perrone, G., 2016. Effect of temperature and water activity on gene expression and aflatoxin biosynthesis in *Aspergillus flavus* on almond medium. Int. J. Food Microbiol. 217, 162–169. https://doi.org/10.1016/j.ijfoodmicro.2015.10.026

García-Cela, E., Ramos, A.J., Sanchis, V., Marin, S., 2012. Ochratoxigenic moulds and effectiveness of grape field antifungals in a climatic change scenario. J. Sci. Food Agric. 92, 1455–1461. https://doi.org/10.1002/jsfa.4726

Garcia, D., Ramos, A.J., Sanchis, V., Marín, S., 2011. Is intraspecific variability of growth and mycotoxin production dependent on environmental conditions? A study with *Aspergillus carbonarius* isolates. Int. J. Food Microbiol. 144, 432–439. https://doi.org/10.1016/j.ijfoodmicro.2010.10.030

Gervais, P., Molin, P., Grajek, W., Bensoussan, M., 1988. Influence of the water activity of a solid substrate on the growth rate and sporogenesis of filamentous fungi. Biotechnol. Bioeng. 31, 457-63. https://doi.org/10.1002/bit.260310510.

Giorni, P., Battilani, P., Magan, N., 2008. Effect of solute, matric potential and temperature on in vitro growth and sporulation of strains from a new population of *Aspergillus flavus* isolated in Italy. Fungal ecology, 1, 102-108. http://dx.doi.org/10.1016/j.funeco.2008.07.001.

International Agency for Research on cancer (IARC), 1993. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. Monographs on the evaluation of carcinogenic risks to humans. Vol. 56, Lyon, France.

Ioannidis, A., Kogkaki, E.A., Natskoulis, P.I., Nychas, G.E., Panagou, E.Z., 2015. Modelling the in fl uence of temperature , water activity and sodium metabisulphite on the growth and OTA production of *Aspergillus carbonarius* isolated from Greek wine grapes. Food Microbiol. 49, 12–22. https://doi.org/10.1016/j.fm.2015.01.006

Keller, N.P. 2019. Fungal secondary metabolism: regulation, function and drug discovery. Nat. Rev. Microbiol. 17(3),167-180. https://doi.org/10.1038/s41579-018-0121-1

Lappa, I.K., Kizis, D., Panagou, E.Z., 2017. Monitoring the Temporal Expression of Genes Involved in Ochratoxin A Production of *Aspergillus carbonarius* under the Influence of Temperature and Water Activity. Toxins, 9(10), 296. https://doi.org/10.3390/toxins9100296

Lasram, S., Oueslati, S., Valero, A., Marin, S., Ghorbel, A., Sanchis, V., 2010. Water Activity and Temperature Effects on Fungal Growth and Ochratoxin A Production by Ochratoxigenic *Aspergillus carbonarius* Isolated from Tunisian Grapes. J. Food Sci. 75, M89–M97. https://doi.org/10.1111/j.1750-3841.2009.01484.x

Leong, S.L., Emmett, R.W., Hocking, A.D., Pitt, J.I., Kazi, B.A., Scott, E.S., 2006. Black *Aspergillus* species in Australian vineyards: from soil to ochratoxin A in wine. Adv. Exp. Med. Biol. 571, 153–171. https://doi.org/10.1007/0-387-28391-9_9

Lind, A.L., Smith, T.D., Saterlee, T., Calvo, A.M., Rokas, A., 2016. Regulation of Secondary Metabolism by the Velvet Complex Is Temperature-Responsive in *Aspergillus*. G3 (Bethesda). 6, 4023–4033. https://doi.org/10.1534/g3.116.033084

Magan, N., Hope, R., Cairns, V., Aldred, D., 2003. Post-harvest fungal ecology : Impact of fungal growth and mycotoxin accumulation in stored grain. Eur. J. Plant Pathol. 7, 723–730. https://doi.org/10.1023/A:1026082425177

Marín S., Bellí N., Lasram S., Chebil S., Ramos A.J., Ghorbel A., Sanchis V., 2006. Kinetics of ochratoxin A production and accumulation by *Aspergillus carbonarius* on synthetic grape medium at different temperature levels. J. Food Sci. 71, 196–200. https://doi.org/10.1111/j.1750-3841.2006.00098.x

Medina, A., Rodríguez, A., Magan, N., 2014. Effect of climate change on *Aspergillus flavus* and aflatoxin B1 production. Front Microbiol. 5, 348. https://doi.org/10.3389/fmicb.2014.00348

Medina, A., Rodríguez, A., Magan, N., 2015. Climate change and mycotoxigenic fungi: Impacts on mycotoxin production. Curr. Opin. Food Sci. 5, 99–104. https://doi.org/10.1016/j.cofs.2015.11.002

Miraglia, M., Marvin, H.J.P., Kleter, G.A., Battilani, P., Brera, C., Coni, E., Cubadda, F., Croci, L., Santis, B. De, Dekkers, S., Filippi, L., Hutjes, R.W.A., Noordam, M.Y., Pisante, M., Piva, G., Prandini, A., Toti, L., Born, G.J. Van Den, Vespermann, A., 2009. Climate change and food safety : An emerging issue with special focus on Europe. Food Chem. Toxicol. 47, 1009–1021. https://doi.org/10.1016/j.fct.2009.02.005

Mitchell, D., Parra, R., Aldred, D., Magan, N., 2004. Water and temperature relations of growth and ochratoxin A production by *Aspergillus carbonarius* strains from grapes in Europe and Israel. J. Appl. Microbiol. 97 (2), 439–445. https://doi.org/10.1111/j.1365-2672.2004.02321.x

O'Brian, G.R., Georgianna, D.R., Wilkinson, J.R., Yu, J., Abbas, H.K., Bhatnagar, D., Clevland, T.E., Nierman, W., Payne, G.A., 2007. The effect of elevated temperature on gene transcription and aflatoxin biosynthesis. Mycologia, 99, 232-239. https://doi.org/10.1080/15572536.2007.11832583

Oueslati, S., Lasram, S., Marin, S., Ramos, A.J., Mliki, A., Sanchis, V., Ghorbel, A., 2010. Alternating temperatures and photoperiod effects on fungal growth and Ochratoxin A production by *Aspergillus carbonarius* isolated from Tunisian grapes. Int. J. Food Microbiol. 139, 210–213. https://doi.org/10.1016/j.ijfoodmicro.2010.02.003

Palmer, J.M., Theisen, J.M., Duran, R.M., Grayburn, W.S., Calvo, A.M., Keller, N.P., 2013. Secondary Metabolism and Development Is Mediated by LlmF Control of VeA Subcellular Localization in *Aspergillus nidulans*. PLoS Genet. 9, e1003193. https://doi.org/10.1371/journal.pgen.1003193

Parra, R., Aldred, D., Archer, D., Magan, N., 2004. Water activity, solute and temperature modify growth and spore production of wild type and genetically engineered *Aspergillus niger* strains. Enzyme Microb. Technol. 35, 232-237. https://doi.org/10.1016/j.enzmictec.2004.04.012.

Perrone, G., Gallo, A., Susca, A., Varga, J., 2008. *Aspergillus* in grapes: Ecology, biodiversity and genomics. In: Varga, J., Samson, R.A. (Eds.), *Aspergillus in the Genomic Era*. Wageningen Academic Publishers, Wageningen, pp. 79-212. https://doi.org/10.3920/978-90-8686-635-9

Perrone G., Gallo A., 2017. *Aspergillus* Species and Their Associated Mycotoxins. In: Moretti A., Susca A. (Eds.), Mycotoxigenic Fungi. Methods in Molecular Biology, Humana Press, New York, pp.33-49. https://doi.org/10.1007/978-1-4939-6707-0_3

Selouane, A., Bouya, D., Lebrihi, A., Decock, C., Bouseta, A., 2009. Impact of some environmental factors on growth and production of ochratoxin A of/by *Aspergillus tubingensis*, *A. niger*, and *A. carbonarius* isolated from moroccan grapes. J. Microbiol. 47, 411–419. https://doi.org/10.1007/s12275-008-0236-6

Somma, S., Perrone, G., Logrieco, A.F., 2012. Diversity of black aspergilli and mycotoxin risks in grape, wine and dried vine fruits. Phytopathol. Mediterr. 51, 131–147. https://doi.org/10.14601/Phytopathol_Mediterr-9888

Stoll, D., Schmidt-Heydt, M., Geisen, R., 2013. Differences in the Regulation of Ochratoxin A by the HOG Pathway in *Penicillium* and *Aspergillus* in Response to High Osmolar Environments.Toxins, 5(7), 1282-1298. https://doi.org/10.3390/toxins5071282.

Vaughan, M.M., Huffaker, A., Schmelz, E.A., Dafoe, N.J., Christensen, S., Sims, J., Martins, V.F., Swerbilow, J., Romero, M., Alborn, H.T., Allen, L.H., Teal, P.E.A., 2014. Effects of elevated [CO2] on maize defence against mycotoxigenic *Fusarium verticillioides*. Plant, Cell Environ. 37, 2691–2706. https://doi.org/10.1111/pce.12337

Visconti, A., Perrone, G., Cozzi, G., Solfrizzo, M., 2008. Managing ochratoxin A risk in the grape-wine food chain. Food Addit. Contam. - Part A Chem. Anal. Control. Expo. Risk Assess. 25, 193–202. https://doi.org/10.1080/02652030701744546

Wang, Y., Wang, L., Wu, F., Liu, F., Wang, Q., Zhang, X., Selvaraj, J.N., Zhao, Y., Xing, F., Yin, W.B., Liu, Y., 2018. A Consensus Ochratoxin A Biosynthetic Pathway: Insights from the Genome Sequence of *Aspergillus ochraceus* and a Comparative Genomic Analysis . Appl. Environ. Microbiol. 17, 84 (19). https://doi.org/10.1128/aem.01009-18

ADDENDUM

In addition, 11.5h/12.5h dark/light current (15/28°C) and increased (18/34°C) temperature cycles of North Apulia area were tested on the ecophysiology, toxin gene expression and OTA production by *A. carbonarius* ITEM 5010. The same experimental approach previously reported in "Material and Methods" of Study I, was followed. Hereafter some of the main results obtained.

- **Lag phase, growth rate and sporulation**

As shown in Figure 12A, the drought stress (0.93 a_w) caused a general increase of the lag phases compared to high water availability (0.99a_w) both at 15/28°C and 18/34°C. Moreover, at 0.93a_w, 15/28°C caused a significantly longer phase (58 h) compared to 18/34°C (38 h). At 0.99a_w, the lag phases exhibit no significant differences when the temperature cycles were changed. As regards growth rate (Figure 12B), at 0.99 a_w, both at 15/28°C and 18/34°C, they were significantly faster than at 0.93 a_w with the highest value observed at 15/28°C (14 mm·day $^{-1}$).

The effect of both temperature and water activity variations, was macroscopically evident comparing the plates at 7 days after inoculation, as shown in Figure 13. Water stress resulted in a less sporulated mycelium compared to 0.99 a_w while temperature variations reflected in a different shape of the colonies.

ANOVA analysis showed that both lag phase and growth rate were significantly influenced ($p<0.001$) by temperature, water activity and their interaction (data not shown).

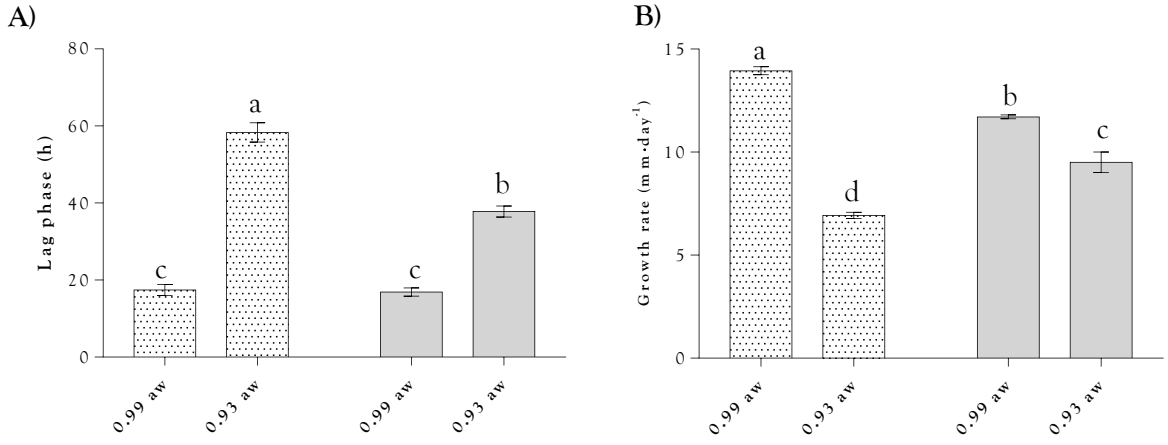


Figure 12. A) Lag phases (h) of *A. carbonarius* ITEM 5010 grown on GJM at 0.99 and 0.93 a_w and incubated at 15/28°C (white bars with dots) and 18/34°C (grey bars), 11.5h/12.5h dark/light photoperiod. **B)** Growth rate (mm·day $^{-1}$) of *A. carbonarius* ITEM 5010 grown on GJM at 0.99 and 0.93 a_w and incubated at 15/28°C (white bars with dots) and 18/34°C (gray bars), 11.5h/12.5h dark/light photoperiod. Letters indicate significant differences (Tukey HSD, $p<0.05$).

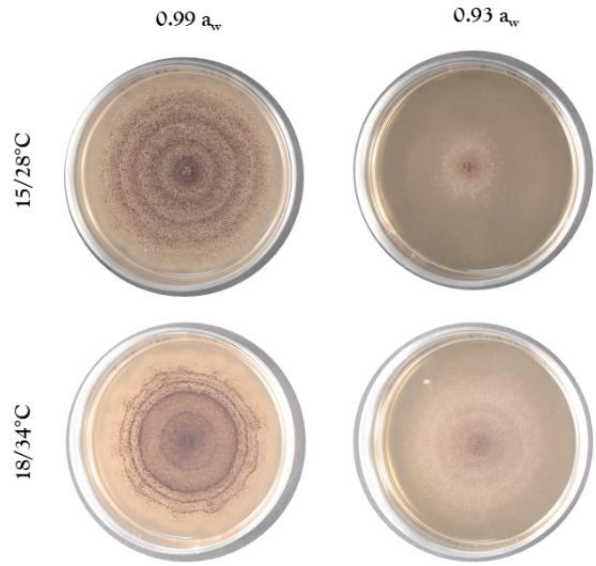


Figure 13. Pictures of *A. carbonarius* ITEM 5010 colonies centrally inoculated taken at 7 dpi at 15/28°C and 18/34°C, 11.5h/12.5h dark/light, 0.99 and 0.93 a_w.

- **Gene expression and OTA production**

The effect of the two alternating day/night temperature on the expression of both biosynthetic and regulatory genes in *A. carbonarius* was analysed after 4 days of growth at 0.99 a_w, which seems to be the most reliable time to detect the activation of the genes involved in OTA biosynthesis, preceding the later increase of OTA production. As shown in Figure 14, a general up-regulation of both structural (*AcOTApks, AcOTAnrps, AcOTAhal* and *AcOTAp450*) and transcription factor (*AcOTAbZIP*) of OTA related genes was observed at 18/34°C compared to 15/28°C. In particular, transcription levels resulted to be almost unchanged for the gene encoding the cytochrome *p450* oxidase protein and 10-fold higher for the NRPS encoding gene. The genes *laeA*, *veA*, and *velB*, belonging to the global transcriptional complex regulating fungal secondary metabolism known as Velvet complex, showed a higher level of expression between 2 and 6-fold at 18/34°C temperature cycle as well (Fig. 14B).

Gene expression analysis performed after 4 days of incubation, was supported by the results of OTA production (Fig. 15).

At 0.99 a_w, the highest OTA production was observed in *A. carbonarius* grown at 18/34°C after 4 days (2478 ng·g $^{-1}$). Under strong water stress conditions (0.93 a_w), *A. carbonarius* appeared unable to produce OTA, whose amount was, under the limit of detection, in any of the temperature conditions tested.

ANOVA analysis evidenced that OTA production was significantly affected by the different temperature conditions (Fig. 15).

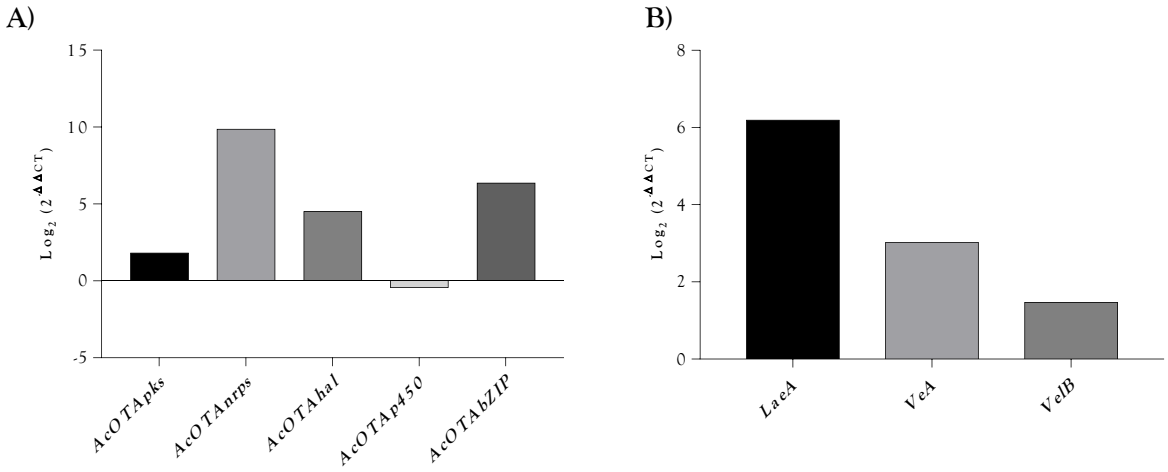


Figure 14. **A)** Fold changes ($Log_2^{(2-\Delta\Delta CT)}$) of *A. carbonarius* OTA structural genes (*AcOTApks, AcOTAnrps, AcOTAhal, AcOTAp450, AcOTAbZIP)* at 18/34°C; **B)** Relative expression of the regulatory Velvet complex genes (*LaeA, VeA, VelB*) of *A. carbonarius* at 18/34°C. For each gene the expression is relative to the expression at 15/28°C, considered as the control, at day 4 after inoculation.

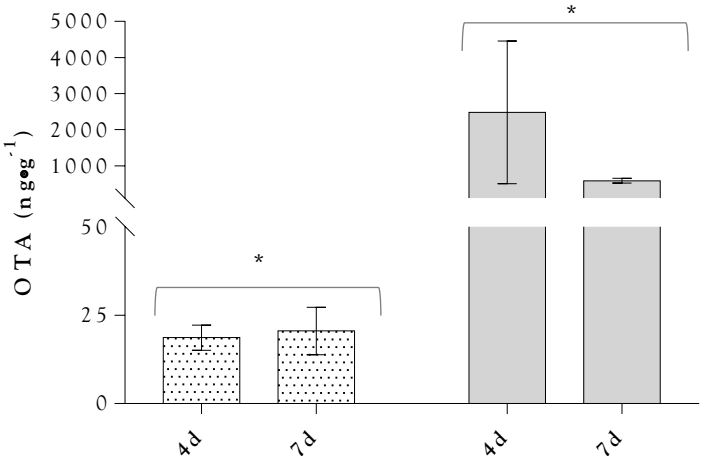


Figure 15. Ochratoxin A (OTA) production (ng·g^{-1}) by *A. carbonarius* ITEM 5010 at 15/28°C (white bars with dots) and 18/34°C (grey bars), 11.5h/12.5h dark/light at 0.99 a_w, after 4 and 7 days. Asterisks indicate significant differences between temperature conditions (p=0.029).

Based on our experimental results, the effect of expected increased temperature (18/34°C) would probably result in a slight decrease of *A. carbonarius* spoilage in the vineyards and in a possible increase of the ochratoxigenic risk in the North Apulia area. In fact, under this condition a significantly higher amount of OTA was detected, especially after 4 days of incubation. Moreover, gene expression analysis supported our chemical results, suggesting the early activation of both structural (*AcOTApks*, *AcOTAnrps*, *AcOTAhal*, *AcOTAbZIP*) and regulatory (*laeA*, *veA*, *velB*) genes of OTA pathway which paralleled the phenotypic OTA production.

Such interesting evidences gave us the hints for more investigations. Indeed, 11.5h/12.5h dark/light 15/28°C and 18/34°C temperature cycles, resulted in low and increased ochratoxigenic risk, respectively, were selected for further experiments in which CO_2, was included. The interacting effect of temperature and CO_2 variations was object of the activities performed during the stay at Cranfield University (United Kingdom) and will be discussed thereafter (Study II).

STUDY II

Interacting climate change factors (CO_2 and temperature cycles) effects on growth, secondary metabolite gene expression and phenotypic ochratoxin A production by *Aspergillus carbonarius* strains on a grape-based matrix

ABSTRACT

Little is known on the impact that climate change (CC) may have on *Aspergillus carbonarius* and Ochratoxin A (OTA) contamination of grapes, especially in the Mediterranean region - a hot spot for the impact of CC scenarios with temperature expected to increase by +2-5°C and CO_2 to double or triple (400 vs 800/1200 ppm). This study examined the effect of (i) current and increased temperature in the alternating 11.5h dark/12.5h light cycle (15/28°C vs 18/34°C), representative of the North Apulia area, South Italy and (ii) existing and predicted CO_2 concentrations (400 vs 1000 ppm), on growth, expression of biosynthetic genes (*AcOTApks*, *AcOTAnrps*, *AcOTAhal*, *AcOTAp450*, *AcOTAbZIP*) and regulatory genes of Velvet complex (*laeA/veA/velB*, "velvet complex") involved in OTA biosynthesis and OTA phenotypic production by three strains of *A. carbonarius*. The experiments made on a grape-based matrix showed that elevated CO_2 resulted in a general stimulation of growth and OTA production. These results were supported by the up-regulation of both structural and regulatory genes involved in the OTA biosynthesis in elevated CO_2 condition. Our work has shown for the first time that elevated CO_2 concentration in the Mediterranean region may result in an increased risk of OTA contamination in the wine production chain.

In *Fungal Biology*, in press

1. INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin produced by *Aspergillus* and *Penicillium* species (Frisvad et al., 2004a,b) responsible for contaminating many foodstuffs including cereals (Scudamore et al., 2004), coffee (Taniwaki et al., 2003), cocoa (Bonvehí, 2004), grapes and derivatives (Abrunhosa et al., 2001; Varga and Kozakiewicz, 2006; Zimmerli and Dick, 1996). Many studies have shown that the *Aspergillus* section *Nigri*, commonly known as "Black Aspergilli", are responsible for OTA contamination of grapes (García-Cela et al., 2016; Palumbo et al., 2011; Battilani et al., 2003). In particular, *A. carbonarius* is considered the major species responsible, with other closely related biseriate species, *A. niger* and *A. welwitschiae* of less importance (Perrone et al., 2008; Cabañes and Bragulat, 2018). OTA is a potent nephrotoxin, classified as a possible human carcinogen (Group 2B) by the International Agency for Research on Cancer (IARC, 1993). Based on toxicological and exposure data, the European Commission, has established maximum levels for the presence of OTA in wine, fruit wine, grape juice, grape nectar and grape must for human consumption (2 $\mu g \cdot kg^{-1}$) and dried vine fruits (10 $\mu g \cdot kg^{-1}$) (EC, 2006 a). OTA contamination of grapes is strongly related to plant phenology, geographical area, meteorological conditions/microclimate and varies from season to season (Visconti et al., 2008; Somma et al., 2012). Indeed, climate represents the key-factor in the agro-ecosystem that influences fungal colonization and mycotoxin production in crops (Magan et al., 2003). Based on the 5th Report of the Intergovernmental Panel on Climate Change (CC) (IPCC, 2014), anthropogenic Green House Gases (GHG) emissions have increased since the pre-industrial era, driven by economic and population growth. Their effects have been one of the dominant causes of the observed global warming since the mid-20th century. Based on recent prediction model, atmospheric concentration of CO_2 is expected to double or triple (from 400 to 800-1200 ppm) and temperature to increase (+2-5°C), in the next 25-50 years.

Such CC scenarios are projected to have a profound effect on the productivity and quality of crop and livestock production world-wide exacerbating food insecurity especially in areas vulnerable to hunger and undernutrition (Wheeler et al., 2013). In addition, food safety could be compromised, with mycotoxins being one of the most important hazards to be most likely affected by CC (Miraglia et al., 2009; Medina et al., 2017). In this regard, numerous studies based on the development of predictive models (Van Der Fels-Klerx et al., 2012, Van Der Fels-Klerx et al., 2016; Battilani et al., 2012; Battilani et al., 2016) have tried to estimate the changes in risks of mycotoxin contamination of staple crops due to possible CC scenarios. These studies mainly focused on an increase in temperature of +2 or +5°C in Europe and the related changes in the

contamination of wheat by deoxynivalenol (DON) (Van Der Fels-Klerx et al., 2012) and of maize by aflatoxin B_1 (AFB_1) (Battilani et al., 2012). However, such studies did not include an evaluation of CO_2 increasing level and the interactions between temperature and elevated CO_2. A study by Medina et al. (2015) showed that interactions between increased temperature (+4°C), elevated CO_2 (350 vs 650 or 1000 ppm CO_2) and drought stress had little effect on growth but significant effects on AFB_1 production in *A. flavus*, as confirmed by gene expression analysis of some structural and regulatory genes involved in toxin biosynthesis. A similar study has been carried out on *A. carbonarius* in relation to changes in temperature and elevated CO_2 on growth and OTA production by Akbar et al. (2016). They found that on coffee-based media and stored coffee beans there was more impact of CC on OTA production in *Aspergillus westerdijkiae* than in *A. carbonarius*. However, no detailed studies have been carried out in relation to grape matrices and different day/night temperature cycles.

An examination of the relative risk of OTA contamination of grapes by the European Commission (2002) reported that there was an increasing gradient of the incidence and OTA levels from Northern (50.3% and 0.18 $\mu g \cdot kg^{-1}$) to Southern (72.3% and 0.64 $\mu g \cdot kg^{-1}$) European countries. Similarly, wines produced in Southern Italy, where climatic conditions favor the growth of OTA-producing fungi in grapes, has generally resulted in higher OTA contamination levels (Visconti et al., 2008) than those produced in Central and Northern Italy. Moreover, in Apulia region (Southern Italy) higher OTA contamination levels have been reported in red wines produced in Southern area (e.g. Primitivo, Negramaro) historically more affected by the presence of OTA than those produced in the Northern area (e.g. Nero di Troia, Aglianico) (Perrone et al., 2007).

In our opinion, the understanding of whether changes in CC scenarios could increase the risk of OTA contamination is of crucial interest in this important wine producing region of Italy. Indeed, the objectives of this study were to examine the effect of two different temperature cycles (15/28 vs 18/34°C, 11.5h/12.5h dark/light), representative of the North Apulia, which is an area not yet affected by severe OTA contamination levels, and interaction with existing and future CO_2 exposure concentrations (400 vs 1000 ppm) on (a) growth rate, (b) gene expression of toxin biosynthesis related genes (*AcOTApks, AcOTAnrps, AcOTAhal, AcOTAp450, AcOTAbZIP)* and the regulatory "Velvet complex" (*laeA/veA/velB*), and (c) phenotypic OTA production, in different strains of *A. carbonarius*.

2. MATERIALS AND METHODS

2.1. Fungal strains

Three OTA-producing strains of *A. carbonarius* (ITEM 7444, ITEM 5010 and ITEM 18515) isolated from Italian wine grapes from Apulia region and held in the Agri-Food Toxigenic Fungi Culture Collection of the Institute of Sciences of Food Production, CNR, Bari (www.ispa.cnr.it/Collection), were selected.

2.2. Media preparation and inoculation

A basal Grape Juice Medium (GJM; 0.99 water activity (a_w)) was prepared by diluting (50% v/v) a commercial pasteurized grape juice (composition per 100 mL: fat, 0.02 g; proteins, 0.3 g; carbohydrates, 16.10 g) with distilled water and supplemented with 1.2% agar (Ioannidis et al., 2015). The a_w of GJM was measured using the AquaLab 4TE water activity meter. The pH of the medium was adjusted to 3.5 using 1M KOH.

Fungal inocula were prepared from fungal colonies grown on Potato Dextrose Agar (PDA) plates for 7 days at 25°C. A sterile loop was used to dislodge conidia and place them into 9 mL of sterile water containing a 0.05% Tween 80 in a 25 mL glass Universal bottle. The concentration was quantified with a haemocytometer and diluted with sterile water to obtain a conidial suspension of 10^5 conidia·mL^{-1} for each *A. carbonarius* strain. Each conidial suspension was used to centrally inoculate the GJM plates previously overlaid with sterile cellophane membranes (8.5 cm diameter). Fungal mycelium was harvested by scraping cellophane membrane surfaces after 4 and 7 days post inoculation (dpi), and stored at -20°C for OTA content analysis or -80°C for RNA extraction. Experiments were carried out with three replicates per treatment and repeated twice.

2.3. Incubation conditions and CO_2 treatment

The *in vitro* agar cultures were placed in 12-L air tight containers and incubated in a dynamic climatic chamber (SANYO Electric Co., Ltd., MLR-350H) at two different 11.5h/12.5h dark/light temperature cycles simulating nowadays (15/28°C) and CC scenario (18/34°C) of the Northern area of the Apulia region. The containers included a beaker (500 mL) of water in order to minimize moisture loss. They were flushed twice a day either with current (400 ppm) or increased (1000 ppm) CO_2 concentrations by using specialty grade mixtures gas cylinders (British Oxygen Company, Guildford, Surrey, UK), with a flow rate of 3 L·min^{-1} in order to renew 3x the air volume of the incubation chambers (Verheecke-Vaessen et al., 2019).

2.4. Growth measurements

Fungal growth was assessed daily until 7 dpi and expressed as the average measurement (mm) of two orthogonal diameters of centrally inoculated colonies. The colony diameters (mm) were plotted against time (days) and the linear regression model was used for calculating the growth rate ($mm \cdot day^{-1}$) and the lag phase prior to growth (hours). The square of the linear correlation coefficient was ≥ 0.98.

2.5. RNA extraction, cDNA synthesis and qRT-PCR

Total RNA was extracted from frozen mycelium by using the Spectrum™ Plant Total RNA kit (Sigma-Aldrich, St Louis, Missouri, U.S.A.), according to the manufacturer's instructions. RNA concentration was determined by using Genova Nano (Jenway, Stone, UK) and its integrity was checked on 1% agarose gel. First strand cDNA was synthesized using 1 µg of total RNA with the Omniscript® Reverse Transcription kit (Qiagen, Hilden, Germany), following the manufacturers' instructions.

The expression levels of the biosynthetic (*AcOTApks, AcOTAnrps, AcOTAhal, AcOTAp450, AcOTAbZIP*) and regulatory "Velvet complex" (*laeA/veA/velB*) genes of *A. carbonarius* were analyzed by using quantitative reverse transcription-PCR (qRT-PCR). The *β-tubulin* gene was used as an internal reference. Nucleotide sequences of primers used in the qPCR assays are shown in the Table 1. qPCR reactions were performed using the Viia 7 Real Time PCR (Thermo Fisher Scientific, Waltham, USA) system programmed to hold at 50°C for 2 min and at 95°C for 2 min and to complete 40 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s. Specificity of the PCR amplifications was confirmed by dissociation curve analysis and qPCR efficiency of each oligonucleotide pair was calculated from each linear regression of standard curves. Real time PCR experiments were conducted in 96-well plates by using SYBR Select 2x MasterMix (Thermo-Fisher Scientific, Waltham, USA) and different concentrations of primers pairs, as reported in Table 8, for each gene. Data analysis was assessed using QuantStudio™ RT-PCR Software (Applied Biosystems, version 1.3).

Relative gene expression was assayed in triplicate for each individual biological replicate. The relative quantification of gene expression was established using the comparative $2^{-\Delta\Delta CT}$ method (Livak and Schimittgen, 2001) and fold changes at 1000 ppm were expressed as $\log_2 (2^{-\Delta\Delta CT})$. Significant differences between fold changes at 1000 ppm vs 400 ppm (control) for each gene, were assessed by using Sidak's multiple comparison test (Table 10).

Table 8. Primers sequences for qRT-PCR used in this study.

Primer	Concentration	Sequence (5'-3')	Reference
RT_OTApks_Ac_FOR	200 nM	CGTGTCCGATACTGTCTGTGA	Gallo et al., 2014
RT_OTApks_Ac_REV	200 nM	GCATGGAGTCCTCAAGAACC	
RT_nrps_Ac_FOR	200 nM	ACGGGTCGCTGCTCTATATC	Ferrara et al., 2016
RT_nrps_Ac_REV	200 nM	ACTCACCACATCAACCACGA	
RT_hal_Ac_FOR	200 nM	GAACGCCAGTAGAGGGACAG	Ferrara et al., 2016
RT_hal_Ac_REV	200 nM	ATGGAGGTGGTGTTGTTGTG	
RT_AcOTAp450_F	200 nM	GTGGTTATCCCGCCCAATAC	Ferrara et al., 2016
RT_AcOTAp450_R	200 nM	TGCCAGATTCATCCCGATAC	
RT_Ac_OTAbZIP_for	250 nM	AATGGAACCAGCATTGATCTC	Ferrara et al., 2016
RT_Ac_OTAbZIP_rev	250 nM	GACCCAAGCATTCGCTCTA	
RT_AClaeA-1F	150 nM	AATGGGACCGCAATGAGTC	This study
RT_AClaeA-2R	150 nM	TCCTGCTCCTGTTCGTCAC	
RT_Ac_veA_FOR	150 nM	GGTGAATGAGACCGAGCA	This study
RT_Ac_veA_REV	150 nM	GCATTGTAGGCGAAGGTGA	
RT_Ac_VelB_For	150 nM	AGTGCGTTCCGACTGACTG	This study
RT_Ac_VelB_Rev	150 nM	TGGACTGATTACCGACATTTACA	
RT3 BT Ac_F	200 nM	CAAACCGGCCAGTGTGGTA	Ferrara et al., 2016
RT3 BT Ac_R	200 nM	CGGAGGTGCCATTGTAAACA	

2.6. Ochratoxin A analysis

For the quantification of OTA in fungal cultures, HPLC-FLD analysis was performed. About 100 mg of freeze-dried mycelium were extracted with 2 mL of a mixture of methanol:acetonitrile:water (30:30:40 v/v/v) by shaking for 2 h at room temperature. After extraction, samples were centrifuged for 15 min at 4500 rpm and 600 µL of supernatant were diluted with 400 µL of acetonitrile:water:acetic acid (35.0:62.5:2.5 v/v/v) and vortexed for 30 s. The diluted extract was filtered (0.22 µm) and 100 µL were injected into the HPLC-FLD apparatus. The mobile phase was an isocratic mixture of acetonitrile:water (45:55 v/v) containing 1% acetic acid. The HPLC-FLD apparatus was a 1100 series liquid chromatography (LC) system comprising a binary pump, an autosampler, and a fluorescence detector (excitation wavelength, 333 nm; emission wavelength, 460 nm) from Agilent Technologies (Waldbronn, Germany). The column used was a Zorbax Eclipse Plus C18, 150 mm × 4.6 mm, 3.5 µm particles (Agilent, USA). The flow rate of the mobile phase was 1 mL·min^{-1}, with a run of 15 minutes. The retention time for OTA was 9.5 minutes. The standard solution of OTA in acetonitrile were purchased from Romer Labs Diagnostic GmbH (Tulln, Austria). Calibration solutions of OTA were prepared in HPLC mobile phase, and calibration curves were prepared in the range of 3.9 ng·mL^{-1} to 1000

ng·mL^{-1}. The volume of injection was 10 µL both for the samples and the standards. The limit of detection and quantification were 1.50 ng·g^{-1} and 5 ng·g^{-1}, respectively.

2.7. Data analysis

Statistical analyses were performed using GraphPad Software (version 7.0, Prism, La Jolla California, USA). After checking the normal distribution of datasets using the Shapiro-Wilk test, the Analysis of Variance (ANOVA) was performed in order to test the effect of each single variable (CO_2, temperature) and their interaction (temperature x CO_2). Furthermore, significant differences among the means were established by using Tukey HSD *post hoc* test ($p \leq 0.05$).

3. RESULTS

3.1. Growth measurements

Figure 16 shows the effect of temperature cycles and exposure to either 400 or 1000 ppm CO_2 on the lag phases (h) prior to growth and the relative growth rates ($mm \cdot day^{-1}$) of the three *A. carbonarius* strains. Overall, there was a reduction in the lag phases when 2.5x higher CO_2 concentration was imposed. Under the combined effect of the temperature cycle of 15/28°C x 1000 ppm, the lag phases of two strains (ITEM 7444, ITEM 18515) were significantly shorter than those observed in the 15/28°C cycle x 400 ppm. The ANOVA analysis (Table 9) showed that CO_2 variation significantly impacted on lag phases prior to growth of all the three *A. carbonarius* strains, while the temperature cycle and its interaction with CO_2 had a significant effect only on the lag phases of the strain ITEM 7444.

Data on the relative growth rates (see Figure 16) showed that the three *A. carbonarius* strains behaved similarly and the exposure to increased CO_2 concentration (1000 ppm) resulted in faster growth compared to the current CO_2 level (400 ppm). In addition, when the temperature cycle was elevated to 18/34°C, growth rates were significantly higher than those observed in the 15/28°C cycle. The different ecophysiological behavior of *A. carbonarius* strains, both in terms of growth and sporulation, under the combined effect of two temperature cycles and CO_2 air concentrations, was evident on 7 days centrally inoculated plates (Figure 17). Statistical analyses showed that single factors (temperature cycle, CO_2) had a significant impact on the growth of all three strains, while their interaction (temperature cycle x CO_2) did not have a significant effect (Table 9).

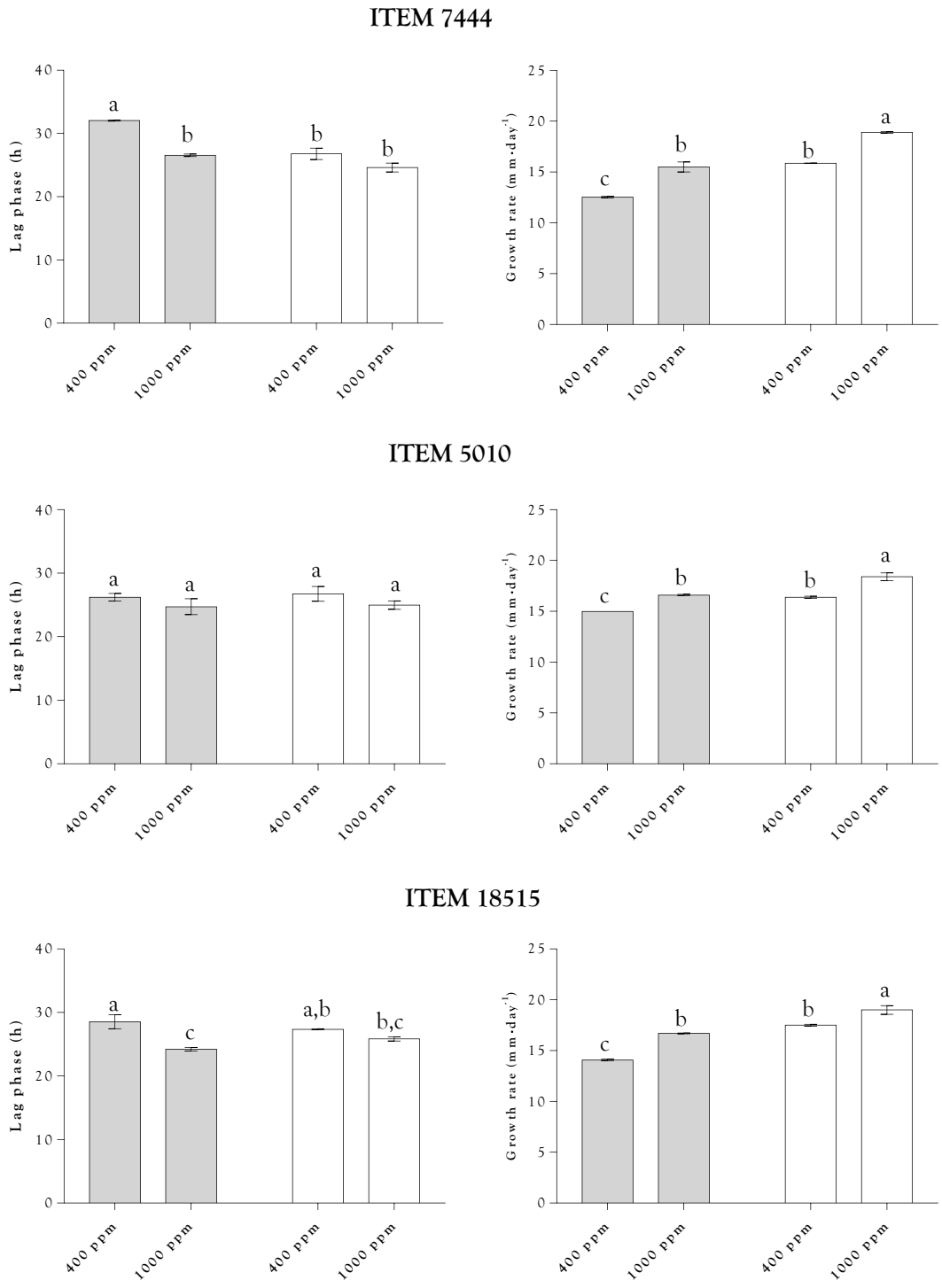


Figure 16. Lag phase (h) and growth rate (mm·day^{-1}) of *A. carbonarius* ITEM 7444, ITEM 5010, ITEM 18515 at 15/28°C (grey bars) and 18/34°C (white bars), 400 vs 1000 ppm. Letters indicate significant differences (Tukey HSD, $p<0.05$).

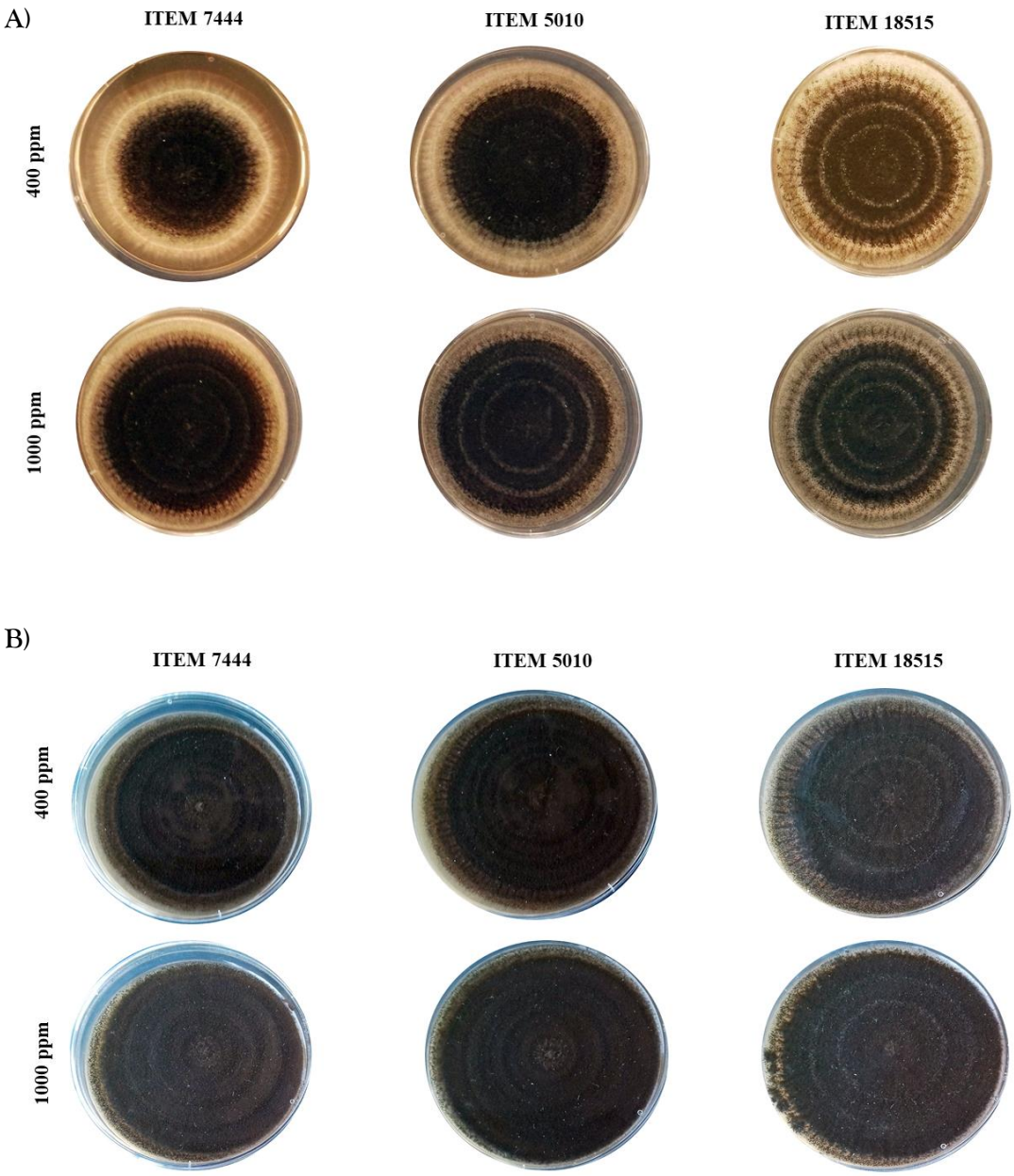


Figure 17. A) Pictures of *A. carbonarius* ITEM 7444, ITEM 5010, ITEM 18515 colonies centrally inoculated taken after 7 days at 15/28°C 11.5h/12.5h dark/light, 400 and 1000 ppm. **B)** Pictures of *A. carbonarius* ITEM 7444, ITEM 5010, ITEM 18515 colonies centrally inoculated taken after 7 days at 18/34°C 11.5h/12.5h dark/light, 400 and 1000 ppm.

Table 9. Summary of ANOVA analysis for lag phase (h) and growth rate (mm·day^{-1}) of *A. carbonarius* ITEM 7444, ITEM 5010 and ITEM 18515 strains in relation to CO_2 (400 vs 1000 ppm) and temperature variations (15/28°C vs 18/34°C).

Strains	Significance			Response
	CO_2	*T*	CO_2 x *T*	
ITEM 7444	**	**	*	Lag Phase (h)
	***	***	ns	Growth rate (mm·day^{-1})
ITEM 5010	*	ns	ns	Lag Phase (h)
	***	***	ns	Growth rate (mm·day^{-1})
ITEM 18515	*	ns	ns	Lag Phase (h)
	***	***	ns	Growth rate (mm·day^{-1})

Levels of significance (*p*-values): 0.12: ns; 0.033: *; 0.002: **; < 0.001: ***
T: temperature; CO_2: carbon dioxide.

3.2. Gene expression and ochratoxin A production

Figure 18 shows the effect of interacting CC factors on the expression of biosynthetic and regulatory genes in the three *A. carbonarius* strains when grown on a grape-based substrate after 4 days incubation. This time point was chosen on the basis of previous studies on the correlation between kinetics of OTA production and transcription levels of OTA biosynthetic genes in *A. carbonarius* (Gallo et al., 2014; Ferrara et al., 2016). Indeed, they reported that genes' activation occurs in the earlier days of incubation and precede the later increase of OTA production. This finding was also reported in *Penicillium nordicum*, another ochratoxin A producer (Geisen et al., 2004).

In elevated CO_2 condition (1000 ppm) and in the 15/28°C temperature cycle a general upregulation of the synthetic OTA genes (*AcOTApks*, *AcOTAnrps*, *AcOTAhal*, *AcOTAp450*, *AcOTAbZIP*) was observed. In particular, for the *A. carbonarius* strain ITEM 5010, there was an increased expression of the gene *AcOTAnrps* exhibiting almost a 5-fold higher transcription level. Under this interacting condition, the gene coding for a *bZIP* transcription factor, was also more than 2-fold overexpressed. This occurred in all three strains examined.

In addition, in the 15/28°C cycle x 1000 ppm CO_2, the regulatory velvet complex genes, *laeA*, *veA* and *velB*, resulted upregulated, with the same trend observed for the key OTA cluster biosynthetic genes. Interestingly, the *veA* gene was a 5-fold upregulated in strain ITEM 7444.

Under the higher 18/34°C temperature cycle, a more variable pattern in the transcriptional profile of all the genes occurred. Under this growth condition, the main OTA cluster genes (*AcOTApks*, *AcOTAnrps*, *AcOTAhal)* were upregulated only in strain ITEM 5010. With regard to

the Velvet complex genes (*laeA/veA/velB*), their expression levels were almost unchanged in all three *A. carbonarius* strains except for the *veA* gene in strain ITEM 7444 strain which showed a >3-fold upregulation in the 18/34°C cycle x 1000 ppm CO_2 treatment.

Figure 19 compares the temporal effect of both temperature cycle x CO_2 exposure concentrations on the OTA production by the three *A. carbonarius* strains. Although the three strains behaved similarly under the different interacting CC factors, intra-species differences in the amounts of OTA detected were observed, with the ITEM 18515 strain being a much higher toxin producer than the other two strains examined. Furthermore, the OTA concentration produced was higher after 4 dpi than after 7 dpi.

In general, higher OTA production occurred in the elevated CO_2 concentration (1000 ppm) treatment, especially in the 15/28°C temperature cycle. In this scenario after 4 dpi, each strain produced higher amounts of OTA, which was significantly different from that detected in current CO_2 (400 ppm) conditions for two out of three strains (ITEM 7444, ITEM 5010). In current CO_2 conditions (400 ppm), no differences were found between the two temperature cycles (15/28°C vs 18/34°C) tested for all three *A. carbonarius* strains. Statistical analysis (Table 11) shows that the temperature cycles significantly influenced OTA production only in strains ITEM 5010 and ITEM 18515, while CO_2 variation and the interaction between CO_2 x temperature cycle had a significant impact on all three *A. carbonarius* strains examined.

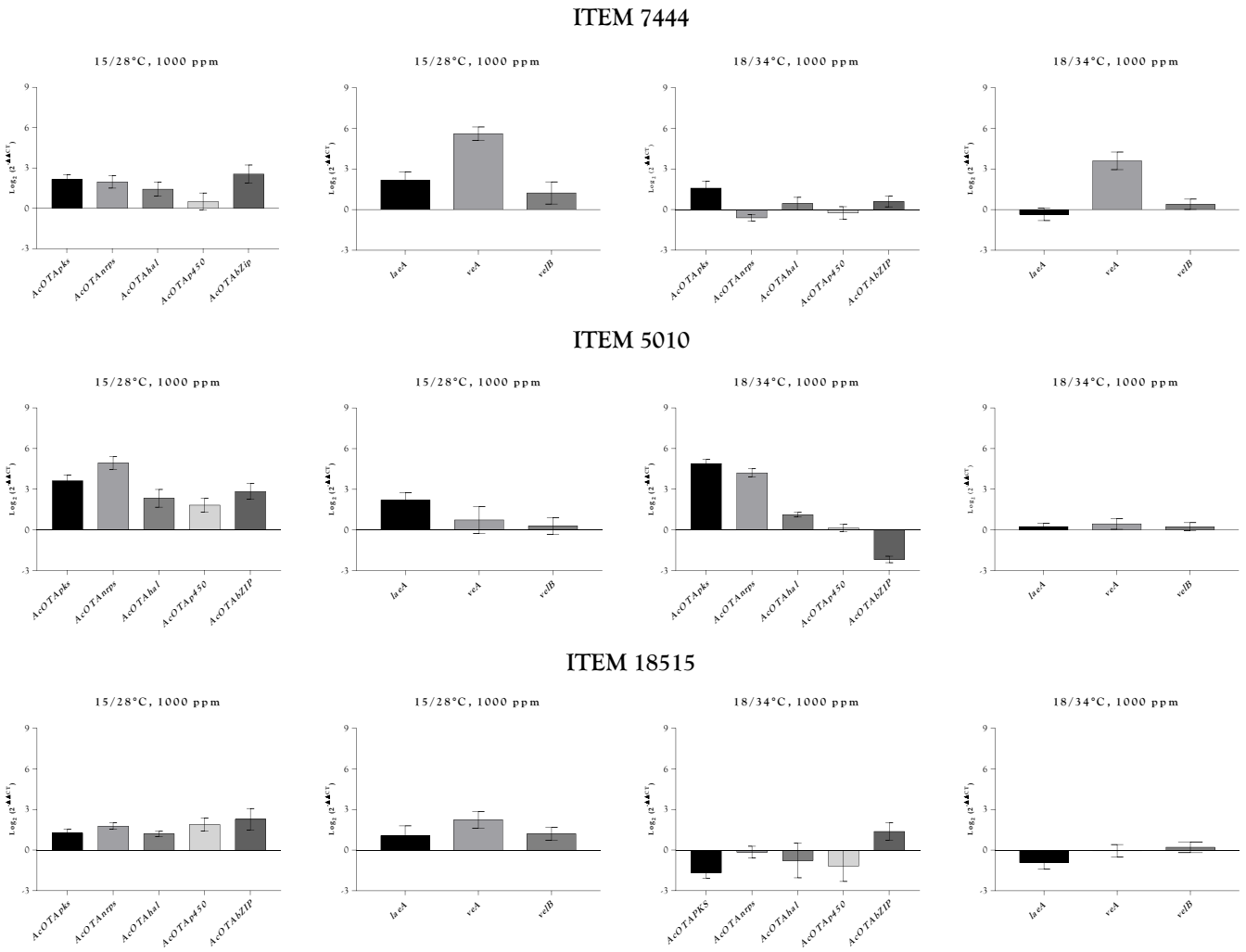


Figure 18. Relative expression at 1000 ppm of OTA biosynthetic (*AcOTApks*, *AcOTAnrps*, *AcOTAhal*, *AcOTAp450*), transcription factor (*AcOTAbZIP*) and regulatory (*laeA/veA/velB*) genes of *A. carbonarius* ITEM 7444, ITEM 5010, ITEM 18515 grown on 0.99 a_w GJM at 15/28°C and 18/34°C 11.5h/12.5h dark/light at 1000 ppm vs 400 ppm (control), after 4 days.

Table 10. Summary of fold changes ($\log_2$ ($2^{-\Delta\Delta CT}$)) of biosynthetic (*AcOTApks*, *AcOTAnrps*, *AcOTAhal*, *AcOTAp450*), transcription factor (*AcOTAbZIP*) and regulatory (*laeA/veA/velB*) genes of *A. carbonarius* ITEM 7444, ITEM 5010, ITEM 18515 at 15/28°C and 18/34°C, 400 ppm vs 1000 ppm.

		Conditions					
		15/28°C			18/34°C		
Strains	Genes	CO_2 (ppm)			CO_2 (ppm)		
		400 (ctrl)	1000	*p*-value	400 (ctrl)	1000	*p*-value
ITEM 7444	*AcOTApks*	0 ± 0.33	2.15 ± 0.34	*	0 ± 0.25	1.58 ± 0.51	*
	AcOTAnrps	0 ± 0.83	1.97 ± 0.46	*	0 ± 0.39	-0.61 ± 0.24	ns
	AcOTAhal	0 ± 0.32	1.42 ± 0.52	ns	0 ± 0.38	0.44 ± 0.46	ns
	AcOTAp450	0 ± 0.43	0.51 ± 0.63	ns	0 ± 0.30	-0.25 ± 0.46	ns
	AcOTAbZIP	0 ± 0.35	2.55 ± 0.67	**	0 ± 0.64	0.59 ± 0.39	ns
	laeA	0 ± 0.77	2.17 ± 0.62	*	0 ± 0.35	-0.35 ± 0.46	ns
	veA	0 ± 0.67	5.60 ± 0.51	***	0 ± 0.55	3.60 ± 0.65	***
	velB	0 ± 0.50	1.23 ± 0.82	ns	0 ± 0.14	0.39 ± 0.38	ns
ITEM 5010	*AcOTApks*	0 ± 0.52	3.62 ± 0.44	***	0 ± 0.23	4.89 ± 0.31	***
	AcOTAnrps	0 ± 0.46	4.93 ± 0.46	***	0 ± 0.20	4.2 ± 0.46	***
	AcOTAhal	0 ± 0.87	2.33 ± 0.65	*	0 ± 0.18	1.1 ± 0.17	**
	AcOTAp450	0 ± 0.80	1.82 ± 0.50	ns	0 ± 0.18	0.13 ± 0.28	ns
	AcOTAbZIP	0 ± 0.92	2.83 ± 0.58	**	0 ± 0.16	-2.19 ± 0.24	***
	laeA	0 ± 0.85	2.21 ± 0.55	*	0 ± 0.29	0.24 ± 0.25	ns
	veA	0 ± 0.69	0.74 ± 1	ns	0 ± 0.14	0.44 ± 0.40	ns
	velB	0 ± 0.85	0.3 ± 0.63	ns	0 ± 0.11	0.23 ± 0.30	ns
ITEM 18515	*AcOTApks*	0 ± 0.34	1.26 ±0.28	ns	0 ± 0.44	-1.66 ± 0.49	*
	AcOTAnrps	0 ± 0.50	1.77 ± 0.24	ns	0 ± 0.51	-0.15±0.65	ns
	AcOTAhal	0 ± 0.24	1.20 ± 0.19	ns	0 ± 0.32	-0.77 ± 0.46	ns
	AcOTAp450	0 ± 1.13	1.89 ± 0.50	*	0 ± 0.43	-1.18±0.93	ns
	AcOTAbZIP	0 ± 1	2.28 ± 0.79	*	0 ± 0.39	1.37 ± 0.64	ns
	laeA	0 ± 0.35	1.06 ±0.73	ns	0 ±0.38	-0.92 ± 0.46	ns
	veA	0 ± 0.47	2.24 ± 0.61	*	0 ±0.64	-0.05 ±0,46	ns
	velB	0 ± 0.57	1.20 ± 0.47	ns	0 ±0.11	0.21 ±0.40	ns

Asterisks indicate significant differences (Sidak, p=0.12: ns; 0.033: *; 0.002: **; < 0.001: ***) at 1000 ppm vs 400 ppm (control) for each gene.

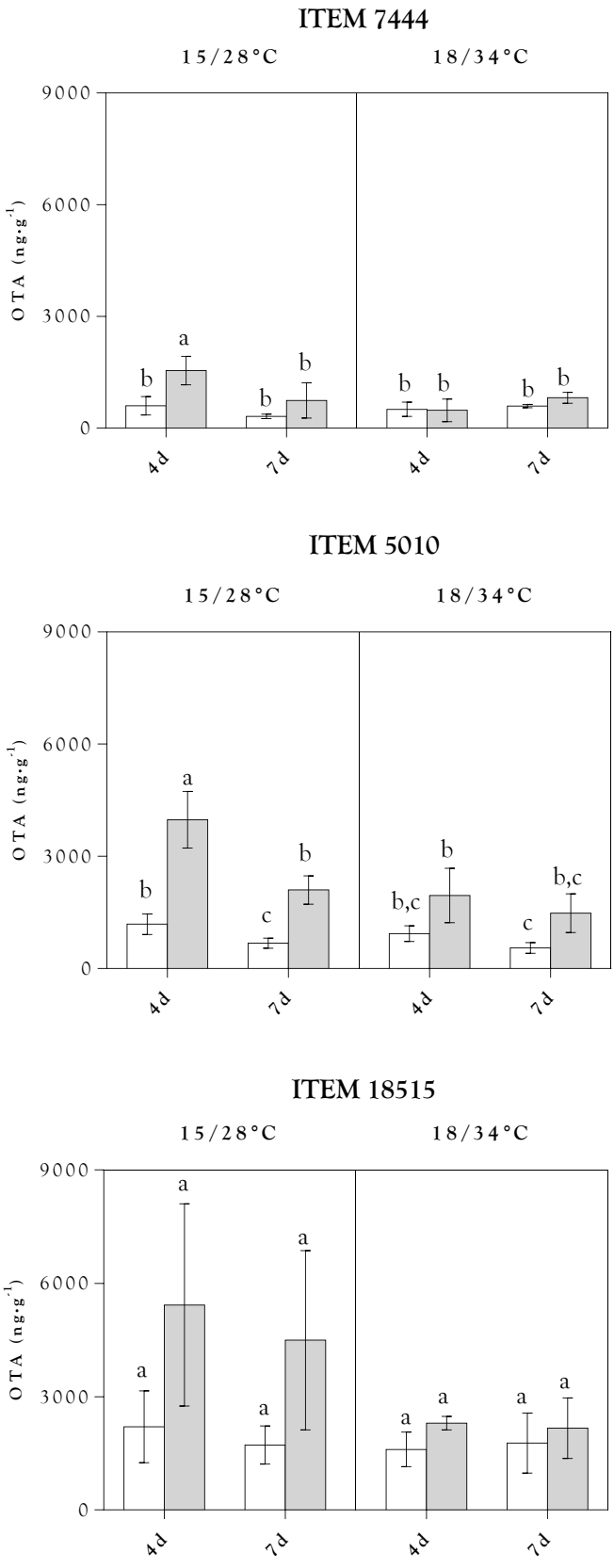


Figure 19. Ochratoxin A production (ng·g^{-1}) by *A. carbonarius* ITEM 7444, ITEM 5010, ITEM 18515 at 15/28°C and 18/34°C 11.5h/12.5h dark/light, 400 ppm (white bars) and 1000 ppm (grey bars) at 4 and 7 dpi. Letters indicate significant differences (Tukey HSD, $p<0.05$).

Table 11. Summary of ANOVA analysis for ochratoxin A production of *A. carbonarius* ITEM 7444, ITEM 5010 and ITEM 18515 strains in relation to CO_2 (400 vs 1000 ppm) and temperature variations (15/28°C vs 18/34°C).

Strains	**Significance**		
	CO_2	*T*	CO_2 *x T*
ITEM 7444	**	ns	*
ITEM 5010	***	**	*
ITEM 18515	*	*	*

Levels of significance (*p*-values): 0.12: ns; 0.033: *; 0.002: **; < 0.001: ***
T: temperature; CO_2: carbon dioxide.

4. DISCUSSION

This is the first study where the effect of interacting CC factors (T x CO_2) have been investigated on growth, relative expression of a range of structural and regulatory genes and OTA production by *A. carbonarius* strains grown on a grape-based matrix. This may be particularly important for the Apulia region, which is considered an important wine producer area in Italy. In particular, we aimed to study the effect of current and projected climate conditions in the North Apulia region, where the OTA risk is now relatively low, selecting two day/night temperature cycles (15/28°C vs 18/34°C, 11.5h/12.5h dark/light), and two CO_2 concentrations (400 vs 1000 ppm).

The effect of day/night temperature associated with photoperiod on the ecophysiology of *A. carbonarius* grown on substrates simulated grape composition has been already investigated. Oueslati et al., 2010, studied the combined effect of three alternating temperatures (20-30°C, 20-37°C, 25-42°C) and 11h/13h light/darkness photoperiod on *A. carbonarius* strains isolated from Tunisian grapes and grown on a Synthetic Nutrient Medium (SNM) at 0.99 a_w. They found that mycelia growth rate was faster at 20-30°C, compared to the other conditions. Similarly, García-Cela et al., 2012, studied the effect of two day/night temperature (20-30°C and 25-37°C) and 16h/8h light/darkness on growth and OTA of two *A. carbonarius* isolated from Spanish grapes and found that 20-30°C cycle resulted in higher risk of contamination. In our study, the different effect of two day/night temperature cycles applied with the same photoperiod, was evident both on mycelial growth rate and sporulation of colonies. In particular, 18/34°C temperature cycle determined a faster growth indicating that the projected +6°C diurnal temperature may be associated to a higher risk of *A. carbonarius* contamination.

In addition, our results, suggested that the expected increase of 2.5x CO_2 atmospheric concentration might increase the *A. carbonarius* colonization rate resulting in a possible increased risk of spoilage in grapes and grape-derived products. Such data constitutes new evidence about the ecophysiology of *A. carbonarius* and differs from previous studies where growth was generally found to be relatively unaffected by elevated CO_2 exposure when compared to mycotoxin production. In this regard, Akbar et al. (2016), suggested that lag phases prior to growth and growth rates of different strains of *Aspergillus* sections *Circumdati* (*A. westerdijkiae*, *A. steynii*, *A. ochraceus*) and *Nigri* (*A. niger*, *A. carbonarius*) grown on a coffee-based media, were generally not significantly stimulated by the effect of increased CO_2 atmospheric concentration (1000 ppm). Similarly, Medina et al. (2015) found that the growth of *A. flavus* on a conducive medium (YES)

was relatively unaffected by the addition of 2x and 3x existing CO_2 levels (350 vs 650/1000 ppm). However, these studies excluded the effect of photoperiods and temperature cycling and in addition, they were conducted on other substrates, differently from us. Thus, the increased growth rate of *A. carbonarius* in the present study was probably different because of the interaction between the temperature cycles and elevated CO_2.

The present study has also examined the impact of such interacting CC factors on the transcriptional profile of key genes related to OTA biosynthesis, and the possible correlation with the phenotypic OTA production. The combination of the current temperature cycle (15/28°C) with elevated CO_2 level (1000 ppm) resulted in an overall upregulation of biosynthetic genes – *AcOTApks*, *AcOTAnrps*, *AcOTAhal*, and in particular – *AcOTAbZIP* encoding for a transcription factor, which were positively correlated with the phenotypic production of OTA under this treatment. Moreover, *laeA*, *veA*, *velB* genes were upregulated following a similar expression pattern of the other structural genes, suggesting that 15/28°C x 1000 ppm CO_2 condition may directly influence the global regulatory velvet complex and thus OTA production. These results are in accordance with Crespo-Sempere et al., 2013 that showed the need of *veA* and *laeA* genes for OTA production in *A. carbonarius*.

In general, information about the expression of mycotoxin related genes under different CC factors are still limited for toxigenic fungi. The limited studies, available until now, reported discordant evidences about the pattern of expression of key genes related to the mycotoxin production and the phenotypic toxin amount detected. In particular, Medina et al. (2015) studied the three-way interacting effects of CC factors (a_w x T x CO_2) on *A. flavus* grown on YES and found that high temperature (37°C) and increasing CO_2 concentration (350 vs 650/1000 ppm) induced the upregulation of both structural (*aflD*) and regulatory (*aflR*) genes which positively correlated with the AFB_1 production. In contrast, Gallo et al. (2016), found that only the structural genes (*aflD*, *aflO*) showed increased expression levels in correlation with higher AFB_1 production when they examined the impact of two-way interacting factors (a_w x T) on *A. flavus* grown on an almond-based medium.

Recently, Verheecke-Vaessen et al. (2019) examined the effect of three-way interacting CC factors (a_w x T x CO_2) on growth, biosynthetic genes and phenotypic T-2 and HT-2 toxin production by *Fusarium langsethiae* on oat-based media and on stored oats. They found that the combinations of a higher temperature (25 vs 30°C) x slight water stress (0.98 a_w) x elevated CO_2

concentration (400 vs 1000 ppm) caused a significant increase in both the expression of biosynthetic toxin genes (*Tri5*, *Tri6*, *Tri16*) and phenotypic production of T-2/HT-2.

With regards to OTA production, very little is known about the impact that interacting CC factors may have on the regulation of the OTA cluster and how such drivers may influence mycotoxin production. Recently, Lappa et al. (2017), monitored the temporal expression of two OTA biosynthetic genes (*AcOTApks*, *AcOTAnrps*) and a regulatory (*laeA*) gene in *A. carbonarius* in relation to temperature and water stress. They reported that while water availability was the key factor affecting OTA production, only temperature acted as the key trigger influencing the transcript levels of the toxin biosynthesis genes.

In the present study, it has been shown, for the first time, that there is an impact of projected CC scenarios on the risk of OTA in a specific area in the Mediterranean region.

Recent studies by the European Commission (2018) pointed out that the Mediterranean basin is already being impacted by CC and extreme weather events, that are drastically changing the landscape of this region. Such CC scenarios are likely to affect the natural ecosystem of important staple crops leading to food/feed safety and security uncertainty (Donatelli et al., 2017). With respect to food safety, mycotoxins are probably one of the major hazards likely to be affected by such changes (Miraglia et al., 2009; Medina et al., 2017). Toxigenic fungi have specific ecological niches for crop colonization and toxin production which reflect their distribution around the world (Van Der Fels-Klerx et al., 2016). Indeed, in hotspot regions for CC it is expected that both pests and diseases may migrate to areas which match their ecological niches and where they are more resilient (Lobell and Gourdji, 2012; Battilani et al., 2016). In addition, there is potential that such CC scenarios will impact on selection or evolution of new fungal genotypes producing different secondary metabolites which may require alternative mitigation strategies than those being used now (Moretti et al., 2019).

On the basis of the predicted CC scenario impacts on the Mediterranean region, the present ecophysiological conditions affecting the colonization by *A. carbonarius* and the OTA contamination in grapes and grape-derived products might change. Under the CC scenarios examined in the present study, we found that the increase of more than 2.5x CO_2 concentration in the North Apulia region, resulted in an increase of colonization rate by *A. carbonarius* and phenotypic OTA production. This was supported also by a molecular analysis of the transcriptional profile of both structural and regulatory genes involved in toxin biosynthesis. Our findings suggest that the predicted CC may have a detrimental effect on the wine production chain in this area. This type of data could be used for the development of predictive models of

the potential impact and possible increase in risks of OTA contamination of grapes and grape-based products in areas already identified as hot spots for CC impacts in the Mediterranean region.

5. REFERENCES

Abrunhosa, L., Paterson, R.R.M., Kozakiewicz, Z., Lima, N., Venâncio, A., 2001. Mycotoxin production from fungi isolated from grapes. Lett. Appl. Microbiol. 32, 240–242. https://doi.org/10.1046/j.1472-765X.2001.00897.x

Akbar, A., Medina, A., Magan, N., 2016. Impact of interacting climate change factors on growth and ochratoxin A production by *Aspergillus* section *Circumdati* and *Nigri* species on coffee. World Mycotoxin J. 9, 863–874. https://doi.org/10.3920/WMJ2016.2041

Battilani, P., Giorni, P., Pietri, A., 2003. Epidemiology of toxin-producing fungi and ochratoxin A occurrence in grape. Eur. J. Plant Pathol. 109, 715–722. https://doi.org/10.1023/A:1026030408338

Battilani, P., Rossi, V., Giorni, P., Pietri, A., Gualla, A., Van der Fels-Klerx, H.J., Booij, C.J.H., Moretti, A., Logrieco, A., Miglietta, F., Toscano, P., Miraglia, M., De Santis, B., Brera, C., 2012. Modelling, predicting and mapping the emergence of aflatoxins in cereals in the EU due to climate change. EFSA report Question No EFSA-Q-2009-00812. EFSA, Parma, Italy.

Battilani, P., Toscano, P., Van der Fels-Klerx, H.J., Moretti, A., Leggieri, M.C., Brera, C., Rortais, A., Goumperis, T., Robinson, T., 2016. Aflatoxin B 1 contamination in maize in Europe increases due to climate change. Sci. Rep. https://doi.org/10.1038/srep24328

Bonvehí, J.S., 2004. Occurrence of ochratoxin A in cocoa products and chocolate. J. Agric. Food Chem. 52, 6347–6352. https://doi.org/10.1021/jf040153w

Cabañes, F.J., Bragulat, M.R., 2018. Black aspergilli and ochratoxin A-producing species in foods. Curr. Opin. Food Sci. 23, 1–10. https://doi.org/10.1016/j.cofs.2018.01.006

Crespo-Sempere, A., Marín, S., Ramos, A., 2013. VeA and LaeA transcriptional factors regulate ochratoxin A biosynthesis in *Aspergillus carbonarius*. Int. J. Food Microbiol. 166, 479-486. https://doi.org/10.1016/j.ijfoodmicro.2013.07.027

Donatelli, M., Magarey, R.D., Bregaglio, S., Willocquet, L., Whish, J.P.M., Savary, S., 2017. Modelling the impacts of pests and diseases on agricultural systems. Agric. Syst. 155, 213–224. https://doi.org/10.1016/j.agsy.2017.01.019

European Commission, 2002. Assessment of dietary intake of ochratoxin A by the population of eu member states. Reports on tasks for scientific cooperation. Available from: http://ec.europa.eu/food/fs/scoop/3.2.7_en.pdf

European Commission, 2006. Commission Regulation (EC) No 1881/2006 of 19 December 2006 on setting the maximum levels for certain contaminants in foodstuffs. Off. J. Eur. Union, L364, 5–24

EU Science Hub, 2018. Worrying effects of accelerating climate change on the Mediterranean Basin. Available at: https://ec.europa.eu/jrc/en/science-update/worrying-effects-accelerating-climate-change-mediterranean-basin

Ferrara, M., Perrone, G., Gambacorta, L., Epifani, F., Solfrizzo, M., 2016. Identification of a Halogenase Involved in the Biosynthesis of Ochratoxin A in *Aspergillus carbonarius* 82, 5631–5641. https://doi.org/10.1128/AEM.01209-16.

Frisvad, J.C., Frank, J.M., Houbraken, J.A.M.P., Kuijpers, A.F.A., Samson R.A., 2004a. New Ochratoxin A producing species of *Aspergillus* section *Circumdati*. Stud. Mycol. 50, 23-43

Frisvad, J.C., Smedsgaard, J., Larsen, T.O., Samson, R.A., 2004b. Mycotoxins, drugs and other extrolites produced by species in *Penicillium* subgenus *Penicillium*. Stud. Mycol. 2004, 201–241

Gallo, A., Solfrizzo, M., Epifani, F., Panzarini, G., Perrone, G., 2016. Effect of temperature and water activity on gene expression and aflatoxin biosynthesis in *Aspergillus flavus* on almond medium. Int. J. Food Microbiol. 217, 162–169. https://doi.org/10.1016/j.ijfoodmicro.2015.10.026.

Gallo, A., Knox, B.P., Bruno, K.S., Solfrizzo, M., Baker, S.E., Perrone, G., 2014. Identification and characterization of the polyketide synthase involved in ochratoxin A biosynthesis in *Aspergillus carbonarius*. Int. J. Food Microbiol. 179, 10-17. https://doi.org/10.1016/j.ijfoodmicro.2014.03.013.

García-Cela, M.E., Marín, S., Reyes, M., Sanchis, V., Ramos, A. J. 2016. Conidia survival of *Aspergillus* section *Nigri*, *Flavi* and *Circumdati* under UV-A and UV-B radiation with cycling temperature/light regime. J. Sci. Food Agric., 96, 2249-2256. https://doi.org/10.1002/jsfa.7343

García-Cela, E., Ramos, A., Marín, S., 2012. Ochratoxigenic moulds and effectiveness of grape field antifungals in a climatic change scenario. J. Sci. Food Agr. 92, 1455-61. 10.1002/jsfa.4726

International Agency for Research on cancer (IARC), 1993. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. Monographs on the evaluation of carcinogenic risks to humans. Vol. 56, Lyon, France.

Ioannidis, A., Kogkaki, E.A., Natskoulis, P.I., Nychas, G.E., Panagou, E.Z., 2015. Modelling the in fl uence of temperature , water activity and sodium metabisulphite on the growth and OTA production of *Aspergillus carbonarius* isolated from Greek wine grapes. Food Microbiol. 49, 12–22. https://doi.org/10.1016/j.fm.2015.01.006

International Panel Climate Change (IPCC) 2014: synthesis report. Contribution of working group I, II, III to the fifth Assessement Report of the Intergovernmental Panel on Climate change. Geneva, Switzerland: Intergovernmental Panel on Climate Change (IPCC): 2014.

Lappa, I.K., Kizis, D., Panagou, E.Z., 2017. Monitoring the Temporal Expression of Genes Involved in Ochratoxin A Production of *Aspergillus carbonarius* under the Influence of Temperature and Water Activity. Toxins. 9, 296-311. https://doi.org/10.3390/toxins9100296

Livak, K.J., Schmittgen T.D., 2001. Analysis of relative gene expression data using Real-Time Quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods. 25, 402-408.

Lobell, D.B., Gourdji, S.M., 2012. The influence of climate change on global crop productivity. Plant Physiol. 160, 1686–1697. https://doi.org/10.1104/pp.112.208298

Magan, N., Hope, R., Cairns, V., Aldred, D., 2003. Post-harvest fungal ecology: Impact of fungal growth and mycotoxin accumulation in stored grain. Eur. J. Plant Pathol. 109,723–730.

Medina, A., Akbar, A., Baazeem, A., Rodriguez, A., Magan, N., 2017. Climate change, food security and mycotoxins: Do we know enough? Fungal Biol. Rev. 31, 143–154. https://doi.org/10.1016/j.fbr.2017.04.002

Medina, A., Rodríguez, A., Sultan, Y., Magan, N., 2015. Climate change factors and Aspergillus flavus : effects on gene expression , growth and aflatoxin production. World Mycotoxin J. 8, 171–179. https://doi.org/10.3920/WMJ2014.1726

Miraglia, M., Marvin, H.J.P., Kleter, G.A., Battilani, P., Brera, C., Coni, E., Cubadda, F., Croci, L., Santis, B. De, Dekkers, S., Filippi, L., Hutjes, R.W.A., Noordam, M.Y., Pisante, M., Piva, G., Prandini, A., Toti, L., Born, G.J. Van Den, Vespermann, A., 2009. Climate change and food safety : An emerging issue with special focus on Europe. Food Chem. Toxicol. 47, 1009–1021. https://doi.org/10.1016/j.fct.2009.02.005

Moretti, A., Pascale, M., Logrieco, A.F., 2019. Mycotoxin risks under a climate change scenario in Europe. Trends Food Sci. Technol. 84, 38–40. https://doi.org/10.1016/j.tifs.2018.03.008

Oueslati, S., Lasram, S., Ramos, A., Marín, S., MLiki, A., Ghorbel, A., 2010. Alternating temperatures and photoperiod effects on fungal growth and Ochratoxin A production by Aspergillus carbonarius isolated from Tunisian grapes. Int. J. Food Microbiol. 139, 210-3. 10.1016/j.ijfoodmicro.2010.02.003.

Palumbo, J., O'Keeffe, T., Vasquez, S., Mahoney, N. 2011. Isolation and identification of ochratoxin A-producing *Aspergillus* section *Nigri* strains from California raisins. Lett. App. Microbiol. 52, 330-336. https://doi.org/10.1111/j.1472-765X.2011.03004.x

Perrone, G., Nicoletti, I., Pascale, M., De Rossi, A., De Girolamo, A., Visconti, A., 2007. Positive correlation between high levels of ochratoxin A and resveratrol-related compounds in red wines. J. Agric. Food Chem. 55, 6807–6812. https://doi.org/10.1021/jf070993l

Perrone, G., Gallo, A., Susca, A., Varga, J., 2008. *Aspergillus* in grapes: Ecology, biodiversity and genomics. In *Aspergillus in the Genomic Era* (pp. 179-212). Wageningen Academic Publishers. https://doi.org/10.3920/978-90-8686-635-9

Scudamore, K.A., Banks, J.N., Guy, R.C.E., 2004. Fate of ochratoxin A in the processing of whole wheat grain during extrusion. Food Addit. Contam. 21, 488–497. https://doi.org/10.1080/02652030410001670166

Somma, S., Perrone, G., Logrieco, A.F., 2012. Diversity of black Aspergilli and mycotoxin risks in grape , wine and dried vine fruits. Phytopathol. Mediterr. 51, 131–147. https://doi.org/10.14601/Phytopathol_Mediterr-9888

Taniwaki, M.H., Pitt, J.I., Teixeira, A.A., Iamanaka, B.T., 2003. The source of ochratoxin A in Brazilian coffee and its formation in relation to processing methods. Int. J. Food Microbiol. 82, 173–179. https://doi.org/10.1016/S0168-1605(02)00310-0

Van der Fels-Klerx, H.J., Goedhart, P.W., Elen, O., Börjesson, T., Hietaniemi, V., Booij, C.J., 2012. Modeling Deoxynivalenol Contamination of Wheat in Northwestern Europe for Climate Change Assessments. J. Food Prot. 75. 1099-106. https://doi.org/10.4315/0362-028X.JFP-11-435.

Van Der Fels-Klerx, H.J., Liu, C., Battilani, P., 2016. Modelling climate change impacts on mycotoxin contamination. World Mycotoxin J. 9, 717–726. https://doi.org/10.3920/WMJ2016.2066

Varga, J., Kozakiewicz, Z., 2006. Ochratoxin A in grapes and grape-derived products. Trends Food Sci. Technol. 17, 72–81. https://doi.org/10.1016/j.tifs.2005.10.007

Verheecke-Vaessen, C., Diez-Gutierrez, L., Renaud, J., Sumarah, M., Medina, A., Magan, N., 2019. Interacting climate change environmental factors effects on Fusarium langsethiae growth, expression of Tri genes and T-2/HT-2 mycotoxin production on oat-based media and in stored oats. Fungal Biol. https://doi.org/10.1016/j.funbio.2019.04.008

Visconti, A., Perrone, G., Cozzi, G., Solfrizzo, M., 2008. Managing ochratoxin A risk in the grape-wine food chain. Food Addit. Contam. - Part A Chem. Anal. Control. Expo. Risk Assess. 25, 193–202. https://doi.org/10.1080/02652030701744546

Wheeler, T., Von Braun, J., 2013. Climate change impacts on global food security. Science, 341, 508-513. https://doi.org/10.1126/science.1239402

Zimmerli, B., Dick, R., 1996. Ochratoxin A in table wine and grape-juice: Occurrence and risk assessment. Food Addit. Contam. 13, 655-68. 10.1080/02652039609374451

STUDY III

Aspergillus carbonarius proteases and their possible activity in ochratoxin A degradation during wine making

ABSTRACT

Aspergillus carbonarius is the main responsible fungus of ochratoxin A (OTA) contamination of grapes and derived products. Recently, the biosynthetic mechanism of this mycotoxin has been mainly elucidated by experiments of knocking out of the key biosynthetic genes. The mutant strain of *A. carbonarius*, in which the Ac*OTAnrps* gene had been disrupted, was unable to produce OTA but retained its ability to degrade OTA into OTα when it was grown in presence of exogenous OTA. Microbial degradation of OTA is due to the enzymatic cleavage of the amide bond between L-β-phenylalanine and OTα by proteolytic proteins. Then, an *in silico* screening has been made on the available genome sequence of *A. carbonarius* ITEM 5010 to identify genes encoding proteases and to investigate their involvement in the OTA degrading activity of *A. carbonarius*. Preliminary transcriptomic analysis allowed selecting protease encoding genes that were expressed at increased level during OTA production. From the analysis of functional domains of the deduced protein sequences, four identified genes encode for aspartic proteases, three for serine proteases and one for a metalloprotease. Wild type and three mutant strains of *A. carbonarius* ITEM 5010 (ΔAc*OTAnrps*, ΔAc*OTApks*, ΔAc*OTAhal*) previously obtained and resulted to be unable to produce OTA, have been incubated in the presence of exogenous OTA with the aim to evaluate the OTA degrading activity of these strains. In addition, expression levels of the identified proteases encoding genes was investigated to evaluate their activation during growth with the aim to establish their involvement in the degradation activity of *A. carbonarius*.

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1. INTRODUCTION

Wines represent the second major source of OTA intake (10-20%) after cereals (40-50%) in human diet (CAC, 1998). *Aspergillus carbonarius*, within the Aspergillus sect. *Nigri*, has been identified as the major cause of contamination in vineyards (Perrone and Gallo, 2017). This mycotoxin is proven to be toxic, being primarily known for its nephrotoxicity and potential carcinogenicity to human (Group 2B) (IARC, 1993). Regulations have been established to limit the risk of exposure to OTA contamination. For wine, it is reported that in high risk years OTA content, could exceed the legislative limit 2 $\mu g \cdot kg^{-1}$ (European Commission, 2006 Reg. 1881) resulting in more than 20-30% of the final products.

Prevention and/or detoxification of contaminated products is then of prime importance in protecting consumer's health. Fungal spoilage and consequent OTA contamination through the grape-wine production chain can be controlled at different points: in the vineyards, harvest and wine processing. Preventive strategies, such as the application of Good Agricultural Practices (GAP), the use of fungicides (e.g. SWITCH®), during cultivation are the most efficient way to limit the fungal growth and mycotoxin contamination in further stages (Abrunhosa et al., 2010). Biocontrol has been also proposed as a strategy to reduce the impact of ochratoxigenic species in vineyards, with yeasts being particularly promising for this scope due to their capacity to colonize plant surfaces for long periods under dry conditions (Ponsone et al., 2011). During harvest, the only efficient way to avoid OTA presence in grapes intended to wine making, is to exclude visible damaged and rotted berries and preserve the integrity of grapes by controlling transport conditions (Visconti et al., 2008). OTA in grapes is transferred to wine during vinification. Differences in this process cause a different occurrence of OTA in wines, with red being more contaminated than white wines due to a longer maceration process (Blesa et al., 2006).

Several decontamination strategies of ochratoxin A in wine have been proposed. Currently, the EU allows the use of fining agents, such as oenological charcoal, as a corrective action against OTA presence by using the lowest possible and most effective doses (Codex Alimentarius, 2007). However, the efficacy of charcoal is directly related to reductions in nutritive and quality parameters of the treated wines limiting their application. The International Code of Oenological Practices suggests the use of chitosan and chitin-glucan not exceeding 500 $g \cdot hL^{-1}$, to reduce OTA in wine (OIV, 2009 a,b).

A better solution to limit the presence of OTA in wine would be biodegradation. The use of microorganisms and/or their enzymes would represent the technology of choice for

decontamination purposes because they preserve nutritive quality of the final products. Numerous microorganisms and some enzymes capable of degrading, adsorbing and detoxifying OTA are reported in literature as possible biodegrading agents (Abrunhosa et al., 2010). The most acknowledged pathway of OTA enzymatic degradation involves the hydrolysis of the amide bond that links the L-β-phenylalanine molecule to the OTα moiety (see figure 20). The degradation product OTα is considered a non-toxic compound, with a 10-times shorter elimination half-life than OTA (Kőszegi et al., 2016). Therefore, this mechanism can be considered a detoxification pathway.

With respect to wine, biodegrading strategies includes the use of some lactic acid bacteria able to efficiently adsorb OTA during wine fermentation (Del Prete et al., 2007). Several studies showed that OTA removal in wine was also possible by using yeast strains, both dead or alive (Scott et al., 2008; Ciconova et al., 2010; Ponsone et al., 2011; Var et al., 2011). Nevertheless, Visconti et al., 2008 reported that the reduction by yeasts or inactivated yeasts walls caused the loss of colour of wines and the efficacy of this treatment is low.

More recently, Solfrizzo et al., 2009, demonstrated that ochratoxin A can be effectively removed from contaminated wine by repassage of wine over uncontaminated grape pomaces obtained from the same or different grape varieties. The experiment was evaluated also at industrial scale and resulted in a useful and environmentally friendly technique for wineries located in high risk regions for OTA contamination (Solfrizzo et al., 2009; Solfrizzo et a., 2019).

In addition, experimental evidences about the efficacy of *A. carbonarius* in biodegrading OTA are also found (Abrunhosa et al., 2002; Bejaoui et al., 2006). In this regard, Gallo et al., 2012, reported that a mutant strain of *A. carbonarius* (Δ*AcOTAnrps*), unable to produce OTA, retained the ability to degrade the mycotoxin into OTα, when exogenous OTA was added to the medium.

A further related study reported the identification of an aspartyl protease encoding gene (*Acap1*) located downstream of the core genes involved in OTA biosynthesis in *A. carbonarius*. The gene is 1367 bp long and the *in silico* analysis of the deduced protein sequence of 421 aa revealed that the AcAP1 protein shows the functional structure of aspartic protease enzymes (Bleve et al., 2017). The finding of this gene within the OTA gene cluster is promising for the possible role of the derived protease in OTA degradation in wines due to the physicochemical properties of these enzymes that allow them to be efficient in acid conditions such as those of wines.

Concurrently, from the *in silico* screening of the *A. carbonarius* genome and from the results of transcriptomic analysis carried out on the fungus grown in different OTA producing conditions, we selected seven protease encoding genes (from *AcproA* to *AcproG*) that were expressed at increased level during OTA production conditions.

Based on these evidences, with the present work we aimed to evaluate i) the ability of three mutant strains (Δ*AcOTApks*, Δ*AcOTAnrps*, Δ*AcOTAhal)* obtained from *A. carbonarius* to degrade OTA into the less toxic compound OTα and ii) the expression levels of the seven proteases encoding genes in order to establish their potential involvement in the OTA degradation process.

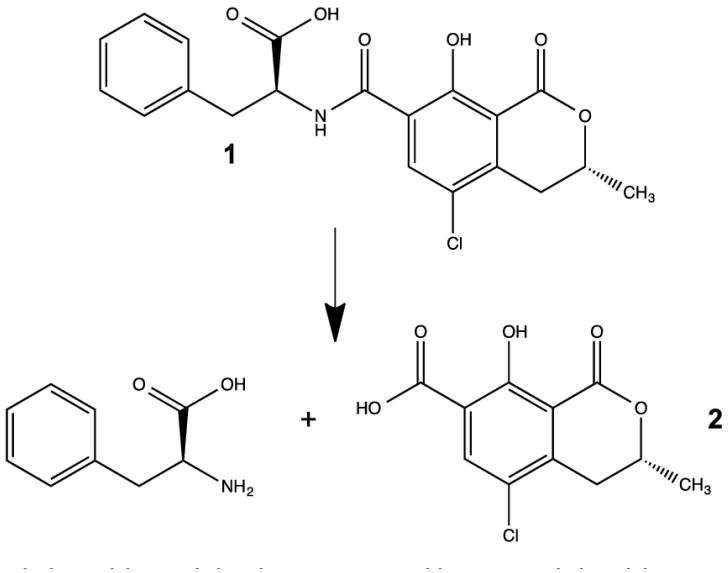

Figure 20. Hydrolysis of the amide bond in OTA (1) to yield OTα (2) and phenylalanine as considered the primary degradation/detoxification pathway for ochratoxin (Haq et al., 2016).

2. MATERIALS AND METHODS

2.1. Fungal strains

The wild-type strain of *A. carbonarius* ITEM 5010 and three mutant strains - Δ*AcOTApks*, Δ*AcOTAnrps*, Δ*AcOTAhal* - previously obtained at the Institute of Food Science Production from ITEM 5010, were used in this study. These mutant strains were deleted for polyketide synthase (*AcOTApks*), non ribosomal-peptide synthase (*AcOTAnrps*) and halogenase (*AcOTAhal*) genes (Perrone and Gallo, 2017) of OTA biosynthesis pathway and for this reason they were unable to produce the mycotoxin.

2.2. Growth conditions

The *A. carbonarius* strains were incubated into 10 mL of Basal Medium (BM, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L $Na_2HPO_4 \cdot 2H_2O$, 1 g/L KCl, 0.3 g/L $(NH_4)_3PO_4$, 0.2 g/L NH_4NO_3, 18 g/L sucrose, 1g/L glucose) in which 50 µL of exogenous OTA (3 µM) were added after 3 days of growth. Liquid cultures and mycelia of each strain were collected after 12h and 24h and stored at -20°C for OTA and OTα analysis and -80°C for RNA extraction, respectively. Blank samples were also prepared with BM plus OTA and the amount was measured at the different time points.

2.3. OTA and OTα analysis

The supernatants of the liquid cultures of the *A. carbonarius* strains tested were filtered through a 0.22 µm pore filter (Sartorius AG, Goettingen, Germany) and 100 µl were injected into a HPLC-FLD apparatus for OTA determination. Direct injection of liquid culture was possible since no interfering peaks eluted at retention times of OTA (10 min) and OTα (8 min).

The HPLC apparatus was an Agilent 1260 Infinity system equipped with a G1312 binary pump, a G1367E autosampler with a 100 µl loop, a G1316C column thermostat set at 30°C, and a fluorescence detector (G1321B) (excitation wavelength, 333 nm; emission wavelength, 460 nm) from Agilent Technologies (Waldbronn, Germany). The column used was a 150 x 4.6 mm i.d., 5 µm, Zorbax C18, (Phenomenex, Torrance, CA, USA) with a 3 mm i.d. and a 0.45 µm pore size guard filter (Rheodyne, Cotati, CA, USA). The mobile phase was an isocratic mixture of acetonitrile:water (45:55 v/v) containing 1% acetic acid. The flow rate of the mobile phase was 1 ml/min. The standard solutions of OTA and OTα were prepared in the mobile phase and calibration curves were prepared in the range from 0.1 to 100 ng/ml.

2.4. RNA extraction, cDNA synthesis and qRT-PCR

Total RNA was extracted from frozen mycelium ground in liquid nitrogen using the RNeasy Plant Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. RNA concentration was determined by using Nanodrop and its integrity was evaluated by doing a 1% agarose gel. First strand cDNA was synthesized using about 3 µg of total RNA, oligo $(dT)_{18}$, random hexamers and SuperScript III Reverse Transcriptase (Invitrogen, San Diego, CA) according to the manufacturer's protocol.

The transcription profiles of seven genes of which three encoding for aspartyl proteases (*AcProA*, *AcProD*, *AcProE*), three for serine proteases (*AcProB*, *AcProC*, *AcProF)*, one for a metalloprotease *(AcProG)* were analysed at 12 and 24h after the addition of exogenous OTA by using real time quantitative reverse transcription-PCR (qRT-PCR). For the amplification reaction, a SYBR Green I assay was performed in the 7500 Real-Time PCR System (Applied Biosystem) with ITAQ UNIVERSYBR GREEN SMX 2500 (BioRad) with 200-250 nM of each primer pairs for target and reference genes. The constitutively expressed β-tubulin gene served as an internal reference to normalize target gene expression. The following amplification conditions were used: an initial denaturation step at 95°C for 2 min, followed by 40 cycles of 10 s at 95°C. 30 s at 60°C and 30 s at 72°C. Specificity of the PCR amplifications was confirmed by dissociation curve analyses. The relative quantification of gene expression was established using the comparative $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). PCR efficiency of each oligonucleotide pair was calculated from each linear regression of standard curves.

3. RESULTS

3.1. OTA biodegradation

The OTA biodegradation by three mutant strains of *A. carbonarius* (ΔAc*OTApks*, ΔAc*OTAnrps*, ΔAc*OTAhal*), unable to produce the mycotoxin, was tested by adding exogenous OTA to the liquid substrate after 3 days of fungal growth. The expected degrading activity was evaluated after 12 and 24h the addition of OTA. Such time points were selected based on a previous work by Gallo et al., 2012 in which a complete OTA degradation occurred after only 2 days of incubation in presence of exogenous OTA with concomitant early detection (after 24h) of the degradation product OTα. These data suggested that the degradation process would have happened in the very first days of incubation and thus gene encoding protease potentially involved in this process are likely to be activated during this period.

In our study, the three *A. carbonarius* mutant strains behaved similarly with a reduction of OTA amount of about 65% after 12h of incubation with supplemented OTA followed by a very low decrease after 24h. In addition, a concomitant slight increase of the degradation product OTα (<1% of the initial amount) was detected already after 12h (Figure 21). Blank samples, represented by the substrate plus OTA, were included in this study to establish the efficacy of our experimental activity.

In order to exclude that most of the mycotoxin would have been retained on the surface of the fungus and that OTα would have remained entrapped, we analysed OTA and OTα contents in the mycelium. Indeed, we found in the mycelium from 3-9% of the initial quantity of OTA introduced in the cultures and we also observed that from 2 to 10% of produced OTα got stuck in the mycelium (data not shown). These results suggested that most of the decrease of OTA was due to a possible enzymatic degrading activity of the fungus.

Figure 22 shows the expression levels of seven proteases encoding genes (*AcProA*, *AcProB*, *AcProC*, *AcProD*, *AcProE*, *AcProF*, *AcProG*) in Δ*AcOTApks*, Δ*AcOTAnrps*, Δ*AcOTAhal A. carbonarius* ITEM 5010 mutant strains. Generally increased levels of transcription after the addition of OTA were observed for some protease genes in two of the mutant strains, Δ*AcOTAnrps* and Δ*AcOTAhal.* In particular, the genes we designated *AcProA* and *AcProB* showed an increase of activation after 12h in Δ*AcOTAnrps.* All the genes here considered reached a higher transcription level after 24h in Δ*AcOTAhal* with a maximum fold change detected for *AcProD*.

The transcripts of four of the proteases encoding genes (*AcProA*, *AcProB*, *AcProC* and *AcProD*) and of an aspartyl protease encoding gene (*Acap1*), identified in a related study by Bleve et al.,

2017, were isolated and cloned in expression vectors for the recombinant expression in *Escherichia coli*. In addition, *Saccharomyces cerevisiae* was used as an alternative expression system for recombinant production of aspartyl protease AcAP1. Experiments of induced protein expression and assays to determine proteolytic activity of the recombinant proteins produced by yeast on plate – using casein, gelatin and skimmed milk as substrates – gave positive results for some clones showing an increased proteolytic activity (data not shown).

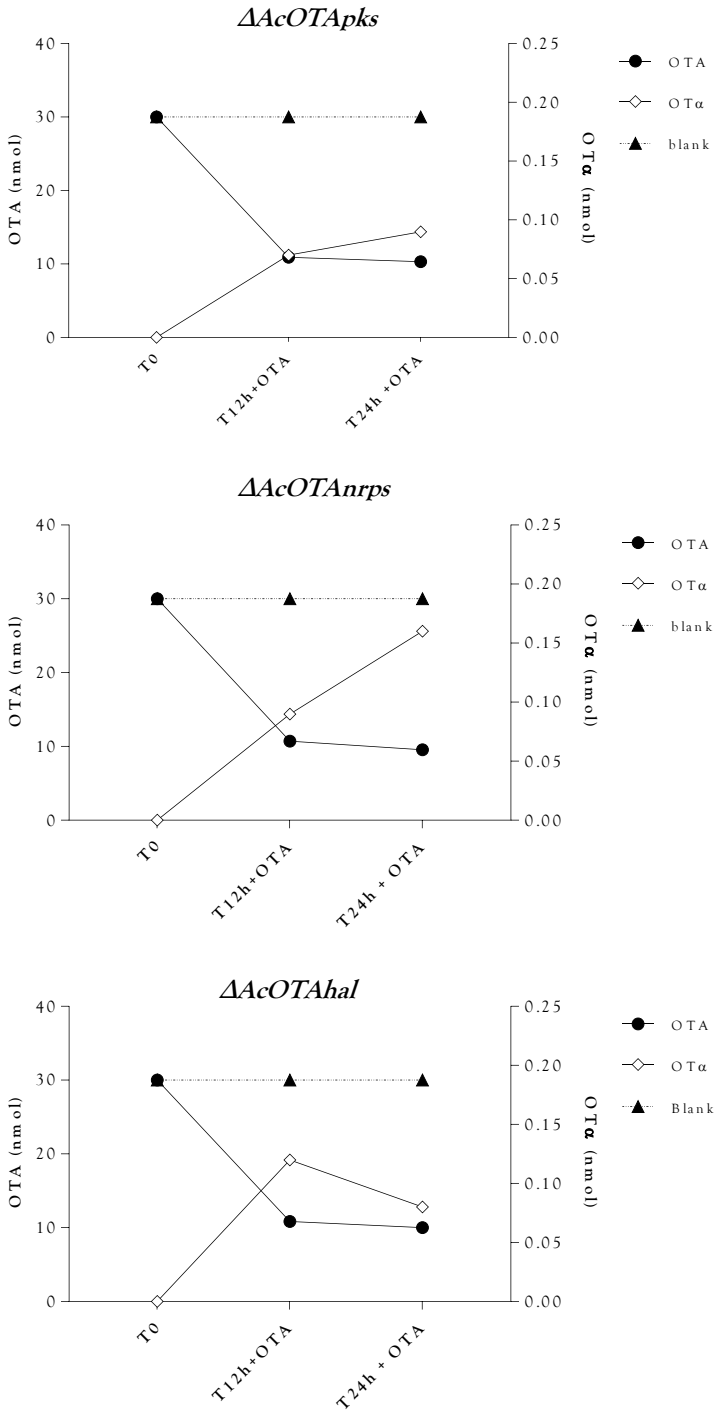

Figure 21. Biodegrading activity of chratoxin A and in OTα at different time points (T0, T12h, T24h) by *A. carbonarius* mutant strains Δ*AcOTApks*, Δ*AcOTAnrps*, Δ*AcOTAhal* after OTA addition at 3d (T0=3d).

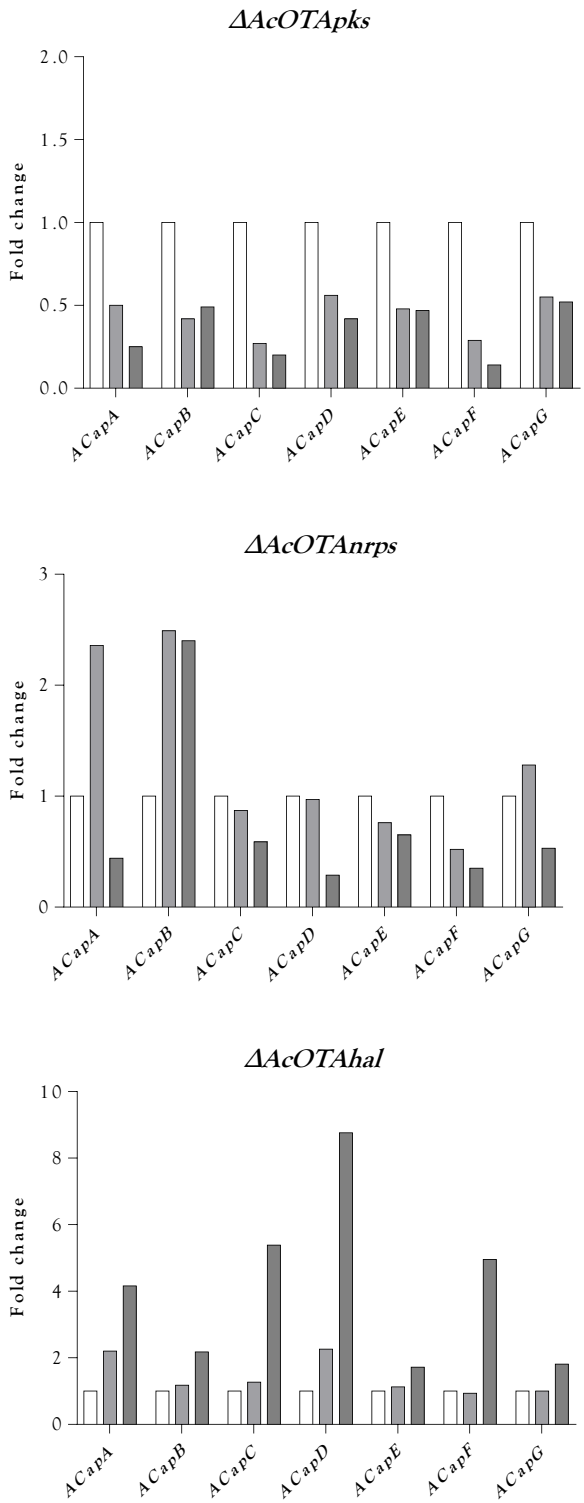

Figure 22. Gene expression of seven proteases encoding genes at T0 (white bars), T12h (light grey bars) and T24h (dark grey bars) after OTA addition by *A. carbonarius* ITEM 5010 mutant strains Δ*AcOTApks*, Δ*AcOTAnrps*, Δ*AcOTAhal*.

4. DISCUSSION

Mycotoxin biotransformation is defined as "the degradation of mycotoxins into non-toxic metabolites by using microorganisms or enzymes" (Boudergue et al., 2009). Biodegradation would represent a valid alternative to the nowadays existing methods due the specific, environmental friendly, effective approach with minor impact on the qualitative parameters of the final product.

In our study, we investigated on the possible activation of protease encoding genes by exploiting the already known ability of *A. carbonarius* to degrade OTA, as possible decontamination strategy of wines.

Our findings confirmed the *A. carbonarius* ability to degrade the mycotoxin demonstrated by a reduction of about 65% of the initial OTA amount in all mutant strains. A concomitant increase of OTα, the OTA metabolism-deriving product, was also observed confirming that the degradation took place. Differently from us, Gallo et al., 2012 found that OTA was completely degraded after only 2 days of incubation with exogenous OTA and that 27% of the initial amount was converted into OTα.

The application of fungi as detoxifying agents has been reported in literature (Abrunhosa et al., 2010). Interestingly, among fungi, black Aspergilli have often been reported to degrade OTA. Abrunhosa et al., 2002 evaluated the biodegradation of ochratoxin A by fungi isolated from grapes. A total of 76 strains, of which 37 black Aspergilli (*A. carbonarius*, *A. ellipticus*, *A. foetidus*, *A. japonicus*, *A. niger*), 25 other *Aspergillus* strains and 14 other genera, were selected among filamentous fungi isolated from Portuguese grapes. Their results showed that the 95% of the black Aspergilli, both non- and OTA producer, degraded more than 80% of the initial amount of OTA and two compounds, of which one was OTα, resulted when ochratoxin A was degraded with carboxypeptidase A suggesting a possible enzymatic action in OTA degradation.

Similarly, Bejaoui et al., 2006 evaluated the biodegradation of OTA by *Aspergillus* section *Nigri* isolated from French grapes. During the first 3 days of incubation, *A. niger* degraded OTA relatively slowly (20%) compared to *A. carbonarius* (55%) and *A. japonicus* (80%). However, after the third day, OTA degradation by *A. niger* became the most rapid and reached the 99% after 5 days. By this time, *A. carbonarius* and *A. japonicus* reduced the levels of OTA by 83% and 89%, respectively. In parallel, the amount of OTα increased until the 9th day of incubation.

With a similar adsorbing approach, bacteria, protozoa and yeasts have been also reported to reduce the bioavailability of OTA.

Due to the excellent OTA-detoxification performance of *Trichosporon mycotoxinivorans* (MTV, 115), it was made into a product named Mycofix® Plus [MTVINSIDE] by Biomin GmbH (Austria) (Hanif et al., 2008) for application at industrial level.

In addition to these physical approaches, mainly based on the adsorption of the mycotoxin, several enzymes have been reported to degrade OTA.

The principal pathway by which enzymes should degrade OTA, involves the hydrolysis of the amide bond that links the L-β-phenylalanine to the dihydroisocumarine molecule, OTα (Abrunhosa et al., 2010). Since OTα is reported to be a less toxic compound than OTA (Harwig, 1974), studies have been done on the possible application of enzymes to decontaminate food products by OTA.

Numerous proteolytic enzymes have been claimed to be able to degrade OTA. Two classes of carboxypeptidases (EC 3.4) have been associated with OTA degradation: carboxypeptidase A (CPA) (EC 3.4.17.1) (Pitout et al., 1969) and carboxypeptidase Y (CPY) (EC 3.4.16.1) (Abrunhosa et al., 2010). The first, isolated from bovine pancreas, was able to perform the degrading reaction with a Km value of 1.5×10^{-4} M at 25°C. The second, isolated from *Saccharomyces cerevisiae*, was demonstrated to hydrolyze OTA with optimum at pH 5.6 and 37°C. However, the hydrolysis reaction was very slow. In fact, after 5 days of incubation, CPY converted 52% of the OTA present in the reaction assay into OTα. This activity is sufficient to reduce significantly levels of OTA during wine fermentation since these processes take several days to complete. Hence, a biodegradation pathway is possible for *S. cerevisiae* in addition to the OTA adsorption phenomenon.

Furthermore, a screening study which included several commercial hydrolases, verified that a crude lipase product from *A. niger* was able to hydrolyze OTA via the amide bond (Stander et al., 2000). Several commercial proteases were also reported to hydrolyze OTA to OTα, such as Protease A from *A. niger* and pancreatin from porcine pancreas. These enzymes showed a significant hydrolytic activity at pH 7.5, which resulted in the cleavage of 87.3% and 43.4% of $1 \mu g \cdot mL^{-1}$ of OTA respectively, after 25 h of incubation (Abrunhosa et al., 2006).

In our study, we aimed to identify some protease encoding genes that could have been activated during OTA degradation and suitable for wine detoxification since the aforementioned enzymes are not efficient at low pH. In this regard, our attention was mainly focused on the identification of genes encoding for aspartyl proteases which are known to exhibit their catalytic

activity in acid conditions. Increased levels of some of the proteases encoding genes suggested that a possible enzymatic mechanism could be involved in OTA biodegradation by *A. carbonarius*.

Although enzymes for decontamination purposes seems to be a promising strategy, due to the compliance of several mandatory requirements of safety, effectiveness, low cost of production and purification, their application is limited. Indeed, the European Commission admit the use of enzymes in food "to perform a technological function in the manufacture, processing, preparation, treatments, packaging, transport or storage of such food". Only recently, acceptability criteria for detoxification treatments, including biotransformation, have been set for commodities intended for animal nutrition (Commission Regulation 2015/786/EU).

Thus, the application of enzymes intended for detoxification is still challenging as it must overcome the gap of knowledge related to the effect that these enzymes exert on the nutritional and organoleptic qualities as well fill the legislative void existing so far on their application as detoxifying agents in foods.

5. REFERENCES

Abrunhosa, L., Paterson, R., Venâncio, A., 2010. Biodegradation of Ochratoxin A for Food and Feed Decontamination. Toxins. 2. 1078-99. https://doi.org/10.3390/toxins2051078

Abrunhosa, L., Santos, L., Venâncio, A., 2006. Degradation of Ochratoxin A by Proteases and by a Crude Enzyme of *Aspergillus niger*. Food Biotechnol. 20, 231-242. http://dx.doi.org/10.1080/08905430600904369

Abunhosa, L., Serra, R., Venâncio, A., 2002. Biodegradation of ochratoxin A by fungi isolated from grapes. J. Agric. Food Chem. 50, 7493-6. https://doi.org/10.1021/jf025747i

Bejaoui, H., Florence, M., Taillandier, P., Lebrihi, A., 2006. Biodegradation of ochratoxin A by *Aspergillus* section *Nigri* species isolated from French grapes: A potential means of ochratoxin A decontamination in grape juices and musts. FEMS Microbiol. Lett. 255, 203-8. https://doi.org/10.1111/j.1574-6968.2005.00073.x

Blesa, J., Soriano, J.M., Moltó, J.C., Mañes, J., 2006. Factors Affecting the Presence of Ochratoxin A in Wines, Crit. Rev. Food Sci. Nutr. 46, 473-478. https://doi.org/ 10.1080/10408390500215803

Bleve, G., Conte, A., Perrone, G., Solfrizzo, M., Mita, G., Logrieco, A.F., Gallo, A., 2017. Recombinant Production of AcAP1 Protease of *Aspergilus carbonarius* to study its involvement in OTA degradation. Abstract in: Report from the 1st MYCOKEY International Conference Global Mycotoxin Reduction in the Food and Feed Chain Held in Ghent, Belgium, 11–14 September 2017. Toxins, 9, 276. https://doi.org/10.3390/toxins9090276

Boudergue, C., Burel, C., Dragacci, S., Favrot, M., Fremy, J., Massimi, C., Prigent, P., Debongnie, P., Pussemier, L., Boudra, H., et al., 2009. Review of mycotoxin-detoxifying agents used as feed additives: mode of action, efficacy and feed/food safety. EFSA Support. Publ. 6. https://doi.org/10.2903/sp.efsa.2009.EN-22

Cicoňova P., Laciakova, A., Mate, D., 2010. Prevention of ochratoxin a contamination of food and ochratoxin a detoxification by microorganisms – a review. Czech J. Food Sci. 28, 465–474. http://doi.org/10.17221/307/2009-CJFS

Codex Alimentarius Commission, 1998. Position paper on ochratoxin A. CX/FAC 99/14

Codex Alimentarius Commission, 2007. Code of Practice for the prevention and reduction of

ochratoxin A contamination in wine. Codex committee on contamination in wine. Codex committee on contaminants in wine. Codex Committee on Contaminants in Foods. CAC/RCP 63-2007. FAO/WHO Joint Publication, January 2007.Available at http://www.codexalimenta- rius.net/download/standards/10750/CXP_063e.pdf

Commission Regulation 2015/786/EU Defining Acceptability Criteria for Detoxification Processes Applied to Products Intended for Animal Feed as Provided for in Directive 2002/32/EC of the European Parliament and of the Council.

Del Prete, V., Rodriguez, H., Carrascosa, A.V., Rivas, B.D.L., Garcia-Moruno, E., Munoz, R., 2007. *In vitro* removal of ochratoxin A by wine lactic acid bacteria. J. Food Prot. 70, 2155–2160. https://doi.org/10.4315/0362-028x-70.9.2155

European Commission, 2006. Commission Regulation (EC) No 1881/2006 of 19 December 2006 on setting the maximum levels for certain contaminants in foodstuffs. Off. J. Eur. Union, L364, 5–24.

Fracchiolla, R., Leo, R., Solfrizzo, M.,Perrone, G., Piemontese, L., Zivoli, R., Ferara, M., Gambacorta, L., Magistà, D., 2019. Processo e impianto per il trattamento di mosti e vini. Patent 102016000083162

Gallo, A., Bruno, K.S., Solfrizzo, M., Perrone, G., Mulè, G., Visconti, A., Baker, S.E., 2012. New insight into the ochratoxin A biosynthetic pathway through deletion of a nonribosmal peptide synthetase gene in *Aspergillus carbonarius*. Appl. Environ. Microbiol. 78, 8208-18. https://doi.org/10.1128/AEM.02508-12

Hanif, N.Q., Muhammad, G., Siddique, M., Khanum, A., Ahmed, T., Gadahai, J. A., Kaukab, G., 2008. Clinico-pathomorphological, serum biochemical and histological studies in broilers fed Ochratoxin A and a toxin deactivator (Mycofix Plus). Br. Poult. Sci. 49, 632–642. https://doi.org/ 10.1080/00071660802295183

Haq, M., Gonzalez, N., Mintz, K., Jaja-Chimedza, A., De Jesus, C. L., Lydon, C., Welch, A., Berry, J. P., 2016. Teratogenicity of Ochratoxin A and the Degradation Product, Ochratoxin α, in the Zebrafish (*Danio rerio*) Embryo Model of Vertebrate Development. Toxins, 8, 40. https://doi.org/10.3390/toxins8020040

Harwig, J. 1974. Ochratoxin A and related metabolites. Mycotoxins (Purchase IFH, eds), 345–367. Elsevier, Amsterdam.

International Agency for Research on cancer (IARC). 1993. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. Monographs on the evaluation of carcinogenic risks to humans. Vol. 56, Lyon, France.

International Organization of Vine and Wine (OIV). Resolution OIV/OENO 338B/2009. Wines-Treatment Using Chitin Glucane. 2009. Available online: http://www.oiv.int/public/medias/3553/f-code-ii-3417.pdf

International Organization of Vine andWine (OIV). Resolution OIV/OENO 338A/2009. Wines-Treatment Using Chitosan. 2009. Available online: http://www.oiv.int/public/medias/3551/f-code-ii-3416.pdf

Kőszegi, T., Poór, M., 2016. Ochratoxin A: Molecular interactions, mechanisms of toxicity and prevention at the molecular Level. Toxins. 8, 111. https://doi.org/10.3390/toxins8040111

Livak, K.J., Schmittgen T.D., 2001. Analysis of relative gene expression data using Real-Time Quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25, 402-408.

Perrone G., Gallo A., 2017. *Aspergillus* Species and Their Associated Mycotoxins. In: Moretti A., Susca A. (eds) Mycotoxigenic Fungi. Methods in Molecular Biology, vol 1542. Humana Press, New York, NY

Pitout, M.J., 1969. The hydrolysis of ochratoxin A by some proteolytic enzymes. Biochem. Pharmacol. 18, 485–491. https://doi.org/10.1016/0006-2952(69)90224-x

Ponsone, M.L., Chiotta, M.L., Combina, M., Dalcero, A., Chulze, S., 2011. Biocontrol as a strategy to reduce the impact of ochratoxin a and Aspergillus section Nigri in grapes. Int. J. Food Microbiol. 151, 70–77. https://doi.org/10.1016/j.ijfoodmicro.2011.08.005

Scott P.M., 2008. Mycotoxins in alcoholic beverages and fruit juices: occurrence and analysis. Food Contaminants, ACS Symposium Series 1001, 170–191. https://dx.doi.org/10.1021/bk-2008-1001.ch009

Solfrizzo, M.,Avantaggiato, G., Panzarini, G., Visconti, A., 2009. Removal of Ochratoxin A from Contaminated Red Wines by Repassage over Grape Pomaces. J. Agric. Food Chem. 58, 317-23. https://doi.org/10.1021/jf9030585.

Stander, M.A., Bornscheuer, U.T., Henke, E., Steyn, P.S.,2000. Screening of commercial hydrolases for the degradation of ochratoxin A. J. Agric. Food Chem. 48, 5736–5739. https://doi.org/10.1021/jf000413j

Var, I., Erginkaya, Z., Kabak, B., 2011. Inhibition of ochratox- in a production of *Aspergillus carbonarius* by yeast species. Czech J. Food Sci. 29, 291–297. https://doi.org/10.17221/179/2009-CJFS

Visconti, A., Perrone, G., Cozzi, G., Solfrizzo, M., 2008. Managing ochratoxin A risk in the grape-wine food chain. Food Addit. Contam. Part A Chem. Anal. Control. Expo. Risk. Assess. 25, 193–202. https://doi.org/10.1080/02652030701744546

CONCLUSIONS AND FUTURE PRESPECTIVES

- **OTA RISK IN RELATION TO CLIMATE CHANGE**

The *in vitro* effect of interacting climate change (CC) factors was investigated for the first time on the ecophysiological behaviour of *A. carbonarius* in order to estimate the possible impact that projected changes may have in the wine production chain.

The main results obtained within this activity led to the following conclusions:

- A possible increase of temperature from 18/31°C to 20/37°C, likely to happen in an area at high risk of OTA contamination, may be associated to a reduction of both *A. carbonarius* spoilage and OTA production (Study I);
- A possible rise of temperature to 18/34°C, likely to happen in currently low risk areas, may be associated to an increase OTA production (Study I, Addendum);
- Elevated CO_2 air concentration (1000 ppm) under different fluxes of day/night temperatures (15/28°C vs 18/34°C), representative of an area not particularly susceptible to OTA contamination, may result in an increase of colonization rate by *A. carbonarius* and phenotypic OTA production (Study II);
- The experimental approach comprehensive of the molecular support not only strengthened the chemical results but also provided new important evidences about the molecular ecology of *A. carbonarius* under the prospected climate change scenario.

Although restricted to an *in vitro* system this type of data provided new relevant evidences that could be used for the development of predictive models to estimate the potential impact and possible increase in risks of OTA contamination of grapes and grape by-products in areas already identified as hot spots for CC impacts in the Mediterranean region.

- **NEW STRATEGIES FOR REDUCING OTA CONTAMINATION**

The OTA enzymatic biodegradation by proteolytic enzymes able to hydrolyse its amide bond can be considered as a detoxification method since OTα and L-β-phenylalanine are considered as less and non-toxic, respectively. The identification in the genome of *A. carbonarius* of genes encoding for proteolytic enzymes, among which a gene encoding for an aspartyl protease within the OTA cluster, resulted to be promising for this scope.

Results of Study III led us to conclude that:

- *A. carbonarius* is able to degrade OTA as demonstrated by the reduction of exogenous OTA and concomitant production of OTα;

- The mechanism by which the biodegradation happens may involve proteolytic enzymes as shown by the increased levels of some protease encoding genes during OTA degradation.

In the attempt to identify and characterize a protease suitable for OTA detoxification in wines, further experiments of cloning and proteolytic assays have been performed.

APPENDIX

COMMUNICATIONS

- "*Aspergillus carbonarius* proteases and their possible activity in ochratoxin A degradation", Gallo A., Ferrara M., Bleve G., **Cervini C.**, Piemontese L., Epifani F., Logrieco A.F., Solfrizzo M., Perrone G. **Abstract/poster**. 1st MYCOKEY International Conference Global Mycotoxin Reduction in the Food and feed chain, 2017, Ghent, Belgium.

- "Climate change effect on ochratoxin A production and gene expression in *Aspergillus carbonarius*", **Cervini C.**, Magistà D., Gallo A., Piemontese L., Solfrizzo M., Perrone G. **Abstract/poster.** 11th International Mycological Congress-Mycological Discoveries for a Better World, 2018, San Juan, Puerto Rico.

- "Impact of interacting climate change factors on the ecophysiology, toxin gene expression and ochratoxin A production by *Aspergillus carbonarius*", **Cervini C.**, Verkeecke-Vaessen C., Garcia-Cela E., Medina A., Magan N., Magistà D., Ferrara M., Gallo A., Perrone G. **Abstract/poster.** International Commission on Food Mycology, 2019, Technical University of Munich, Freising, Germany.

- *"Aspergillus* from grapes: ochratoxin A risk in relation to climate change and new strategies for reducing contamination", **Oral communication**, XXIV Workshop on the Developments in the Italian PhD research on food science technology and biotechnology, 2019, Firenze, Italy. Evaluation: 89/100 (very good level).

ARTICLES

- "Effects of temperature and water activity change on ecophysiology of ochratoxigenic *Aspergillus carbonarius* in field-simulating conditions", **Cervini, C.**, Gallo, A., Piemontese, L., Magistà, D., Logrieco, A.F., Ferrara, M., Solfrizzo, M., Perrone, G., 2019, *International Journal of Food Microbiology*, 315: 108420, https://doi.org/10.1016/j.ijfoodmicro.2019.108420;

- "Interacting climate change factors (CO_2 and temperature cycles) effects on growth, secondary metabolite gene expression and phenotypic ochratoxin A production by *Aspergillus carbonarius* strains on a grape-based matrix", **Cervini, C.**, Verkeecke-Vaessen, C., Ferrara, M., García-Cela, E., Magistà, D., Medina, A., Gallo, A., Magan, N., Perrone, G., 2019, *Fungal Biology*, in press, https://doi.org/10.1016/j.funbio.2019.11.001.