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Ochratoxin A and aflatoxin producing fungi in raisins and dried figs: detection methods and evaluation of novel strategies to control this contamination

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Abstract

The contamination by Ochratoxin A (OTA) and Aflatoxin B1 (AfB1) in raisins and dried figs is mainly related to the presence of *Aspergillus species.* This contamination poses risks to human and animal health due to the high toxicity and carcinogenicity of these compounds. In this project, different aspects of this problem were considered. The first one consisted of a survey of raisin and dried fig samples, collected from different regions in Algeria and Turkey, for the presence of some OTA and AfB1 producing fungi and for OTA and AfB1 contamination. Both the fungal and mycotoxin contaminations were found in most of the collected samples, with the prevalence of *Aspergillus carbonarius,* which is the main OTA producing species. In some cases, OTA levels were above the maximum tolerable level established by EU directives, underlining the need to pass legislation to regulate the amount of maximum tolerable limit of OTA content in raisins and dried fruits in Algeria. Then, an extraction protocol for raisins and dried figs suitable for direct use in a *nor1*-gene specific loop-mediated isothermal amplification (LAMP) was set to enable rapid and sensitive detection of several aflatoxin-producing species in a single analysis. The tested LAMP assay, after using a minimal sample preparation, was able to detect the presence of aflatoxin-producing fungi belonging to *Aspergillus* section *Flavi* in dried figs and raisins. The developed LAMP assay may be considered as a rapid and sensitive tool for surveillance and quality control in the food industry. After that, a comparison between the effects of the antifungal protein PgAFP as a natural control mean, and potassium sorbate (E-202) on the growth of *Aspergillus carbonarius* and its OTA production was carried out, considering both the eco-physiological and transcriptional levels (gene expression). The results showed that PgAFP significantly controls OTA production, whereas E-202, although able to decrease *Aspergillus carbonarius* growth, can cause a significant increase in OTA production by the fungus. It can be concluded that PgAFP, a biological product with antifungal activity, is safer to use as food preservative compared to E-202. In the last part of this study, the effects of the treatment using different concentrations of ozone (O_3) on *Aspergillus carbonarius* conidia germinability, and on OTA and AfB1 degradation, both as pure molecules and when present in raisins, was investigated. O_3 significantly controlled the germinability of both OTA-producing and non-OTA-producing *Aspergillus carbonarius* conidia. It was also able to degrade a significant percentage of OTA and AfB1. These

results highlight the potential of O_3 to control the contamination by mycotoxigenic fungi and by mycotoxins.

Keywords: Ochratoxin A, Aflatoxin, *Aspergillus*, *Aspergillus carbonarius*, raisins, dried figs, detection, control, ozone, PgAFP, E-202.

Sommario

La contaminazione da parte di Ocratossina A (OTA) e di aflatossina B1 in uva passa e fichi secchi è principalmente dovuta alla presenza di specie fungine appartenenti al genere *Aspergillus*. Questa contaminazione costituisce un rischio per la salute umana e animale a causa della elevata tossicità e cancerogenicità di questi composti. In questo studio sono stati considerati alcuni aspetti di questo problema. In primo luogo, è stata valutata la presenza di isolati appartenenti ad alcune delle specie capaci di produrre OTA e AfB1 e la eventuale contaminazione da parte di questi metaboliti nei campioni di uva passa e fichi secchi raccolti da diverse regioni in Algeria e in Turchia. La maggior parte dei campioni raccolti è risultata contaminata sia da funghi micotossigeni che dalle micotossine considerate, con prevalenza di *Aspergillus carbonarius* che è il principale produttore di OTA. In alcuni casi, i livelli di OTA sono risultati al di sopra del massimo livello tollerabile dalla legislazione dell'UE. Questo evidenzia la necessità di emanare una normativa per regolare il limite massimo tollerabile di OTA in uvetta e frutta secca in Algeria.

In seguito, è stato elaborato un protocollo di estrazione da uva passa e fichi secchi per l'uso diretto al fine di effettuare una LAMP (loop-mediated isothermal amplification)-pcr specifica per *nor1*, allo scopo di consentire il rilevamento rapido e sensibile di diversi isolati produttori di aflatossina in una singola analisi. Il saggio elaborato utilizzando la tecnica LAMP, ha permesso di rilevare la presenza di funghi produttori di aflatossina appartenenti al genere *Aspergillus* sezione *Flavi* sia nei fichi secchi che nell'uva passa utilizzando una minima preparazione del campione. Il saggio LAMP sviluppato può costituire uno strumento rapido e sensibile per la sorveglianza e il controllo di qualità nell'industria alimentare.

A questo punto, è stato effettuato un confronto tra gli effetti della proteina antifungina PgAFP, un mezzo di controllo naturale, e del sorbato di potassio (E-202), sulla crescita di *Aspergillus carbonarius* e sulla produzione di OTA. Il confronto è stato effettuato sia a livello eco-fisiologico che trascrizionale (valutando l'espressione genica). I risultati

hanno mostrato che PgAFP controlla significativamente la produzione di OTA, mentre E-202, pur essendo in grado di diminuire la crescita di *Aspergillus carbonarius*, può causare un aumento significativo della produzione di OTA nel fungo. Si può concludere che PgAFP, un prodotto biologico con attività antifungina, potrebbe essere usato come conservante alimentare con maggiore sicurezza rispetto a E-202.

Nell'ultima parte di questo studio, sono stati valutati gli effetti del trattamento con diverse concentrazioni di ozono (O_3) sulla germinabilità dei conidi di *Aspergillus carbonarius*, e sulla degradazione di OTA e AfB1, sia come molecole pure che in uva passa. O_3 ha permesso di controllare significativamente la germinabilità dei conidi di *A. carbonarius* sia produttore di OTA che non produttore di tossina. Il trattamento con ozono ha permesso anche di degradare una percentuale significativa di OTA e AfB1. Questi risultati evidenziano il potenziale di O_3 nel controllo dei funghi micotossigeni e delle micotossine già prodotte.

Parole chiave: Ocratossina A, aflatossina, *Aspergillus*, *Aspergillus carbonarius*, uva passa, fichi secchi, controllo, ozone, PgAFP, Sorbato di potassio.

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Chapter I: Literature review

I.1 Introduction

Drying of fruit and vegetables is one of the oldest and the most important forms of food preservation methods. It is a well-known process that originated in antiquity, since it has a great effect on product quality. The major objective in drying agricultural products is the reduction of the moisture content to a level which does not allow the development of microorganisms, which guarantee a safe storage over an extended period (Cohen and Yang, 1995; Doymaz, 2005). Among the possible contaminants, fungi are the most difficult to prevent as they may grow at a level of water activity (a_w) as low as 0.65 (Beuchat, 1983). Moreover, dried fruits can be contaminated by molds that can produce toxic secondary metabolites: mycotoxins, that can enter the food chain by contaminating crops still in the field, during storage, or even later (Fredj et al., 2009). Studies regarding the presence of mycotoxins on dried fruits have revealed that those most commonly found are aflatoxin (AfB1) and ochratoxin A (OTA) (Trucksess and Scott, 2008). In general, aflatoxins and OTA can be biosynthesized by different species of fungi mainly belonging to the genus *Aspergillus*, which are able to grow in several food commodities (Battilani et al., 2004; Varga et al., 2011).

This chapter provides background on the study, which focuses on raisins and dried figs in Algeria and Turkey. The chapter includes discussion of pathogen and mycotoxin contamination of raisins and dried figs (in particular OTA and Aflatoxin B1 and their toxicity), *Aspergillus* spp.'s production of these toxins, and the environmental conditions favourable to their development. Then, detection methods, and the most important control means used to face and control this contamination are discussed. The chapter concludes with a summary of the objectives of this thesis.

I.2 Importance of raisins and dried figs

The fig tree, *Ficus carica L.*, is one of the oldest cultivated trees and one of the most important fruit species grown in the Mediterranean region, with Turkey, Egypt, and Algeria being the main fig-producing countries in the world. Production in Algeria was about 131,798 tonnes in 2016 (FAO, 2016). Figs are highly valued due to their high fiber, mineral, and polyphenol content (Heperkan et al., 2012; Michailides, 2003). Figs can be eaten raw or after drying. Dried figs are very nutritious and healthy, and an important

source of carbohydrates, essential amino acids, vitamins A, B1, B2 and C as well as minerals that are used in industrial food products (Javanmard, 2010; Solomon et al., 2006).

Grape is one of the world's major fruit crops. Turkey and Algeria are among the most important grape producers in the world. In 2016 Turkey and Algeria produced 4 million tonnes, and 570,972 tonnes respectively (FAO, 2016). Grapes are the fruit of the ideal energy because they contain easily digestible sugars (glucose and fructose), vitamins C and B, minerals and a significant amount of flavonoids with antioxidant and cardioprotective effects (Vicente et al., 2009; Williamson and Carughi, 2010). Most grapes come from cultivars of *Vitis vinifera*, the European grapevine native to the Mediterranean region and Central Asia, whereas minor amounts of fruit and wine come from American and Asian species. Grapes have different destinations: eaten fresh as table grapes, used for wine production, and dehydrated to produce dried grapes (raisins). These can be used for direct consumption or as an ingredient in cereal bars, biscuits, cookies, puddings and breads. As grapes, raisins contain different polyphenols with antioxidant properties such as resveratrol and quercetin. These compounds also are studied for their anti-inflammatory, anti-cancer, blood cholesterol-lowering activities. Raisins in particular are dense sources of minerals such as calcium, iron, manganese, magnesium, copper, fluoride, and zinc (Vicente et al., 2009; Williamson and Carughi, 2010).

I.3 Pathogens and mycotoxins contamination on raisins and dried figs

Among dried fruits, raisins, prunes, figs, apricots are typically produced in the Mediterranean region, due the suitable environmental conditions. However, the same environmental parameters that promote the development of these crops are conducive to the growth of several plant pathogens and pests. Once the fruits have been dried, most pathogens no longer find the conditions necessary to grow, with the exception of fungal contaminants. Some fungal contaminants can synthesise toxic secondary metabolites (mycotoxins), which may accumulate in the colonised tissues. (Logrieco et al., 2003; Tsitsigiannis et al., 2012). The term mycotoxin comes from the Greek "mycos" which means fungus and from the Latin "toxicum" which means poison. Mycotoxins can enter the food chain starting from the field, after harvest, during storage, or even later, during the storage of the processed products, given favorable conditions of temperature and humidity (Fredj et al., 2009).

According to the Food and Agricultural Organization of the United Nations (FAO), up to 25% of the world's food crops have been estimated to be significantly contaminated with mycotoxins (WHO, 1999), and they are considered part of the most significant food contaminants in terms of impact on public health, food security and many national economies (Steyn, 1995; Pitt, 2000). Mycotoxins are harmful to animals and humans as they may cause serious diseases that vary, for humans, from allergic responses to immunosuppression and cancer. There are over 300 reported mycotoxins (Geisen, 1998, Kong et al., 2016), among these aflatoxins, ochratoxin A, fumonisins, certain trichothecenes, and zearalenone are thought to be particularly damaging to public health worldwide (Pitt, 2000), because they induce diverse and powerful biological effects. Some are carcinogenic (aflatoxins, ochratoxins and fumonisins), mutagenic (aflatoxins and sterigmatocystin), teratogenic (aflatoxins), estrogenic (zearalenone), haemorrhagic (trichothecenes), immunotoxic (aflatoxins and ochratoxins), nephrotoxic (ochratoxins), hepatotoxic (aflatoxins), and dermotoxic (trichothecenes) (Steyn, 1995).

The majority of the mycotoxins are produced by some species of widespread mould genera: e.g. *Aspergillus, Pencillium* and *Fusarium*, which are naturally present in the ambient air, in soil and on the cultures. Thus, these toxins can contaminate feeds and foods such as meat, cheese, spices, fruits, and grapes and also some beverages (Petzinger and Weidenbach, 2002; Yiannikouris and Jouany, 2002).

The human ingestion of mycotoxins is due to both the consumption of plant-based foods (bread, fruit, corn) contaminated with toxigenic fungi and with the produced toxins, as well as to the consumption of meat or animal products (milk, cheese) from animals fed with contaminated feed. Animal products, even if they are free from fungal contamination, can contain mycotoxins, since these secondary metabolites, once synthesized, are distributed and can even concentrate in tissues regardless of the presence of the producer fungi. Toxicity syndromes caused by the intake of mycotoxins are known as mycotoxicoses (Steyn, 1995).

I.4 Ochratoxin A: backgrounds and toxicity

OTA was discovered in 1965 as a metabolite of *Aspergillus ochraceus* by Van der Merwe et al., (1965) during a large research program designed to identify new mycotoxins. There are three main forms of ochratoxin: A, B and C (Fig. I-1). Ochratoxins

B and C are OTA metabolites that are much less toxic than the A form consequently, ochratoxin A (OTA) has received the most attention (Pateraki, 2008).

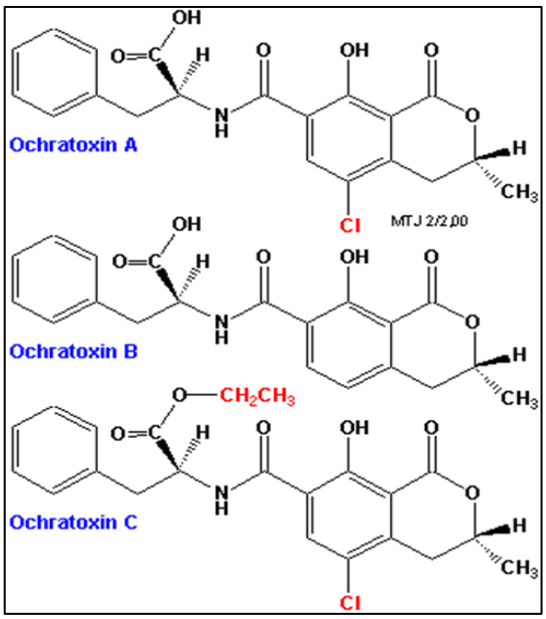


Figure I-1: Differences in structure of ochratoxins A, B, C
http://www.biosite.dk/leksikon/ochratoxin.htm

OTA is an inhibitor of protein synthesis in both *in vivo* and *in vitro* models. In the presence of peroxidases, the phenoxyl radical is formed from OTA (Sorrenti et al., 2013), then, in the presence of glutathione, the phenoxyl radical may be converted to OTA again, but at the expense of the formation of a superoxide anion radical $O_2^{-\cdot}$ which forms H_2O_2, which, in turn, can induce a Fenton reaction resulting in the appearance of a hydroxyl radical ($OH^{\cdot}$) that increases oxidative damage. Furthermore, the phenoxyl radical may directly form C8-deoxyguanosine-adducts.

Although glomerular filtration of OTA is strongly limited due to its albumin binding, the small filtrated and secreted fraction is partially reabsorbed, which can help to account for the accumulation of the toxin in the kidney tubule cells (Ringot et al., 2006).

OTA has been associated with the aetiology of Balkan Endemic Nephropathy (BEN) (Krogh et al., 1977; Radić et al., 1997) and the development of tumours in the urinary tract in humans (Wafa et al., 1998). OTA has been classified in 1993 by the International Agency for Research on Cancer (IARC) as a possible human carcinogen (group 2B) (IARC, 1993).

OTA can occur in different kinds of foods, and high concentrations have been detected in the few studies carried out on dried vine fruits (Abarca et al., 2003). Most of

the information available about OTA contamination of grapes comes from studies carried out in southern European countries such as Spain, Italy, Greece and Turkey (Covarelli et al., 2012). In particular, high levels of OTA were reported in regions with a Mediterranean climate (Cerain et al., 2002).

I.5 Ochratoxin A: producing fungi and conducive conditions

OTA was originally described as a metabolite of *Aspergillus ochraceus*, so the toxin was named Ochratoxin (Van der Merwe et al., 1965). In subsequent years, several other *Aspergillus* and *Penicillium* species were described as producers of this toxin, including strains from *Aspergillus* species section *Circumdati* (*A. ochraceus, A. alliaceus, A. ostianus, A. sclerotiorum, A. sulphureus, A. melleus,* and *A. petrakii*) (Ciegler, 1972; Hesseltine et al., 1972), *A. glaucus* (section *Aspergillus*), *A. niger, A. awamori, A. foetidus,* and *A. carbonarius* (section *Nigri*) (Abarca et al., 1994; Téren et al., 1996), and *Penicillium verrucosum* (Pitt, 1987).

Ochratoxin A (OTA) in raisins is mainly related to the presence of *Aspergillus* section *Nigri* (black Aspergilli), which are common soil inhabitants wherever saprophytic colonization and survival on crop debris results in the production of large numbers of spores which can become air-borne and contaminate ripening crops (Samson et al., 2004). Among the black Aspergilli group, *A. carbonarius* has been reported as the fungus capable of producing the highest amount of toxin, moreover the percentage of isolates able to synthesize OTA is very high (Astoreca et al., 2010; Heenan et al., 1998; Cabañes et al., 2002; Serra et al., 2006; Abarca et al., 2003). *A. carbonarius* conidia, together with those coming from other black Aspergilli, are present on the berry skin from fruit setting, increase in amount from early veraison to harvest, and peak at ripening; however, the incidence of colonised berries is highly related to climatic conditions during the ripening stage and to their geographical location (Somma et al., 2012).

Ochratoxin A producers, which are some *Penicillium* and several black *Aspergillus* species, are a group of species that differ in their ecological niches, in the affected commodities, and in the frequency of their occurrence in different geographical regions. The presence of OTA as a contaminant of grapes, grape products, or even figs and dried figs depends on conducive microclimate conditions which can facilitate germination, germ tube extension, establishment and mycelial colonisation to occur. The most important factors governing these components of the life cycle of micro-organisms are

water availability, temperature and the interaction of microorganisms with the nutrient status of the food matrix. (Magan and Aldred, 2005).

In temperate and cold climates, Ochratoxin A is produced generally by *Penicillium verrucosum* because it grows only at temperatures below 30°C and at water activity as low as 0.8 almost exclusively in cereal and cereal products. In warmer and tropical climates, Ochratoxin A is produced mainly by *Aspergillus* (Pitt and Hocking, 1997) and beside cereals, it is quite commonly found in fruits, particularly in grapes (Battilani et al., 2003). In general, this toxin is produced over a very wide pH range (Esteban et al., 2006). Optimum temperatures for the growth of A. section *Nigri* are reported between 30°C and 37°C, and the growth at temperatures below 15°C is uncommon.

The presence of berries damaged by abiotic or biotic causes, provides preferential entries for black *Aspergilli*, and, in these conditions, their efficiency in producing OTA increases (Battilani et al., 2004). High OTA levels occur in grapes severely damaged by the grape moth, *Lobesia botrana*, particularly in Mediterranean areas. Black *Aspergilli* are opportunistic fungi that develop particularly on damaged berries at ripening, they are present in bunches starting from the setting stage, colonising most berries after early veraison, and they occur and produce OTA on grapes from veraison to harvest (Visconti et al., 2008). In addition, other factors related to grape variety and crop management, such as training system, irrigation and phytosanitary treatments, which influence the ecosystem of the vine, have an effect on OTA accumulation (Lasram et al., 2012).

Very high levels of OTA contamination have been reported on raisins worldwide (Meyvaci et al., 2005; Aksoy et al., 2007; Palumbo et al., 2011; Battilani et al., 2006). After sun drying, the occurrence of *A. carbonarius* becomes higher than that recorded at harvesting time. (Valero et al., 2005). During the drying period grapes are subjected to dehydration until they acquire the desired sugar content and the a_w drops from 0.95 until to 0.75. In these conditions, only few micro-organisms such as black Aspergilli and in particular *A. carbonarius* can survive. In general, black Aspergilli are less susceptible than other species to the germicidal UV rays and the strong sunlight heating due to their thick, heavily melanised pigmented spores (Hocking et al., 2007; Rotem and Aust 1991; Valero et al., 2005; Covarelli et al., 2012; Magan and Aldred, 2005). The increase of black *Aspergillus* during the drying period and the presence of OTA producer strains may explain the higher levels of OTA in dehydrated grapes.

Contamination by OTA in dried figs was reported in lower incidence rates in numerous surveys. The presence of both aflatoxins and OTA in the same sample has also been reported (Bayman et al., 2002; Bircan, 2009; Drusch and Ragab 2003; Drusch and Aumann, 2005; Özay and Alperden, 1991; Senyuva et al., 2005; Trucksess and Scott, 2008).

I.6 Aflatoxin B1: backgrounds and toxicity

Aflatoxins (AFs), were first discovered after an outbreak of a disease of turkeys of unknown etiology in England in 1960. The disease was called Turkey "X" disease and was eventually attributed to a toxic groundnut meal imported from Brazil (Blount, 1960 and 1961). There are a number of different types of aflatoxins (Fig. I-2), Aflatoxin B1, B2, G1 and G2 are found predominantly in food, whereas M1 and M2 aflatoxins are found primarily in animal tissues and fluids (milk, urine) as the hydroxylated metabolic products of aflatoxins B1 (AfB1) and B2 (AfB2) respectively (Richard, 2007). Special interest has been paid to aflatoxins due to their high occurrence and toxicity. The most toxic aflatoxin known, AfB1, is cited as a group I carcinogen by the International Agency for Research on Cancer (IARC, 1993). When AfB1 is ingested, it is absorbed by the intestine and carried to the liver. There, AfB1 is activated and metabolized by cytochromes p450 (CYP) of hepatocytes to AfB1-8,9-epoxide. The 8,9-epoxide is known to react with DNA at the guanine residues of specific sites, one of these being the third base position of codon 249 of the p53 gene. A dose-dependent relationship between dietary AfB1 intake and codon 249ser p53 mutations was observed in hepatocellular carcinoma from Asia, Africa and North America (Moss et al., 2002). AfB1 is considered as the most powerful hepatocarcinogenic compound not produced by human activites but produced by a living organism (Cullen and Newberne, 1994; Newberne and Butler, 1969; Shank et al., 1972; Peers et al., 1976). Acute aflatoxicosis results in death; chronic aflatoxicosis results in cancer, immune suppression, and other "slow" pathological conditions (Hsieh, 1988). aflatoxins are an imminent threat for the safety of food and feed, worldwide (Milićević et al., 2010).

Among the latest outbreak of acute human aflatoxicosis the ones occurred in Kenya's eastern and central provinces in 2004 as a result of the ingestion of contaminated commercial maize caused the death of 125 people (Lewis et al., 2005; Probst et al., 2007).

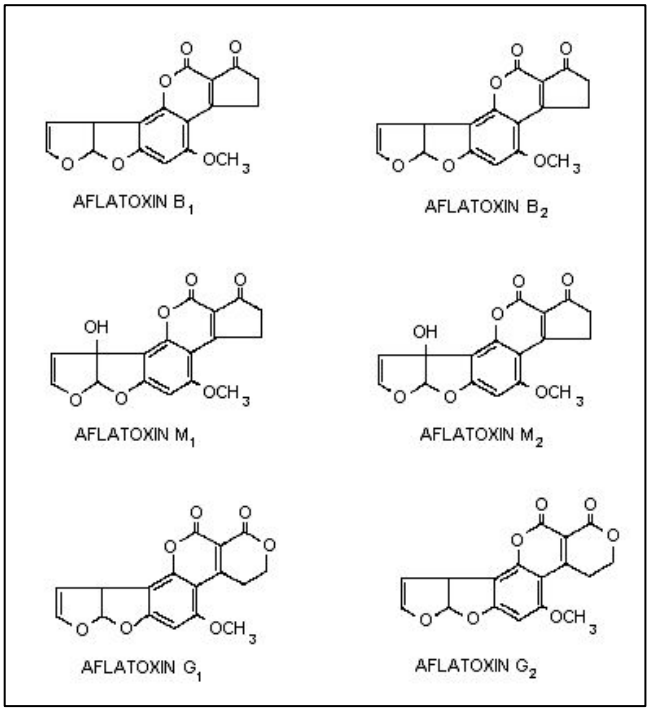


Figure I-2: Chemical structures of the main aflatoxins.
http://www.food-info.net/uk/tox/afla.htm

Aflatoxigenic fungi can occur in food commodities such as corn, peanuts, cottonseed, nuts, spices, figs, and dried fruits (Pitt and Hocking, 1997). Aflatoxins can occur in crops in the field before harvest, as well as during the postharvest phases (Santini and Ritieni, 2013).

I.7 Aflatoxin B1: Producing fungi and conducive conditions

Aflatoxigenic species occur in three sections of the fungal genus *Aspergillus*, i.e. sections *Flavi*, *Nidulantes*, and *Ochraceorosei* (Varga et al., 2011). Among these, section *Flavi* currently contains 15 species capable of producing a wide array of toxic compounds (Varga et al., 2015). In particular, *Aspergillus flavus* is common and widespread and is most often found when certain crops are grown under stressful conditions. High temperatures and drought stress affect the physiology of plants, and stressed plants may be more susceptible to fungal infection and aflatoxin production (Klich, 2007a). This mold occurs in soil, decaying vegetation, hay, and grains undergoing microbiological deterioration and invades all types of organic substrates whenever and wherever the conditions are favorable for its growth.

Food and feed raw materials are especially susceptible to invasion by aflatoxigenic *Aspergillus* spp. under warm and dry climatic conditions. Aflatoxins can be produced at

all stages of commodity processing, including growth in the field, post-harvest, transportation, storage and processing **(**Cotty and Jaime-Garcia, 2007; Ellis et al., 1991). Once conditions of temperature, drought stress and inoculum levels are met, *A. flavus* can enter the plant through a number of portals, this variability has made the development of control measures more difficult. Insect damage is associated with increased aflatoxin in all affected crops. The insects damage plant tissue, thereby creating entry portals for the fungus (Lee et al., 1977; Klich, 2007a). Some insect pests such as the dried fruit beetle or vinegar flies that are active at fruit ripening stage, may act as vectors in transferring the aflatoxigenic fungi to the fruit cavity. In addition to the important effect of environmental factors, and insects for aflatoxin contamination, cultural practices, soil management the susceptibility of varieties play an important role (CAC, 2008).

Typical Mediterranean products such as dried fruits (raisins, prunes, figs, apricots) may be contaminated by aflatoxins (Juan et al., 2008; Karaca and Nas, 2006; Ozer et al., 2012). In particular, figs are at high potential risk of aflatoxin contamination. The high sensitivity of this fruit is due to its juicy and pulpy skin, to the high concentration of sugar, and to its morphology, since it has a natural opening (ostiole) from which fungi and insects can enter and colonize the interior cavity of the fruit. Thus, toxigenic fungi may grow and synthesize aflatoxins on the outer surface or inside the internal cavity even if no damage occurs on the skin (CAC, 2008; Doster et al., 1996). The critical periods for aflatoxin formation in dried fig fruits starts with the ripening of figs on the tree, continues during the over-ripening period, when they lose water, shrivel and fall down onto the ground and until they are fully dried on drying trays (CAC, 2008; Kanapitsas et al., 2015; Valero et al., 2007).

Aflatoxin formation is affected by a large number of biotic and abiotic factors in the environment of the fungus. And it is well known that optimum environomental conditions for fungal growth can be different compared to optimum conditions needed for mycotoxins biosynthesis. Klich, (2007b) reported that the maximum aflatoxin levels were at water activities of 0.95–0.96 and temperatures of 21° and 30°C, and at water activity of 0.89 at 37°C.

I.8 Detection of toxigenic fungi

Classical methods of identification and detection of mycotoxin-producing fungi in food and food raw materials are based on macroscopic and microscopic features that

include cultivation on appropriate media as well as micro- and macro- morphological analysis (Klich, 2002; Raper and Fennell, 1965). These methods are time-consuming, labor and cost intensive and require considerable mycological expertise and dedicated lab facilities. Moreover, misidentification can occur because some fungi may be poorly characterised (Beuchat, 1993; Patiño et al., 2005; Raper and Fennell, 1965). Therefore, rapid and simple methods for the identification of mycotoxigenic fungi are desirable to detect and monitor their presence.

As an alternative to microbiological techniques, molecular methods such as PCR and real-time PCR, which target DNA by amplifying housekeeping genes, have been considered for rapid and accurate diagnosis because of their high specificity and sensitivity (Accensi et al., 1999; Cruz and Buttner, 2008; Patiño et al., 2005; Perrone et al., 2004; Rodríguez et al., 2012; Schmidt et al., 2003). In general, PCR-based assays in food or feed evaluations need at least some kind of DNA clean up and concentration prior to analysis, because a very small amount of material is recovered. This implies that the screening of a high number of isolated pure cultures, or fungal colonies from plate counts can generally not be analyzed by PCR without intermediate cultivation and DNA isolation**.** Moreover, the detection of contaminants directly from infected commodities by PCR is mostly inhibited by the complex microbiota often containing closely related species and a complex mixture of compounds that may affect the efficiency and sensitivity of a PCR assay (Niessen and Vogel, 2010; Rossen et al., 1992)

As an alternative to PCR-based analysis, loop-mediated isothermal amplification (LAMP) has been described as an easy and rapid diagnostic tool (Notomi et al., 2000). LAMP method has attracted much attention, numerous reports have been recorded to evaluate several pathologies including fungal diseases worldwide (Fu et al., 2011; Temple and Johnson 2011). The use of LAMP method enables to conduce all reactions under isothermal conditions. Compared to conventional PCR and real-time PCR assays, expensive equipment is not necessary to obtain a high level of precision, and fewer preparation steps are required. The LAMP method has the advantage of speed and simplicity in detection of fungal microorganisms compared with the classic diagnostic methods (Hara-Kudo et al., 2005; Ushikubo, 2004). The possibility to detect target DNA in materials that are directly added to the master mix is a great advantage that most LAMP assays have over PCR because the system has a much lower sensitivity to inhibition by sample matrix components compared to PCR-based methods (Francois et al., 2011; Kaneko et al., 2007).

Four primers are used for LAMP with six different binding sites in the target DNA which are required to bind properly in order to initiate DNA amplification (Notomi et al., 2000). This higher number of binding sites enables highly specific amplification of target DNA. Several methods have been reported to detect LAMP products: visual observation of turbidity derived from magnesium pyrophosphate formation, real-time turbidimeter, as well as colorimetric LAMP assays using DNA or metallic ions binding dyes (Goto et al., 2009; Tomita et al., 2008; Tomlinson et al., 2010).

The first LAMP reaction for the detection of a mycotoxin-producing fungus; *Fusarium graminearum*, was reported by Niessen and Vogel (2010). LAMP assays for the specific detection of mycotoxigenic fungi, in particular for aflatoxigenic *Aspergillus* spp. have been developed and applied (Luo et al., 2014), but no available assay for the rapid and specific detection of a broad spectrum of different aflatoxin producing species in a wide range of different food commodities has been reported, to date, to our knowledge. Concerning ochratoxigenic *Aspergillus* spp., the development of LAMP assays for the specific detection of *A. carbonarius* and *A. niger* clade was reported by Storari et al., (2013).

I.9 Detection of Ochratoxin A and Aflatoxin B1

The toxicity of these mycotoxins has prompted the adoption of regulatory limits which, in turn, implies the development of suitable validated and official analytical methods and rapid screening tests for cost-effective food control on a large scale. Commonly used methods to analyze mycotoxins are thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) with UV or fluorescence detection (FD), and enzyme immunoassays (ELISA) (Monaci et al., 2004; Rahmani et al., 2009).

Different analytical methods require a purification process in order to obtain a sample suitable for the analytical system. In general, thin layer chromatography can be conducted on a raw extract, while to perform an HPLC analysis, a purified sample is needed. Among the most used purification strategies we mention the immune-affinity purification, or the solid phase extraction (Alshannaq and Yu, 2017; Shephard, 2008)

Thin-layer chromatography (TLC), is still regarded as a cheap and effective technique for ochratoxin A estimation, particularly because of its low cost and adaptability. This technique can be used for the separation, purity assessment, and preliminary identification of different compounds.

High-performance liquid chromatography (HPLC), remains the most used quantitative method for determination of ochratoxin A. Most HPLC methods use a reversed phase column and an acidic mobile phase, so the carboxyl group of the mycotoxin is in the undissociated form.

A number of recent papers deal with immunoassay techniques, especially various enzyme-linked immunosorbent assays (ELISA). Detection limits for HPLC and ELISA are quite comparable, but the ELISA technique suffers from false positive results, because of cross-reactions, and, more importantly, false-negative results, especially from tissue extracts (Monaci et al., 2004)

I.10 Control methods

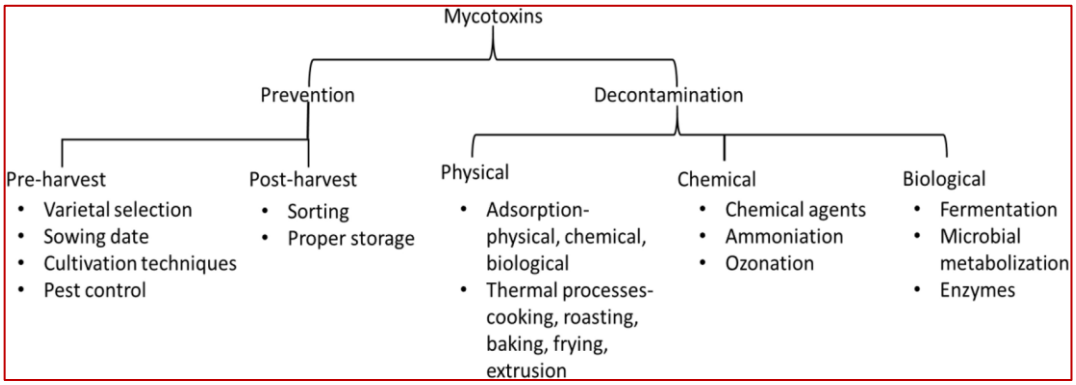
The occurrence of mycotoxinogenic moulds in foods is potentially dangerous for public health and also constitutes a major economic problem. Several strategies, including chemical, physical and biological control methods have been put in place trying to limit the presence of these toxic metabolites in food.

The ideal control method would be easy to use, economical and would not generate toxic compounds or alter other food quality parameters such as nutrient content (Amézqueta et al., 2009). The Hazard Analysis and Critical Control Point (HACCP) approach to processing mycotoxin contaminated commodities should be considered. Certain treatments have been found to reduce the level of specific mycotoxins, but no single method has been developed that is equally effective against the wide variety of mycotoxins which may co-occur in different commodities (Varga and Tóth, 2005). These methods can be applied at pre- or post-harvest level. However, in most of the cases preharvest and post-harvest preventive strategies have to be supplemented with control strategies for the elimination and/or the detoxification (inactivation) of mycotoxins.

As pre-harvest control, mycotoxin contamination can be reduced by following correct agronomic management that involves the use of resistant varieties, timing of planting, irrigation and pruning, the use of particular training systems in order to maintain a correct balance between vegetative growth and fruiting, good canopy management to facilitate adequate sunlight interception, photosynthesis and good air circulation. Furthermore, careful visual inspection to remove inoculum sources such as weeds, agricultural residues or dirty farming materials and also the use of physical and chemical agents that can assist in minimising the development of Aspergilli**.** Because mechanical damage can occur

during harvesting, extra care during this phase to keep such harms to a minimum can significantly reduce contamination. Environmental factors that favour Aspergilli infection include high soil or air temperature, drought stress, nitrogen stress, crowding of plants and conditions that aid dispersal of conidia during silking (CAST, 1989; Covarelli et al., 2012; Hocking et al., 2007; Kabak et al., 2006; Robens, 1990).

During post-harvest, storage and processing are the major and the most critical phase when contamination can be prevented. Fungus can survive in residues left in storage and feeding facilities and thereby, produce mycotoxins under such conditions. In addition, improper packaging or spoiled foodstuffs can cause mycotoxin contamination during this stage. Control can be exerted by improving the drying and storage conditions, as well as by the treatment of the harvest by physical and chemical antifungal agents. Environmental conditions, in particular moisture and temperature, have to be well-controlled. General maintenance and effective hygiene of storage facilities are key factors; storage areas must be clean and disinfected in order to avoid future product deterioration and for the prevention of pests and water ingress. Appropriate packaging is often successful way of excluding insects and moulds (Amézqueta et al., 2009; CAC, 2003; Choudhary and Kumari, 2010; FAO/WHO/UNEP, 1999; Hocking et al., 2007; Kabak et al., 2006; Magan and Aldred, 2007).



(Pankaj and Keener, 2017)

Figure I-3: Summary of the prevention and decontamination strategies for mycotoxins

I.10.1 Biological control

From the food safety point of view, biological control seems nowadays to be the most promising approach for mycotoxins control, this method has emerged as a promising approach for the control of mycotoxins in crops, during both pre-and post-harvest phases. This strategy is accomplished by using microbial antagonists either alone or as part of an

integrated control strategy to reduce pesticide inputs (Tsitsigiannis et al., 2012). Horn et al. (2001) reported that biological control of aflatoxin-producing *A. flavus* and *A. parasiticus* isolates by applying non-toxigenic *A. flavus* isolates in peanut fields was promising. Another technique is the fermentation with non-toxigenic microorganisms in food production as for *A. Terreus* (Dzhavakhiya and Voinova, 2004). In optimal conditions, this procedure can result in a mycotoxin-free food or feed (Santini and Ritieni, 2013). Concerning OTA contamination, biological control may constitute a strategy for controlling both the growth of fungi and the production of OTA. Some bacteria and fungi have shown detoxifying properties in *in vivo* assays (Fuchs et al., 2008). According to Bleve et al. (2006) antagonistic yeasts isolated from the epiphytic flora associated with grapes appear to control *A. carbonarius* and *A. niger*. *Bacillus thuringiensis* was also found to significantly inhibit the growth of OTA-producing fungi on grapes (Bae et al., 2004).

I.10.2 Physical control

The majority of mycotoxins are heat-stable, so heat treatment as physical mean usually applied in food technology, does not have a signicant effect on mycotoxin contamination (Bata and Lásztity, 1999). Treatment with UV irradiation showed several limitations in the effectiveness and environmental sustainability. Among physical treatments ozone has recently received a particular consideration as an antimicrobial agent for the inactivation of bacteria, fungi, viruses and protozoa for disinfecting water, in the food industry, and for reuse of waste water (Guzel-Seydim et al., 2004). Several studies indicated that ozone is an effective treatment for increasing shelf-life and decreasing fungal deterioration during post-harvest, without producing hazardous residues (Gabler et al., 2010; Palou et al., 2002).

I.10.3 Chemical control

The use of chemical agents was for many years the election strategy to prevent fungal contamination in grapes, but the recent concerns about the possible presence of residues in food and in the environment, have made this strategy less appropriate. In addition, the general public demand for a reduced use of chemical preservatives or additives in food or feed (Brul and Coote, 1999) have limited the use of chemicals. Moreover, some moulds have become resistant to certain chemical treatments and some preservatives. For example, some *Penicillium* spp. can grow in the presence of potassium sorbate and other

moulds possess the ability to degrade sorbate (Nielsen and de Boer, 2000; Schnürer and Magnusson, 2005). The reduction of such moulds in food production is thus of primary importance and there is great interest in developing efficient and safe strategies for this purpose.

I.11 Ozone treatment

There is an increasing demand for safe, environmental friendly novel products to control microbial contaminations without leaving toxic chemical residues in the food and in the environment. Ozone treatment has given promising results for important problems in the food industry, including mycotoxins. It is a strong oxidizer that can be used for the inactivation of various microorganisms and the degradation of chemical contaminants. The activity of ozone is mainly due to its oxidative properties which cause the oxidation of sulfhydryl groups, which are abundant in microbial enzymes, and of the polyunsaturated fatty acids of the cell membrane.

The reactivity against unsaturated chemical bonds is also responsible for its mycotoxin degradation mechanism, since molecules with exposed double bonds are found to be more susceptible to degradation (Freitas-Silva and Venâncio, 2010). Decomposition products do not represent any hazard for the treated materials (Karaca and Velioglu, 2014; Kells et al., 2001) and ozone does not cause loss of nutrients or sensory qualities in foods if correctly employed (Cullen et al., 2009). However, there are not many reports on the use of ozone against filamentous fungi or mycotoxins.

Among the few studies that have focused on the use of ozone in fungi inactivation, the one conducted by Antony-Babu and Singleton (2009) suggests that ozone exposure is effective to prevent the spread of *Aspergillus nidulans* and *A. ochraceus,* and the one conducted by Iacumin et al. (2012) showed that ozonated air is able to prevent both the growth of *A. ochraceus* OTA-producing strain and the biosynthesis of this metabolite during the ripening of Milano-like sausages. In addition, Ames et al. (2013) found that ozone controlled the germination of conidia of *A. alternata, A. flavus, A. niger, P. digitatum, P. expansum.*

The first study focusing on mycotoxin degradation in contaminated agricultural kernels by ozone application appeared in the late 1960s. Dwarakanath et al. (1968) reported that gaseous ozone reduced total aflatoxins by 91% (from 214 to 20 ppb) in 2 hours. Although these preliminary results were promising, the use of ozone in mycotoxin degradation was not again tested until 20 years later. In the first work done on the use of

ozone for the degradation of mycotoxins by McKenzie et al. (1997) they found that ochratoxin A and aflatoxin B1 and AfG1 can be significantly and rapidly degraded using 2% weight ozone.

I.12 Thesis objectives

The main objectives of this study are:

1. To carry out a survey of *Aspergillus* spp. OTA and AfB1 producers in dried figs and raisins from Algeria and Turkey, using different methods for their detection and molecular identification, and to set an extraction protocol for raisins and dried figs suitable for direct use in LAMP, in order to detect aflatoxin-producing fungi belonging to *Aspergillus* section *Flavi* rapidly and precisely;
2. To investigate, at eco-physiological and transcriptional levels, the effect of potassium sorbate (E-202) and PgAFP protein from *Penicillium chrysogenum* on the growth of *Aspergillus carbonarius* and on ochratoxin A production in raisins.
3. To study the efficacy of ozone to control Ochratoxin A and Aflatoxin B1 contamination and the viability of some of the toxigenic fungi;

I.13 References

Abarca, M.L., Accensi, F., Bragulat, M.R., Castella, G. and Cabanes, F.J., 2003. *Aspergillus carbonarius* as the main source of ochratoxin A contamination in dried vine fruits from the Spanish market. *Journal of Food Protection*, 66(3), pp.504-506.

Abarca, M.L., Bragulat, M.R., Castella, G. and Cabanes, F.J., 1994. Ochratoxin A production by strains of *Aspergillus niger* var. *niger*. *Applied and Environmental Microbiology*, 60(7), pp.2650-2652.

Accensi, F., Cano, J., Figuera, L., Abarca, M.L. and Cabanes, F.J., 1999. New PCR method to differentiate species in the *Aspergillus niger* aggregate. *FEMS Microbiology Letters*, 180(2), pp.191-196.

Aksoy, U., Eltem, R., Meyvaci, K.B., Altindisli, A. and Karabat, S., 2007. Five-year survey of ochratoxin A in processed sultanas from Turkey. *Food Additives and Contaminants*, 24(3), pp.292-296.

Alshannaq, A. and Yu, J.H., 2017. Occurrence, Toxicity, and Analysis of Major Mycotoxins in Food. *International Journal of Environmental Research and Public Health*, 14(6), p.632.

Ames, Z.R., Feliziani, E. and Smilanick, J.L., 2013. Germination of fungal conidia after exposure to low concentration ozone atmospheres. *Postharvest Biology and Technology*, 83, pp.22-26.

Amézqueta, S., González-Peñas, E., Murillo-Arbizu, M. and de Cerain, A.L., 2009. Ochratoxin A decontamination: A review. *Food Control*, 20(4), pp.326-333.

Antony-Babu, S. and Singleton, I., 2009. Effect of ozone on spore germination, spore production and biomass production in two *Aspergillus* species. *Antonie van Leeuwenhoek*, 96(4), p.413.

Astoreca, A., Barberis, C., Magnoli, C. and Dalcero, A., 2010. *Aspergillus carbonarius* growth and ochratoxin A production on irradiated dried grapes under different water activity and temperature conditions. *World Mycotoxin Journal*, 3(2), pp.175-182.

Bae, S., Fleet, G.H. and Heard, G.M., 2004. Occurrence and significance of Bacillus thuringiensis on wine grapes. *International Journal of Food Microbiology*, 94(3), pp.301-312.

Bata, Á. and Lásztity, R., 1999. Detoxification of mycotoxin-contaminated food and feed by microorganisms. *Trends in Food Science & Technology*, 10(6), pp.223-228.

Battilani, P., Giorni, P. and Pietri, A., 2003. Epidemiology of toxin-producing fungi and ochratoxin A occurrence in grape. *European Journal of Plant Pathology*, 109(7), pp.715-722.

Battilani, P., Giorni, P., Bertuzzi, T., Formenti, S. and Pietri, A., 2006. Black aspergilli and ochratoxin A in grapes in Italy. International *Journal of Food Microbiology*, 111, pp.S53-S60.

Battilani, P., Logrieco, A., Giorni, P., Cozzi, G., Bertuzzi, T. and Pietri, A., 2004. Ochratoxin A production by *Aspergillus carbonarius* on some grape varieties grown in Italy. *Journal of the Science of Food and Agriculture*, 84(13), pp.1736-1740.

Bayman, P., Baker, J.L., Doster, M.A., Michailides, T.J. and Mahoney, N.E., 2002. Ochratoxin production by the *Aspergillus* ochraceus group and *Aspergillus alliaceus*. *Applied and Environmental Microbiology*, 68(5), pp.2326-2329.

Beuchat, L.R., 1983. Influence of water activity on growth, metabolic activities and survival of yeasts and molds. *Journal of Food Protection*, 46(2), pp.135-141.

Beuchat, L.R., 1993. Selective media for detecting and enumerating foodborne yeasts. *International Journal of Food Microbiology*, 19(1), pp.1-14.

Bircan, C., 2009. Incidence of ochratoxin A in dried fruits and co-occurrence with aflatoxins in dried figs. *Food and Chemical Toxicology*, 47(8), pp.1996-2001.

Bleve, G., Grieco, F., Cozzi, G., Logrieco, A. and Visconti, A., 2006. Isolation of epiphytic yeasts with potential for biocontrol of *Aspergillus carbonarius* and *A. niger* on grape. *International Journal of Food Microbiology*, 108(2), pp.204-209.

Blount, W.P., 1960. A new turkey disease problem in England characterised by heavy mortality. *British Oil & Cake Mills*, 27, pp.1-3.

Blount, W.P., 1961. Turkey "X" disease. *Turkeys*, 9(2), pp.52-55.

Brul, S. and Coote, P., 1999. Preservative agents in foods: mode of action and microbial resistance mechanisms. *International Journal of Food Microbiology*, 50(1), pp.1-17.

Cabañes, F.J., Accensi, F., Bragulat, M.R., Abarca, M.L., Castellá, G., Minguez, S. and Pons, A., 2002. What is the source of ochratoxin A in wine?. *International Journal of Food Microbiology*, 79(3), pp.213-215.

CAC (Codex Alimentarius Commission), 2003. Code of practice for the prevention and reduction of mycotoxin contamination of cereals, including annexes on ochratoxin A, zearalenone, fumonisins and trichothecenes. *CAC/RCP*, pp.51-2003.

CAC (Codex Alimentarius Commission), 2008. Code of practice for the prevention and reduction of aflatoxin contamination in dried figs. Doc. Ref. CAC/RCP, pp.65-2008.

CAST, 1989. Mycotoxins: Economic and Health Risks. United States Council for Agricultural Science and Technology (CAST), Ames, Iowa, Report No 116, pp 1-91.

Cerain, A.L.D., González-Peñas, E., Jiménez, A.M. and Bello, J., 2002. Contribution to the study of ochratoxin A in Spanish wines. *Food Additives & Contaminants*, 19(11), pp.1058-1064.

Choudhary, A.K. and Kumari, P., 2010. Management of mycotoxin contamination in preharvest and post-harvest crops: present status and future prospects. *Journal of Phytology*, 2(7).

Ciegler, A., 1972. Bioproduction of ochratoxin A and penicillic acid by members of the *Aspergillus ochraceus* group. *Canadian Journal of Microbiology*, 18(5), pp.631-636.

Cohen, J.S. and Yang, T.C., 1995. Progress in food dehydration. *Trends in Food Science & Technology*, 6(1), pp.20-25.

Cotty, P.J. and Jaime-Garcia, R., 2007. Influences of climate on aflatoxin producing fungi and aflatoxin contamination. *International Journal of Food Microbiology*, 119(1), pp.109-115.

Covarelli, L., Beccari, G., Marini, A. and Tosi, L., 2012. A review on the occurrence and control of ochratoxigenic fungal species and ochratoxin A in dehydrated grapes, non-fortified dessert wines and dried vine fruit in the Mediterranean area. *Food Control*, 26(2), pp.347-356.

Cruz, P. and Buttner, M.P., 2008. Development and evaluation of a real-time quantitative PCR assay for *Aspergillus flavus*. *Mycologia*, 100(5), pp.683-690.

Cullen, J.M., Newberne, P.M., 1994. Acute hepatotoxicity of aflatoxins. In: Eaton DL, Groopman J.D, eds. The toxicology of aflatoxins: human health, veterinary, and agricultural significance. San Diego: *Academic Press*. p 3–26.

Cullen, P.J., Tiwari, B.K., O'Donnell, C.P. and Muthukumarappan, K., 2009. Modelling approaches to ozone processing of liquid foods. *Trends in Food Science & Technology*, 20(3), pp.125-136.

Doster, M.A., Michailides, T.J. and Morgan, D.P., 1996. *Aspergillus* species and mycotoxins in figs from California orchards. *Plant Disease*, 80(5), pp.484-489.

Doymaz, I., 2005. Sun drying of figs: an experimental study. *Journal of Food Engineering*, 71(4), pp.403-407.

Drusch, S. and Aumann, J., 2005. Mycotoxins in fruits: Microbiology, occurrence, and changes during fruit processing. *Advances in Food and Nutrition Research*, 50, pp.33-78.

Drusch, S. and Ragab, W., 2003. Mycotoxins in fruits, fruit juices, and dried fruits. *Journal of Food Protection*, 66(8), pp.1514-1527.

Dwarakanath, C.T., Rayner, E.T., Mann, G.E. and Dollear, F.G., 1968. Reduction of aflatoxin levels in cottonseed and peanut meals by ozonization. *Journal of the American Oil Chemists' Society*, 45(2), pp.93-95.

Dzhavakhiya, V.V. and Voinova, T.M., 2004. Optimization of fermentation conditions for high lovastatin producing mutant 45–50 of fungus *Aspergillus terreus*. *Biotechnology and industry. Nova Science Publisher Inc., New York*, pp.81-87.

Ellis, W.O., Smith, J.P., Simpson, B.K., Oldham, J.H. and Scott, P.M., 1991. Aflatoxins in food: occurrence, biosynthesis, effects on organisms, detection, and methods of control. *Critical Reviews in Food Science & Nutrition*, 30(4), pp.403-439.

Esteban, A., Abarca, M.L., Bragulat, M.R. and Cabañes, F.J., 2006. Effect of pH on ochratoxin A production by *Aspergillus niger* aggregate species. *Food Additives and Contaminants*, 23(6), pp.616-622.

FAO/WHO/UNEP (1999). Mycotoxin prevention and decontamination, corn - a case study. Third Joint FAO/WHO/UNEP International Conference on Mycotoxins. 6 b: 1-11.

FAO: Faostat (2016). *Agriculture Data*. Available at: http://faostat.fao.org/default.aspx (accessed 10 September 2017).

Francois, P., Tangomo, M., Hibbs, J., Bonetti, E.J., Boehme, C.C., Notomi, T., Perkins, M.D. and Schrenzel, J., 2011. Robustness of a loop-mediated isothermal amplification reaction for diagnostic applications. *FEMS Immunology & Medical Microbiology*, 62(1), pp.41-48.

Fredj, S.M.B., Chebil, S. and Mliki, A., 2009. Isolation and characterization of ochratoxin A and aflatoxin B1 producing fungi infecting grapevines cultivated in Tunisia. African *Journal of Microbiology Research*, 3(9), pp.523-527.

Freitas-Silva, O. and Venâncio, A., 2010. Ozone applications to prevent and degrade mycotoxins: a review. *Drug Metabolism Reviews*, 42(4), pp.612-620.

Fu, S., Qu, G., Guo, S., Ma, L., Zhang, N., Zhang, S., Gao, S. and Shen, Z., 2011. Applications of loop-mediated isothermal DNA amplification. *Applied Biochemistry and Biotechnology*, 163(7), pp.845-850.

Fuchs, S., Sontag, G., Stidl, R., Ehrlich, V., Kundi, M. and Knasmüller, S., 2008. Detoxification of patulin and ochratoxin A, two abundant mycotoxins, by lactic acid bacteria. *Food and Chemical Toxicology*, 46(4), pp.1398-1407.

Gabler, F.M., Smilanick, J.L., Mansour, M.F. and Karaca, H., 2010. Influence of fumigation with high concentrations of ozone gas on postharvest gray mold and fungicide residues on table grapes. *Postharvest Biology and Technology*, 55(2), pp.85-90.

Geisen, R., 1998. PCR methods for the detection of mycotoxin-producing fungi. *Application of PCR in mycology. CAB International, New York, 1998*, pp.243-266.

Goto, M., Honda, E., Ogura, A., Nomoto, A. and Hanaki, K.I., 2009. Short technical reports. Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue. *Biotechniques*, 46, pp.167-172.

Guzel-Seydim, Z.B., Greene, A.K. and Seydim, A.C., 2004. Use of ozone in the food industry. *LWT-Food Science and Technology*, 37(4), pp.453-460.

Hara-Kudo, Y., Yoshino, M., Kojima, T. and Ikedo, M., 2005. Loop-mediated isothermal amplification for the rapid detection of Salmonella. *FEMS microbiology letters*, 253(1), pp.155-161.

Heenan, C.N., Shaw, K.J. and Pitt, J.I., 1998. Ochratoxin A production by *Aspergillus carbonarius* and *A. niger* isolates and detection using coconut cream agar. *Journal of Food Mycology*, 1, pp.67-72.

Heperkan, D., Moretti, A., Dikmen, C.D. and Logrieco, A.F., 2012. Toxigenic fungi and mycotoxin associated with figs in the Mediterranean area. *Phytopathologia Mediterranea*, pp.119-130.

Hesseltine, C.W., Vandegraft, E.E., Fennell, D.I., Smith, M.L. and Shotwell, O.L., 1972. Aspergilli as ochratoxin producers. *Mycologia*, pp.539-550.

Hocking, A.D., Su-lin, L.L., Kazi, B.A., Emmett, R.W. and Scott, E.S., 2007. Fungi and mycotoxins in vineyards and grape products. *International Journal of Food Microbiology*, 119(1), pp.84-88.

Horn, B.W., Greene, R.L., Sorensen, R.B., Blankenship, P.D. and Dorner, J.W., 2001. Conidial movement of nontoxigenic *Aspergillus flavus* and *A. parasiticus* in peanut fields following application to soil. *Mycopathologia*, 151(2), pp.81-92.

Hsieh, D., 1988. Potential human health hazards of mycotoxins. In: Natori, S., Hashimoto, K., Ueno, Y. (Eds.), *Mycotoxins and Phytotoxins.* Third Joint Food and Agriculture Organization/W.H.O./United Nations Environment Program International Conference of Mycotoxins. *Elsevier*, Amsterdam, The Netherlands, pp. 69–80.

Iacumin L., Manzano M., Comi G. 2012. Prevention of *Aspergillus ochraceus* growth and Ochratoxin a contamination of sausages using ozonated air. *Food Microbiology*, 29(2), pp. 229-32.

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 1993. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. World Health Organization, International Agency for Research on Cancer.

Javanmard, M., 2010. Occurrence of mould counts and *Aspergillus* species in Iranian dried figs at different stages of production and processing. *Journal of Agricultural Science and Technology, 12*, pp.331-338.

Juan, C., Zinedine, A., Molto, J.C., Idrissi, L. and Mañes, J., 2008. Aflatoxins levels in dried fruits and nuts from Rabat-Salé area, Morocco. *Food Control*, 19(9), pp.849-853.

Kabak, B., Dobson, A.D. and Var, I.I.L., 2006. Strategies to prevent mycotoxin contamination of food and animal feed: a review. *Critical Reviews in Food Science and Nutrition*, 46(8), pp.593-619.

Kanapitsas, A., Batrinou, A., Aravantinos, A. and Markaki, P., 2015. Effect of γ-radiation on the production of aflatoxin B 1 by *Aspergillus parasiticus* in raisins (*Vitis vinifera L.*). *Radiation Physics and Chemistry*, 106, pp.327-332.

Kaneko, H., Kawana, T., Fukushima, E. and Suzutani, T., 2007. Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. *Journal of Biochemical and Biophysical Methods*, 70(3), pp.499-501.

Karaca, H. and Nas, S., 2006. Aflatoxins, patulin and ergosterol contents of dried figs in Turkey. *Food Additives and Contaminants*, 23(05), pp.502-508.

Karaca, H. and Velioglu, Y.S., 2014. Effects of ozone treatments on microbial quality and some chemical properties of lettuce, spinach, and parsley. *Postharvest Biology and Technology*, 88, pp.46-53.

Kells, S.A., Mason, L.J., Maier, D.E. and Woloshuk, C.P., 2001. Efficacy and fumigation characteristics of ozone in stored maize. *Journal of Stored Products Research*, 37(4), pp.371-382.

Klich, M.A., 2002. Indentification of common *Aspergillus* species. *Centraalbureau voor Schimmelcultures.* Utrecht, The Netherlands, 116 pp.

Klich, M.A., 2007a. *Aspergillus flavus*: the major producer of aflatoxin. *Molecular Plant Pathology*, 8(6), pp.713-722.

Klich, M.A., 2007b. Environmental and developmental factors influencing aflatoxin production by *Aspergillus flavus* and *Aspergillus parasiticus*. *Mycoscience*, 48(2), pp.71-80.

Kong, D., Liu, L., Song, S., Suryoprabowo, S., Li, A., Kuang, H., Wang, L. and Xu, C., 2016. A gold nanoparticle-based semi-quantitative and quantitative ultrasensitive paper sensor for the detection of twenty mycotoxins. *Nanoscale*, 8(9), pp.5245-5253.

Krogh, P., Hald, B., Pleština, R. and Čeović, S., 1977. Balkan (endemic) nephropathy and foodborn ochratoxin A: *preliminary results of a survey of foodstuffs*. *APMIS*, 85(3), pp.238-240.

Lasram, S., Oueslati, S., Mliki, A., Ghorbel, A., Silar, P. and Chebil, S., 2012. Ochratoxin A and ochratoxigenic black *Aspergillus* species in Tunisian grapes cultivated in different geographic areas. *Food Control*, 25(1), pp.75-80.

Lee, L.S., Cucullu, A.F., Pons, W.A. and Russell, T.E., 1977. Separation of aflatoxin-contaminated cottonseed based on physical characteristics of seed cotton and ginned seed. *Journal of the American Oil Chemists' Society*, 54(3), pp.A238-A241

Lewis, L., Onsongo, M., Njapau, H., Schurz-Rogers, H., Luber, G., Kieszak, S., Nyamongo, J., Backer, L., Dahiye, A.M., Misore, A. and DeCock, K., 2005. Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in eastern and central Kenya. *Environmental Health Perspectives*, 113(12), p.1763.

Logrieco, A., Bottalico, A., Mulé, G., Moretti, A. and Perrone, G., 2003. Epidemiology of toxigenic fungi and their associated mycotoxins for some Mediterranean crops. *European Journal of Plant Pathology*, 109(7), pp.645-667.

Luo, J., Taniwaki, M.H., Iamanaka, B.T., Vogel, R.F. and Niessen, L., 2014. Application of loop-mediated isothermal amplification assays for direct identification of pure cultures of *Aspergillus flavus*, *A. nomius*, and *A. caelatus* and for their rapid detection in shelled Brazil nuts. *International Journal of Food Microbiology*, 172, pp.5-12.

Magan, N. and Aldred, D., 2005. Conditions of formation of ochratoxin A in drying, transport and in different commodities. *Food Additives and Contaminants*, 22(s1), pp.10-16.

Magan, N. and Aldred, D., 2007. Post-harvest control strategies: minimizing mycotoxins in the food chain. *International Journal of Food Microbiology*, 119(1), pp.131-139.

McKenzie, K.S., Sarr, A.B., Mayura, K., Bailey, R.H., Miller, D.R., Rogers, T.D., Norred, W.P., Voss, K.A., Plattner, R.D., Kubena, L.F. and Phillips, T.D., 1997. Oxidative

degradation and detoxification of mycotoxins using a novel source of ozone. *Food and Chemical Toxicology*, 35(8), pp.807-820.

Meyvaci, K.B., Altindisli, A., Aksoy, U., Eltem, R., Turgut, H., Arasiler, Z. and Kartal, N., 2005. Ochratoxin A in sultanas from Turkey I: Survey of unprocessed sultanas from vineyards and packing-houses. *Food Additives and Contaminants*, 22(11), pp.1138-1143.

Michailides T.J., 2003. Diseases of fig. In: *Diseases of Tropical Fruit Crops* (R.C. Ploetz, ed.), CAB International Wallingford, UK, pp. 253–273.

Milićević, D.R., Škrinjar, M., Baltić, T., 2010. Real and perceived risks for mycotoxin contamination in foods and feeds: challenges for food safety control. *Toxins* 2, 572-592.

Monaci, L., Tantillo, G. and Palmisano, F., 2004. Determination of ochratoxin A in pig tissues by liquid–liquid extraction and clean-up and high-performance liquid chromatography. *Analytical and Bioanalytical Chemistry*, 378(7), pp.1777-1782.

Moss, M.O., 2002. Risk assessment for aflatoxins in foodstuffs. *International Biodeterioration & Biodegradation*, 50(3), pp.137-142.

Newberne, P.M. and Butler, W.H., 1969. Acute and chronic effects of aflatoxin on the liver of domestic and laboratory animals: a review. *Cancer Research*, 29(1), pp.236-250.

Nielsen, P.V. and De Boer, E., 2000. Food preservatives against fungi. *Introduction to Food-and Airborne Fungi* (eds.: RA Samson, ES Hoekstra, JC Frisvad and O. Filtenborg), pp.357-363.

Niessen, L. and Vogel, R.F., 2010. Detection of Fusarium graminearum DNA using a loop-mediated isothermal amplification (LAMP) assay. *International Journal of Food Microbiology*, 140(2), pp.183-191.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. and Hase, T., 2000. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, 28(12), pp.e63-e63.

Özay, G. and Alperden, I., 1991. Aflatoxin and Ochratoxin—a contamination of dried figs (Ficus carina L) from the 1988 crop. *Mycotoxin Research*, 7(2), pp.85-91.

Ozer, H., Oktay Basegmez, H.I. and Ozay, G., 2012. Mycotoxin risks and toxigenic fungi in date, prune and dried apricot among Mediterranean crops. *Phytopathologia Mediterranea*, pp.148-157.

Palou, L., Crisosto, C.H., Smilanick, J.L., Adaskaveg, J.E. and Zoffoli, J.P., 2002. Effects of continuous 0.3 ppm ozone exposure on decay development and physiological responses of peaches and table grapes in cold storage. *Postharvest Biology and Technology*, 24(1), pp.39-48.

Palumbo, J.D., O'Keeffe, T.L., Vasquez, S.J. and Mahoney, N.E., 2011. Isolation and identification of ochratoxin A-producing *Aspergillus* section *Nigri* strains from California raisins. *Letters in Applied Microbiology*, 52(4), pp.330-336.

Pankaj, S.K., Shi, H. and Keener, K.M., 2017. A review of novel physical and chemical decontamination technologies for aflatoxin in food. *Trends in Food Science & Technology*, 71, pp.73-83.

Pateraki, M.G., 2008. Ecology of *A. carbonarius* and ochratoxin A production in vine fruits and control in the production chain.

Patiño, B., González-Salgado, A., González-Jaén, M.T. and Vázquez, C., 2005. PCR detection assays for the ochratoxin-producing *Aspergillus carbonarius* and *Aspergillus ochraceus* species. *International Journal of Food Microbiology*, 104(2), pp.207-214.

Peers, F.G., Gilman, G.A. and Linsell, C.A., 1976. Dietary aflatoxins and human liver cancer. A study in Swaziland. *International Journal of Cancer*, 17(2), pp.167-176.

Perrone, G., Susca, A., Stea, G. and Mule, G., 2004. PCR assay for identification of *Aspergillus carbonarius* and *Aspergillus japonicus*. *European Journal of Plant Pathology*, 110 (2004), pp. 641-649.

Petzinger, E. and Weidenbach, A., 2002. Mycotoxins in the food chain: the role of ochratoxins. *Livestock Production Science*, 76(3), pp.245-250.

Pitt, J.I. and Hocking, A.D., 1997. Fungi and Food Spoilage Blackie Academic & Professional. *New South Wales*, Australia.

Pitt, J.I., 1987. Penicillium viridicatum, Penicillium verrucosum, and production of ochratoxin A. *Applied and Environmental Microbiology*, 53(2), pp.266-269.

Pitt, J.I., 2000. Toxigenic fungi and mycotoxins. *British Medical Bulletin*, 56(1), pp.184-192.

Probst, C., Njapau, H. and Cotty, P.J., 2007. Outbreak of an acute aflatoxicosis in Kenya in 2004: identification of the causal agent. *Applied and Environmental Microbiology*, 73(8), pp.2762-2764.

Radić, B., Fuchs, R., Peraica, M. and Lucić, A., 1997. Ochratoxin A in human sera in the area with endemic nephropathy in Croatia. *Toxicology Letters*, 91(2), pp.105-109.

Rahmani, A., Jinap, S. and Soleimany, F., 2009. Qualitative and quantitative analysis of mycotoxins. *Comprehensive Reviews in Food Science and Food Safety*, 8(3), pp.202-251.

Raper, K.B. and Fennell, D.I., 1965. The genus *Aspergillus*. *Williams and Wilkins, Baltimor*e, MD.

Richard, J.L., 2007. Some major mycotoxins and their mycotoxicoses-An overview. *International Journal of Food Microbiology*, 119(1), pp.3-10.

Ringot, D., Chango, A., Schneider, Y.J. and Larondelle, Y., 2006. Toxicokinetics and toxicodynamics of ochratoxin A, an update. *Chemico-biological Interactions*, 159(1), pp.18-46.

Robens, J.F., Huff, W. and Richard, J., 1990. A Perspective on Aflatoxin in Field Crops and Animal Food Products in the United States (ARS-83). *US Department of Agriculture: Washington*, DC, pp.1-157.

Rodríguez, A., Rodríguez, M., Luque, M.I., Martín, A. and Córdoba, J.J., 2012. Real-time PCR assays for detection and quantification of aflatoxin-producing molds in foods. *Food Microbiology*, 31(1), pp.89-99.

Rossen, L., Nørskov, P., Holmstrøm, K. and Rasmussen, O.F., 1992. Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. *International Journal of Food Microbiology*, 17(1), pp.37-45.

Rotem, J. and Aust, H.J., 1991. The effect of ultraviolet and solar radiation and temperature on survival of fungal propagules. *Journal of Phytopathology*, 133(1), pp.76-84.

Samson, R.A., Houbraken, J.A.M.P., Kuijpers, A.F., Frank, J.M. and Frisvad, J.C., 2004. New ochratoxin A or sclerotium producing species in *Aspergillus* section *Nigri*. *Studies in Mycology*, 50, pp.45-61.

Santini, A. and Ritieni, A., 2013. Aflatoxins: risk, exposure and remediation. In *Aflatoxins-Recent Advances and Future Prospects*. InTech.

Schmidt, H., Ehrmann, M., Vogel, R.F., Taniwaki, M.H. and Niessen, L., 2003. Molecular typing of *Aspergillus ochraceus* and construction of species specific SCAR-primers based on AFLPs. *Systematic and Applied Microbiology*, 26(1), pp.138-146.

Schnürer, J. and Magnusson, J., 2005. Antifungal lactic acid bacteria as biopreservatives. *Trends in Food Science & Technology*, 16(1), pp.70-78.

Senyuva, H.Z., Gilbert, J., Ozcan, S. and Ulken, U., 2005. Survey for co-occurrence of ochratoxin A and aflatoxin B1 in dried figs in Turkey by using a single laboratory-

validated alkaline extraction method for ochratoxin A. *Journal of Food Protection*, 68(7), pp.1512-1515.

Serra, R., Mendonça, C. and Venâncio, A., 2006. Ochratoxin A occurrence and formation in Portuguese wine grapes at various stages of maturation. *International Journal Of Food Microbiology*, 111, pp.S35-S39.

Shank, R.C., Bhamarapravati, N., Gordon, J.E. and Wogan, G.N., 1972. Dietary aflatoxins and human liver cancer. IV. Incidence of primary liver cancer in two municipal populations of Thailand. *Food and Cosmetics Toxicology*, 10(2), pp.171-179.

Shephard, G.S., 2008. Determination of mycotoxins in human foods. *Chemical Society Reviews*, 37(11), pp.2468-2477.

Solomon, A., Golubowicz, S., Yablowicz, Z., Grossman, S., Bergman, M., Gottlieb, H.E., Altman, A., Kerem, Z. and Flaishman, M.A., 2006. Antioxidant activities and anthocyanin content of fresh fruits of common fig (*Ficus carica* L.). *Journal of Agricultural and Food Chemistry*, 54(20), pp.7717-7723.

Somma, S., Perrone, G. and Logrieco, A.F., 2012. Diversity of black Aspergilli and mycotoxin risks in grape, wine and dried vine fruits. *Phytopathologia Mediterranea*, pp.131-147.

Sorrenti, V., Di Giacomo, C., Acquaviva, R., Barbagallo, I., Bognanno, M. and Galvano, F., 2013. Toxicity of ochratoxin A and its modulation by antioxidants: A review. *Toxins*, 5(10), pp.1742-1766.

Steyn, P.S., 1995. Mycotoxins, general view, chemistry and structure. *Toxicology Letters*, 82, pp.843-851.

Storari, M., Rohr, R.V., Pertot, I., Gessler, C. and Broggini, G.A., 2013. Identification of ochratoxin A producing *Aspergillus carbonarius* and *A. niger* clade isolated from grapes using the loop-mediated isothermal amplification (LAMP) reaction. *Journal of Applied Microbiology*, 114(4), pp.1193-1200.

Temple, T.N. and Johnson, K.B., 2011. Evaluation of loop-mediated isothermal amplification for rapid detection of Erwinia amylovora on pear and apple fruit flowers. *Plant Disease*, 95(4), pp.423-430.

Téren, J., Varga, J., Hamari, Z., Rinyu, E. and Kevei, F., 1996. Immunochemical detection of ochratoxin A in black *Aspergillus* strains. *Mycopathologia*, 134(3), pp.171-176.

Tomita, N., Mori, Y., Kanda, H. and Notomi, T., 2008. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nature Protocols*, 3(5), pp.877-882.

Tomlinson, J.A., Dickinson, M.J. and Boonham, N., 2010. Detection of Botrytis cinerea by loop-mediated isothermal amplification. *Letters in Applied Microbiology*, 51(6), pp.650-657.

Trucksess, M.W. and Scott, P.M., 2008. Mycotoxins in botanicals and dried fruits: a review. *Food Additives and Contaminants*, 25(2), pp.181-192.

Tsitsigiannis, D.I., Dimakopoulou, M., Antoniou, P.P. and Tjamos, E.C., 2012. Biological control strategies of mycotoxigenic fungi and associated mycotoxins in Mediterranean basin crops. *Phytopathologia Mediterranea*, pp.158-174.

Ushikubo, H., 2004. Principle of LAMP method--a simple and rapid gene amplification method. *Uirusu*, 54(1), pp.107-112.

Valero, A., Marín, S., Ramos, A.J. and Sanchis, V., 2005. Ochratoxin A-producing species in grapes and sun-dried grapes and their relation to ecophysiological factors. *Letters in Applied Microbiology*, 41(2), pp.196-201.

Valero, A., Marín, S., Ramos, A.J. and Sanchis, V., 2007. Effect of preharvest fungicides and interacting fungi on *Aspergillus carbonarius* growth and ochratoxin A synthesis in dehydrating grapes. *Letters in Applied Microbiology*, 45(2), pp.194-199.

Van der Merwe, K.J., Steyn, P.S. and Fourie, L., 1965. 1304. Mycotoxins. Part II. The constitution of ochratoxins A, B, and C, metabolites of *Aspergillus ochraceus* wilh. *Journal of the Chemical Society* (Resumed), pp.7083-7088.

Varga, J. and Tóth, B., 2005. Novel strategies to control mycotoxins in feeds: A review. *Acta Veterinaria Hungarica*, 53(2), pp.189-203.

Varga, J., Baranyi, N., Chandrasekaran, M., Vágvölgyi, C. and Kocsubé, S., 2015. Mycotoxin producers in the *Aspergillus* genus: an update. *Acta Biologica Szegediensis*, 59(2), pp.151-167.

Varga, J., Frisvad, J.C. and Samson, R.A., 2011. Two new aflatoxin producing species, and an overview of *Aspergillus* section *Flavi*. *Studies in Mycology*, 69, pp.57-80.

Vicente, A.R., Manganaris, G.A., Sozzi, G.O. and Crisosto, C.H., 2009. Nutritional quality of fruits and vegetables. *Postharvest handling: A systems approach*, pp.57-106.

Visconti, A., Perrone, G., Cozzi, G. and Solfrizzo, M., 2008. Managing ochratoxin A risk in the grape-wine food chain. *Food Additives and Contaminants*, 25(2), pp.193-202.

Wafa, E.W., Yahya, R.S., Sobh, M.A., Eraky, I., El-Baz, M., El-Gayar, H.A., Betbeder, A.M. and Creppy, E.E., 1998. Human ochratoxicosis and nephropathy in Egypt: a preliminary study. *Human & Experimental Toxicology*, 17(2), pp.124-129.

Williamson, G. and Carughi, A., 2010. Polyphenol content and health benefits of raisins. *Nutrition Research*, 30(8), pp.511-519.

Yiannikouris, A. and Jouany, J.P., 2002. Mycotoxins in feeds and their fate in animals: a review. *Animal Research*, 51(2), pp.81-99.

Chapter II: Investigation on dried figs and raisins from Algeria and Turkey for OTA, AfB1, and toxigenic *Aspergillus* spp. contamination.

II.1 Introduction

In grapes and raisins, black Aspergilli and in particular *A. carbonarius* has been identified as the key species responsible for OTA contamination since most of the strains belonging to this fungal species are able to produce high concentrations of OTA (Cabañes et al., 2002; Battilani et al., 2003). In addition, raisins are at greater risk of OTA contamination due to the increased rate of *A. carbonarius* during drying (Gómez et al., 2006; Valero et al., 2005; Magan and Aldred, 2005). Several published surveys and reports have addressed the epidemiology, ecology, and distribution of black Aspergilli occurring in grapes and grape products. Most of the surveys are from the Mediterranean region and *A. carbonarius* with its Ochratoxin A production is more frequently isolated in southern Mediterranean areas (Greece, Portugal, Spain, Southern Italy and Turkey) (Akdeniz et al., 2013; Aksoy et al., 2007; Battilani et al., 2006; Blesa et al., 2004; Lucchetta et al., 2010; Meyvaci et al., 2005; Serra et al., 2003; Stefanaki et al., 2003; Tjamos et al., 2004). However, Ochratoxin contamination and producing fungi in grape and grape products have never been reported in Algeria, although OTA contamination was reported in wheat in this country in 2008 by Riba et al. (2008).

Aflatoxins are the most toxic mycotoxins. They are potent carcinogens, teratogens, genotoxics and mutagens that pose severe hazards to animal and human health (Gourama and Bullerman, 1995; Klich, 2007; Richard, 2008), and they are produced predominantly by *Aspergillus flavus* and *Aspergillus parasiticus* (Bennett and Papa, 1988). Aflatoxigenic fungi are found in various food commodities including figs (Oktay et al., 2011; Buchanan et al., 1975). In the literature, the occurrence of Aflatoxin in dried fig fruits in Algeria has not been extensively investigated. However, Aflatoxin in Algeria was reported in several other commodities such as: apricot kernels (Imperato et al., 2011), pistachios (Fernane et al. 2010), wheat (Riba et al., 2010) and some common spices including aniseed, black pepper, cinnamon, coriander, cumin, ginger, red pepper, saffron, sweet cumin, and sweet pepper (Azzoune et al., 2016).

As regards the occurrence of OTA producing fungi in Algeria, few papers have been published; Riba et al. (2008) found a high percentage of ochratoxigenic *Aspergillus spp.* in wheat, whereas Khalef et al. (1993) reported a high incidence of chronic interstitial

nephropathies of unknown aetiology and for which OTA was suspected to play a role. However, in Algeria, there are no data on the occurrence of ochratoxigenic and aflatoxigenic spp. in raisins and dried figs, and little is known about levels of OTA and Aflatoxin contamination in these commodities.

In Algeria the maximum tolerable level for Aflatoxin contamination is regulated to 20 ppb for total Aflatoxins (B_1+B_2+G_1+G_2) and 10 ppb for AfB1, whereas Ochratoxin contamination has not been regulated (FAO, 2004).

The present study was carried out to isolate and to identify *Aspergillus* spp. OTA or AfB producers from raisins and dried figs using different means, and to evaluate the potential occurrence of OTA and AfB contamination in Algeria particularly.

II.2 Materials and methods

II.2.1 Raisin samples collection

The sampling procedure and drying of grapes were carried out by the technical institute of fruit trees and grapevine in Mascara region (Algeria). Ten samples were collected randomly after drying in 2013 and 2014 from three different regions in Algeria and from four grape varieties (Table II-1). The samples were placed in plastic bags and transported to the laboratories where they were refrigerated at 4°C until use. In 2014, five additional samples were provided from Manisa region in Turkey, which is the main grape-producing region (Figure II-1). Sampling and drying of the grapes were carried out between the month of September and October.

Table II-1: Regions and sampled varieties from raisin

Country	Sampling regions	Varieties	Number of samples	Year of sampling
Algeria	Biskra	Sultanine	1	2013
		Sultanine	1	2015
	Mostaganem	Sultanine	2	2013
		Supernova seedless	2	2015
	Mascara	Centennial	2	2013
		Sublime seedless	2	2013
Turkey	Alaşehir/Manisa	Sultana seedless	2	2014
	Sarıgöl/Manisa		2	2014
	Ahmetli/Manisa		1	2014

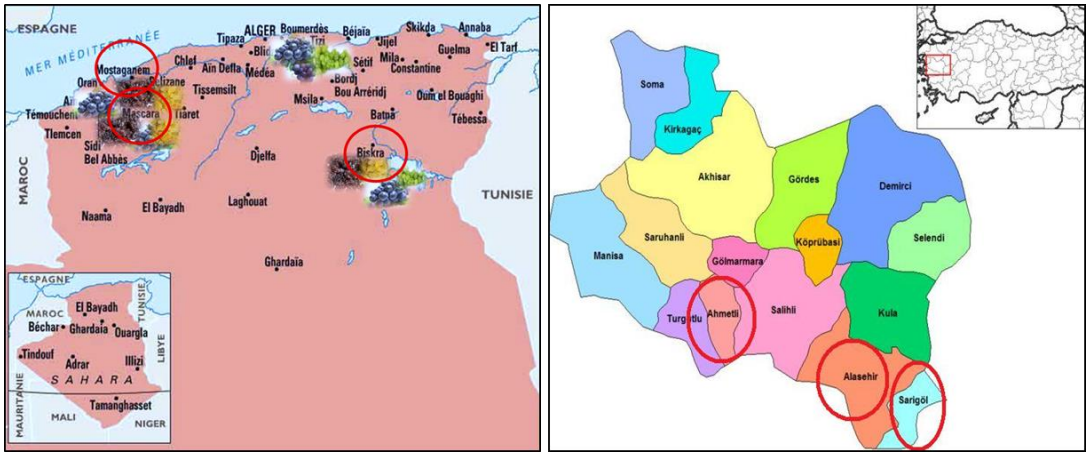


Figure II-1: Regions in Algeria and Tukey from which raisin samples were collected.

Drying procedures operation is necessary to ensure the product quality. Sugar levels required in the fresh grapes is 22 % (220g/L must). Ripe bunches of grapes free of all impurities (leaves, rotten or dried grapes), were manually harvested and placed in perforated shelves. Then bunches were placed in a cold soaking solution (containing 6-8 Kg potassium carbonate (K_2CO_3) and 0.5 L in 100L cold water) for 4-5 minutes and then removed and left to drain on a wooden or metallic platform. Then the grapes were spread over a slightly sloping concrete platform for 7 to 10 days during which the grapes were regularly turned over. The drying procedures are shown in Figure II-2.

Figure II-2: Raisin: harvesting and drying procedure.

II.2.2 Dried fig samples collection

Dried fig samples were obtained from local farmers in Béni Maouch region (Bejaia) in Algeria, where they performed the Sampling and the drying procedures during the month of September (Fig. II-3). After harvesting, the figs were placed on woody drying racks (Fig. II-4), and dried in a traditional way, under the sun for 3 to 4 days until reaching a relative humidity (RH) < 25% and sugar content > 45%. They were covered with a plastic cover during the night and in case of rain. Different collected varieties are shown in Table II-2.

Table II-2: Regions and sampled varieties of dried figs.

Region	Variety	Samples
Beni Maouch (Béjaïa)	Taranimt	1
	Azenjal	3
	Thamrouith	8

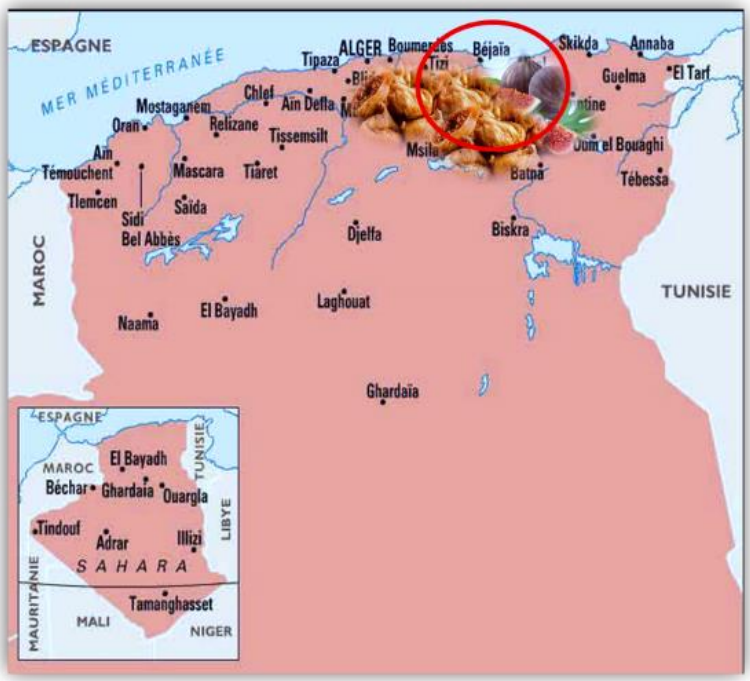


Figure II-3: Regions in Algeria from which raisin samples were collected.

Figure II-4: Harvesting and drying procedure of dried figs.

II.2.3 Isolation of *Aspergillus* species from raisins and dried figs

The isolation of *Aspergillus spp.* was performed using two different media: NYDA (Nutrient Yeast Dextrose Agar) and DRBC (Dichloran Rose Bengal Chloramphenicol).

The protocol for fungal isolation on DRBC medium was as follows: from each sample**,** about 20 grams of raisins or one whole dried fig were placed in a sterile, special

plastic bag for the homogenizer with 50ml sterile tap water and were then subjected to homogenization for 30 seconds. The obtained suspension was serially diluted (from 10^{-1} to 10^{-3}) and aliquots (100µl) were spread on DRBC agar medium. The DRBC composition for 1L of medium is: Glucose 10g; Peptone 5g; Potassium dihydrogen phosphate KH_2PO_4 1g; Agar 15g; Magnesium sulphate $MgSO_4$ 0.5g; Bengal rosa 0.025g; Chloramphenicol 0.1g; Dichloran 0.002g; and up to 1L of medium (pH adjusted to 5.6). Plates were incubated at 24°C for 7 days and daily checked until fungal colonies became evident (Fig. II-5).

The protocol for fungal isolation on NYDA medium was as follows: for each sample**,** about 200 grams of raisins or dried figs were placed in a beaker with 500ml sterile distilled water, and subjected to orbital shaking (270 rpm) for 1h at 25°C. The obtained suspension was serially diluted (from 10^{-1} to 10^{-5}) and aliquots (100µl) were spread in Petri dishes amended with NYDA medium. NYDA composition for 1L of medium: Nutrient broth 8g; Yeast extract 6g; Dextrose 10g; Agar 20 g. The medium was made selective for the growth of fungi by adding 250mg of Streptomycin sulphate and 250mg of Ampicillin. Plates were incubated at 24°C for 7 days (Fig. II-6).

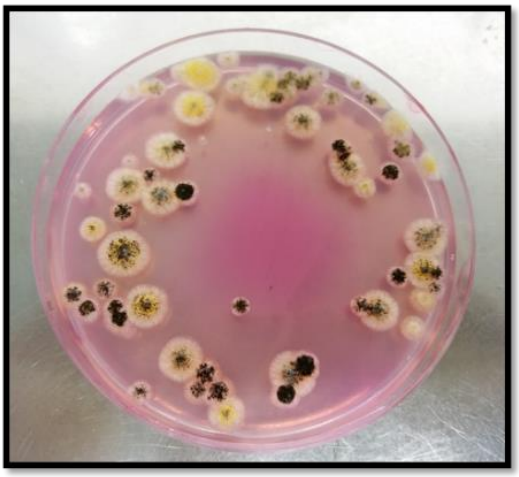


Figure II-5: Fungal isolates from raisins/ dried figs on the DRBC medium

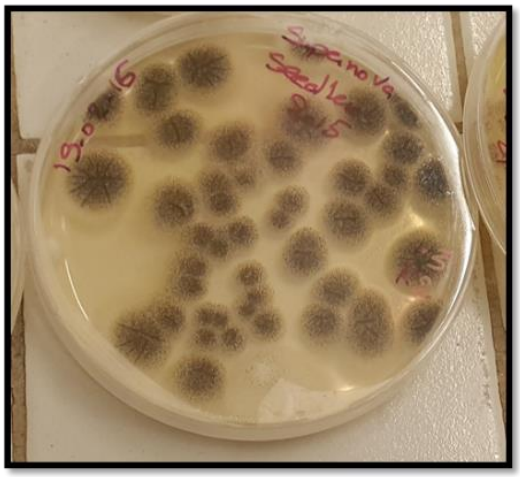


Figure II-6: Fungal isolates from raisins/ dried figs on the NYDA medium

After incubation, the plates were observed under a colony counter (WTW, model BZG 30) and all the developed molds that belonged to *Aspergillus* genera according to their morphological characteristics, following the rules and the descriptions provided by Pitt and Hocking (1997); Raper and Fennell (1965); Samson et al. (2004), were counted and CFUs were evaluated. Then one or more representative colonies from each group having the same morphological characteristics were chosen and transferred to a Petri dish

with MEA (malt extract agar) or PDA (Potato Dextrose Agar) medium and subjected to purification.

To obtain a pure single-spore culture, some spores coming from PDA or MEA cultures were placed in 1.5mL Eppendorf filled with 1 ml sterile distilled water containing tween 0.1% and vortexed. An amount of 20 µl was spread on PDA medium plates. When small colonies appeared, they were transferred to a new PDA plate, as shown in Figure II-7.

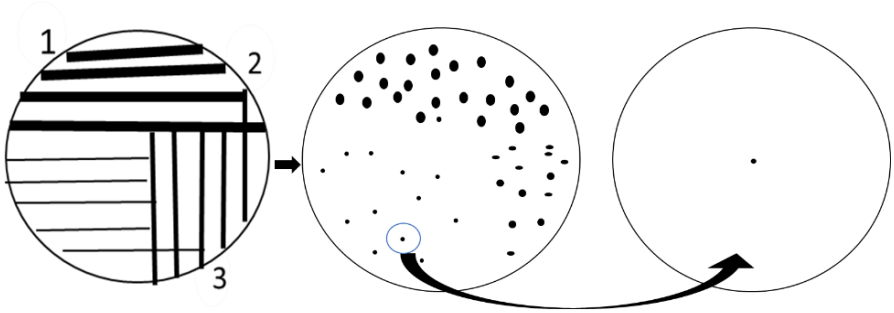


Figure II-7: Spreading scheme for 20µl of conidia suspension and transferring to a new PDA plate.

II.2.4 Morphological identification of black *Aspergillus* spp.

Morphological identification of mainly *Aspergillus carbonarius* and *Aspergillus niger* species was carried out following the guide lines given by Pitt and Hocking (1997); Raper and Fennell (1965); Samson et al. (2004). The major and remarkable macroscopic features in species identification are: the colony diameter, texture, color and the appearance of culture reverse. The microscopic characteristics include: conidial heads color and length, vesicles shape and seriation, conidia size and morphology.

II.2.5 Molecular identification of some black *Aspergillus* spp.

II.2.5.1 DNA extraction from fungal mycelium

After purification, some representative fungal strains thought to belong to *Aspergillus carbonarius* or *Aspergillus niger* were grown in Erlenmeyer flasks containing 25ml of PDB (Potato Dextrose broth) medium. The cultures were subjected to orbital shaking (170 rpm) for 7 days at 25°C, then the mycelium was collected by filtration on paper filters (Whatman n°4). Fungal mycelium was used for DNA extraction based on the commercial kit DNeasy® Plant Mini kit (Qiagen, Solna, Sweden), but modified in the cell lysis. In particular, fungal mycelium (100mg frozen mycelium), 4µl RNAse A

(100mg ml^{-1}, Qiagen), 400µl AP1 lysis buffer (provided by the DNeasy® Plant Mini Spin Column kit), two sterile iron balls (Ø 5mm) and 25 mg of acid washed glass beads (425-600µm diameter) were used. The tubes were run in a FastPrep® Cell Disrupter (MM400, Retsch®) for 1min at speed level 5.0. The lysate was incubated in a heating block at 65°C for 10min and thereafter treated according to the protocol supplied with the DNeasy® Mini Spin Column kit (Qiagen). At the end the spin column containing the DNA was transferred to a new 1,5 ml micro-centrifuge tube and 100 µL buffer AE used for elution were added, incubated for 5min at room temperature, and centrifuged for 1 min at 6000*g (this last step was repeated twice). DNA concentration was then determined by spectrophotometer (UV-1800, Shimadzu) at λ = 260 – 280nm. Finally the DNA was stored at –20°C for further PCR assays.

II.2.5.2 PCR amplifications for black *Aspergillus* spp. Strains

Our efforts were mainly focused on the occurrence of *A. carbonarius* and *A. niger*. Two species-specific primers were designed on the basis of ITS (internal transcribed spacers of rDNA units) sequence comparisons obtained from *Aspergillus* strains and were tested in a number of strains from different origins and hosts (González-Salgado et al., 2005; Patiño et al., 2005). All genomic DNAs used in this work were tested for suitability for PCR amplification using primers ITS4 and ITS6. The two sets of primers were:

- ITS1/NIG: (5’ TCCGTAGGTGAACCTGCGG 3’) and (5’ CCGGAGAGAGGGGACGGC 3’) respectively for *A. niger* (González-Salgado et al., 2005);
- CAR1/CAR2: (5’GCATCTCTGCCCCTCGG3’) and (5’GGTTGGAGTTGTCGGCAG 3’) respectively for *A. carbonarius* (Patiño et al., 2005).

All amplification reactions were conducted in volumes of 25 µL containing: 2 µL of genomic DNA from each sample, 5 µL of 5x PCR buffer (Promega, Madison WI-U.S.A), 12.3 µL of sterile distilled water, 1 µL of 100 mM of dNTPs buffer (Promega, Madison WI-U.S.A), 2.5 µL of 25 mM $MgCl_2$ (Promega, Madison WI-U.S.A), 1 µL each of forward and reverse primers of 10mM and 0.2 µL of GoT*Taq*® DNA polymerase (5 U/µL Promega, Madison WI-U.S.A) for both *A. carbonarius* and *A. niger* reactions. The PCR amplification protocol used for *A. carbonarius* was as follows: 1 cycle of 5s at 95°C, 30 cycles of 10s at 95°C (denaturation), 25s at 59°C (annealing), 15s at 72°C (extension) and finally 1 cycle of 5 min at 72°C. In the case of *A. niger* the PCR program was as follows: 1 cycle of 4 min 30s at 95°C, 25 cycles of 30s at 95°C (denaturation), 2 s at 62°C (annealing), 40s at 72°C (extension) and finally 1 cycle of 5 min at 72°C. Amplicons were

separated in 1.2% agarose gel in TAE buffer (1x), previously stained with 2µl Gel red nucleic acid stain, and observed under UV.

II.2.5.3 Real-time LAMP (loop-mediated isothermal amplification) for the detection of *Aspergillus carbonarius*

Two LAMP primer sets were designed by Storari et al. (2013), based on the polyketide synthase genes involved or putatively involved in ochratoxin A (OTA) biosynthesis in *A. carbonarius*.

Table II-3: List of AcPKS LAMP Primer sequences

Primers		Sequence (5′-3′)
***A. carbonarius* – AcPKS**	FIP	GCTGCAATGCACCCGGTAGTTGAAGACGTGGAGGGCTTCT
	BIP	AAGTCACGTCAAAAGCCCTGGTGATTCCTTGGGAGGTTGGTC
	LF	GCTGGCCGAAAAGATCGCTAA
	LB	CCCCTCTGCTATGAAGTCCG
	F3	TGGTGGTACGAATGCACAC
	B3	CGAAATGACAAACAGGCGGT
	FIP	CCTGCGCCACCTTCCAAGTGCGATTCGCCCCTCTATGTTG

Instead of the conventional detection method used by Storari et al. (2013)**,** which was a colorimetric detection by using Hydroxynaphthol blue (HNB) as the indicator for DNA amplification, in this work the same primers sequences were used for a Real-time LAMP assay. The real-time LAMP assays for *A. Carbonarius* detection was performed with a Bio-Rad IQ™ 5 thermocycler (Bio-Rad Laboratories), employing a reaction volume of 25 µL containing 1x Isothermal Master-Mix (OptiGene Ltd. Horsham) with a ds-DNA binding dye, 1.6 Mm/l of each internal primer (FIP and BIP), 0.2 Mm/l of each external primer (F3 and B3), 0.8 Mm/l of each loop primer (LF and LB), and 2 µL template DNA. LAMP amplifications were carried out at 65°C for a period of 30 minutes and the real-time fluorescence value was measured. For each reaction series, a negative control was included.

II.2.6 Assessment of the contamination of the collected samples by OTA and AfB1

II.2.6.1 Ochratoxin A and Aflatoxin B1 extraction from strains isolated from raisins and dried figs

Work on Aflatoxin B1 (AfB1) and Ochratoxin A (OTA) extraction and analysis were done at the Institute of Molecular Biology and Pathology-CNR in Rome. In order to evaluate OTA contamination, the isolated strains were cultured in Czapek Dox yeast

(0.5%) liquid medium in static condition at 25°C for 10 days. Then 35mL were taken from the liquid culture and each sample was extracted using the same volume of a mixture of ethyl acetate and formic acid (99:1 v/v) (Fig. II-8). The extraction process was repeated twice, and the extracts were collected and dried at 30°C under an airflow. The samples were then redissolved in 100 µL of methanol for HPLC analysis.

For AfB1 extraction, the protocol was the same, except that instead of ethyl acetate, chloroform was used as solvent.

Figure II-8: Aflatoxin B1 and Ochratoxin A extraction procedures from *Aspergillus* cultures.

II.2.6.2 Aflatoxin B1 and Ochratoxin A extraction from raisins and dried figs

Extraction of AfB1 and OTA from raisins and dried figs was carried out by mincing around 50g of raisins/dried figs with 80% distilled water (1:0.8 v/v) using a blender (Fig. II-9). Each amount of 45g of that slurry was then extracted by adding 30 ml of a mixture of methanol: water: orthophosphoric acid (0,1M) (70:20:10 v/v/v) and conducting an orbital agitation (150 rpm) for 1 hour. The extraction was repeated twice. The extracts were collected and centrifuged at 4000 rpm for 15 minutes, then cleansed by means of paper filters (Whatman n°4) to obtain a clear liquid. Methanol was evaporated using an air stream. 12.5mL of the concentrated extracts were diluted with 100 mL of PBS (Phosphate buffered saline) buffer 0.1M in the case of raisin extraction, while for dried figs extraction the whole volume was used without dilution. The resulting samples were divided in two parts, one to be purified using the specific AflaStar (for AfB1) and one to be purified using the specific OchraStar (for OTA) immuno-affinity columns (Romer Labs, Austria). For OTA extraction, the sample was loaded to the IAC column, once it

passed through, the IAC column was washed with PBS buffer (20 ml) and eluted with 2ml of methanol acidified with 1% of 0.1M acetic acid. For AfB1 extraction the raw extract from the raisins was passed through the IAC columns AflaStar Rohmer and the washing step after loading the sample in the IAC column was conducted using 20 mL of distilled water instead of 20 ml of PBS buffer. The extracts were concentrated under an air flow and re-dissolved in 200 µl of methanol. An aliquot of 20µL was analysed by HPLC.

Figure II-9: Aflatoxin B1 and Ochratoxin A extraction procedures from raisins and dried figs.

II.2.6.3 High Performance Liquid Chromatography (HPLC) analysis

After purification, the extracts were analysed by High Performance Liquid Chromatography (HPLC) (Agilent 1260, Milan, Italy) (Fig. II-10), equipped with a diode array detector. An Agilent YMC-Triart C18 column (150x4,6mm; 3.0 mm) equipped with a pre-column filter was used as a stationary phase, and a mobile phase of acetonitrile: water: acetic acid (99:99:2, v/v/v). The analyses were conducted under isocratic conditions at a flow rate of 0.8 ml/min.

The quantification of AfB1 and OTA was performed using the external standard method by comparing the peak values obtained by the pure standard with those having the same retention time obtained when analysing the samples. the wavelength for OTA and AfB1 are 333nm and 363nm respectively. The limit of detection (LD) and quantification (LQ) were 0.5 and 1.5 ng for OTA, and 1 and 3 ng for AfB1. The limit of detection (LD) and quantification (LQ) for OTA were 0.03 and 0.13 µg/Kg, and 0.04 and 0.12 µg/Kg for AfB1.

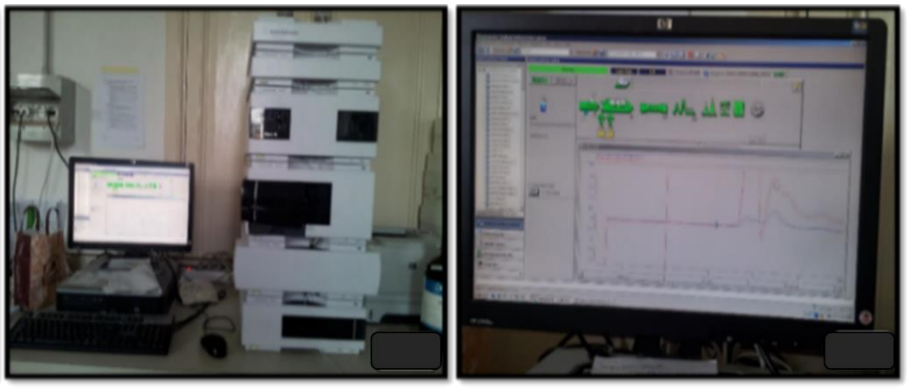


Figure II-10: Ochratoxin A and aflatoxin B1 analysis by HPLC.

II.2.7 LAMP assay for the detection of aflatoxin producers within *Aspergillus* section *Flavi.* from dried figs and raisins.

The research project described here was set up under the supervision of Prof. Dr. Martin Ludwig Niessen Technical Microbiology, Technical University of Munich (Germany), in the context of a doctoral internship abroad as intended in the PhD program.

The goal of the study was to detect aflatoxin producers within *Aspergillus* section *Flavi* in dried figs and raisins from Algeria and Turkey, setting the extraction protocol for direct use in isothermal detection assays LAMP.

The outcome of this research was published in the International Journal of Food Microbiology: Niessen, L., Bechtner, J., Fodil, S., Taniwaki, M.H. and Vogel, R.F., 2017. LAMP-based group specific detection of aflatoxin producers within *Aspergillus* section *Flavi* in food raw materials, spices, and dried fruit using neutral red for visible-light signal detection.

II.2.7.1 Primers for LAMP assay

A set of LAMP primers (Table II-4) was designed by Prof. Dr. Martin Ludwig Niessen (Niessen et al., 2017) based on the multiple alignment of partial sequences of the norsolorinic acid reductase gene (*nor1*, syn. *AflD*) of *A. arachidicola*, *A. bombycis*, *A. caelatus*, *A. flavus*, *A. minisclerotigenes*, *A. nomius*, *A. oryzae*, *A. parasiticus* and *A. pseudotamarii*.

Table II-4: List of *nor1* LAMP Primer sequences

Primer name	Sequence 5´ > 3´
F3-nor1 ID8	ACT GCG ACT CGG AAA GYG A
B3-nor1 ID8	GGA CTG CTG CAG CAT CAG
FIP-nor1 ID8	GGC CCA AAG TTC TGC GCC AT-C CAG ACA TTG CGG GAR GA
BIP-nor1 ID8	ACC ATG CCC CTC GAR CAT CT-G CGG GTT GCC TGA AAC AG
LF-nor1 ID92	ACY ACC ACG TCC AAG TGC
LB-nor1 ID92	TGA TGG TCA AYA TGT ATG CTC C

II.2.7.2 Preparation of template DNA for LAMP analysis from raisins and dried figs

For raisins, a LAMP template was prepared from whole raisins, 2 g of each sample were transferred into a sterile 15 mL Falcon tube (Sarstedt, Numbrecht, Germany) and vigorously hand mixed with 5 mL of sterile deionized water containing 1 % (v/v) Tween 20. For dried figs, a LAMP template was prepared by chopping a whole fig from each sample into small pieces (between 15 to 25g), transferring them into a sterile 50 mL Falcon tube and vigorously hand mixing them with 20 mL of sterile deionized water containing 1 % (v/v) Tween 20. Subsequently, 2 mL of the supernatant from raisin and dried fig samples were transferred to a sterile 2 ml reaction vessel and centrifuged for 2 min at 11,000 x g. Tween 20 was removed by washing the resulting pellet three times, each with 1 mL of sterile deionized water with intermediate centrifugation, as described previously, before suspending the extract in 500 µL of sterile deionized water.

For direct addition to the LAMP assays, 300µL of the extract isolated from contaminated samples were shaken vigorously with a vortex at maximum speed after addition of sterile glass beads (0.4 g of 0.5 mm diameter, 0.2 g of 1.25-1.65 mm diameter) for 10 min. The resulting supernatant was directly used as template in LAMP reactions.

II.2.7.3 DNA amplification

LAMP mastermix for neutral red-based detection was set up containing the following components per 25 µL of reaction volume: 1.9 µL of 10x MOPS buffer (200 mM MOPS, 100 mM KCl, 100 mM $(NH_4)_2SO_4$), 1 µL $MgCl_2$ (200 mM), 3.5 µl dNTP-mix (10 mM each GATC), 2.6 µL primer-mix (1.6 mM each FlP-nor1 ID8 and BIP-nor1 ID8, 0.8 mM each LF-nor1 ID92 and LB-nor1 ID92, 0.2 mM each F3-nor1 ID8 and B3-nor1 ID8; see Table II-4 for primer sequences), 8.5 µL of sterile deionized water, 1.5 µL neutral red

(2.5 mM in 1x amonium sulfate buffer, pH 8.7), 1 µL of *Bst* polymase (8 U/µL, New England Biolabs, Frankfurt, Germany) and 5 µL of template DNA in sterile deionized water. Buffer, deionized water and neutral red solutions were filter sterilized through Filtropur S 0.2 µm syringe tip filters (Sarstedt, Nümbrecht, Germany) or reconstituted with sterile deionized water (deoxynucleotides, primers) and stored at 4°C. *Bst* polymerase were stored at -20°C. To avoid contamination, two different sets of pipettes were used in separate rooms for the preparation of LAMP master mix and addition of template DNA, respectively. Sterile pipet tips with filters were used for all pipetting. LAMP reactions were incubated in a Mastercycler® Gradient thermal cycler (Eppendorf, Hamburg, Germany) at 64 °C for neutral red-based signal detection for 60 min.

II.3 Results

II.3.1 Morphological identification of *Aspergillus* strains, from raisins and dried figs

A mycological survey of the 15 samples of raisins collected during 2013, 2014 and 2015 indicated the presence of filamentous fungi (62,243 isolates) with predominance of *Aspergillus* genus and especially those belonging to the Section *Nigri* (Black Aspergilli) which represented 87% (Fig. II-11). The remaining 12% (7,700 isolates), were the green and yellow Aspergilli: *Aspergillus flavus*, *Aspergillus parasiticus*, and *Penicillium* spp. isolates. Few representative *A. flavus* and *A. parasiticus* isolates were chosen to be studied during this work.

We focused mainly on the identification of the *Aspergillus carbonarius* and *Aspergillus niger* species, thus some representative black *Aspergillus* isolates (103 isolates) were chosen to be identified based on morphological characteristics following the guidelines given by Samson et al. (2004); Pitt and Hocking (1997) and Raper and Fennell (1965). We were able to identify 14% as *A. carbonarius* and 45% *A. niger*, the rest were other black *Aspergillus* species such as *A. awamori* and *A. foetidus*,

Fungal isolation from the 12 samples of dried figs on both NYDA and DRBC media, revealed the predominance of mostly yeast, mainly two species that were identified using MALDI-TOF MS analysis as *Zygosaccharomyces rouxii* and *Candida lactiscondensi*. Only in 3 dried fig samples (two from Thamrouith and one Taranimt varity), 25 isolates of filamentous fungi were found. They were identified as green and yellow Aspergilli (*A. flavus* and *A. parasiticus*) and *Penicillium* spp. No black Aspergilli were isolated.

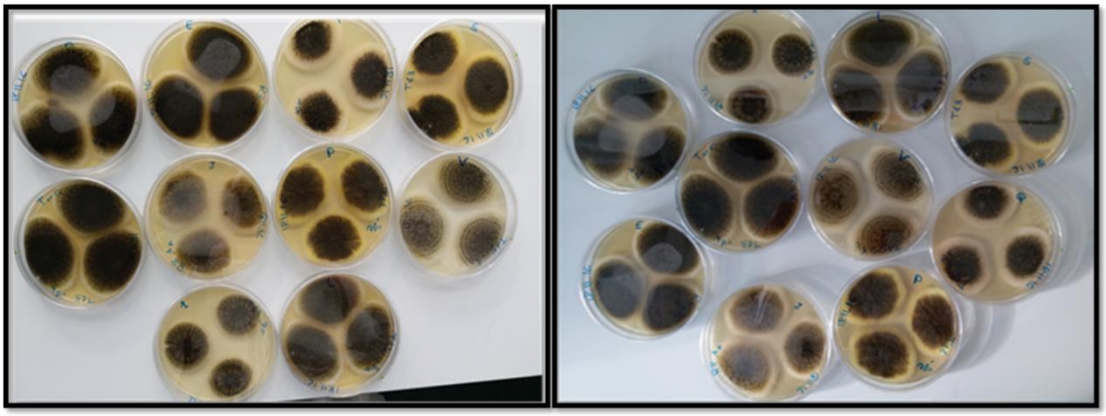


Figure II-11: Some black *Aspergillus* isolates from raisins on Malt extract agar medium.

II.3.2 Isolated *Aspergillus* strains by variety of raisins and dried figs

As shown in Table II-5, the highest number of *Aspergillus* isolates coming from Algerian raisins, was attributed to the samples coming from Mostaganem region, which is situated on the coast, and especially to the two varieties of supernova seedless with up to 25,000 isolates. This was followed by the Sultanine variety with 3,000 isolates in the same region. The same variety Sultanine, grown in Biskra region accounted for only 50 isolates.

The Centennial variety from Mascara region, had the lowest number of contamination with *Aspergillus* species, due probably to a characteristic in the variety itself, since in the same region and year, other varieties (Sublima seedless and Sultanine) had 450 and 200 isolates respectively. Also, we noticed that Sultanine variety from Mascara region, had a difference in the number of isolates between 2013 (200 isolates) and 2014 (60 isolates).

Concerning raisin samples coming from Turkey, the highest number of isolates was attributed to two samples of Sultana seedless from Alaşehir in Manisa region, with 2300 and 9300 isolates, compared to the other samples coming from Sarıgöl and Ahmetli regions.

Table II-5: Number of isolates per raisin and dried fig samples

Samples	Code	Variety	Number of Isolates	Sampling regions	Sampling year
Raisins	**R1**	Sultanine	3000	Mostaganem	2013
	R2	Sultanine	50	Biskra	2013
	R3	Sultanine	200	Mascara	2013
	R4	Sublima seedless	450	Mascara	2013
	R5	Sublima seedless	430	Mascara	2013
	R6	Centennial	3	Mascara	2013
	R7	Centennial	50	Mascara	2013
	R8	Sultanine	60	Mascara	2014
	R9	Supernova seedless	25000	Mostaganem	2015
	R10	Supernova seedless	20000	Mostaganem	2015
	T11	Sultana seedless	9300	Alaşehir (Manisa)	2014
	T12	Sultana seedless	700	Sarıgöl (Manisa)	2014
	T13	Sultana seedless	400	Sarıgöl (Manisa)	2014
	T14	Sultana seedless	2300	Alaşehir (Manisa)	2014
	T15	Sultana seedless	300	Ahmetli (Manisa)	2014
Dried figs	**F6**	Thamrouith	8	Beni Maouch (Béjaïa)	2015
	F7	Thamrouith	3	Beni Maouch (Béjaïa)	2015
	F8	Taranimt	14	Beni Maouch (Béjaïa)	2015

II.3.3 Molecular Identification of *Aspergillus carbonarius* and *Aspergillus niger* isolated from raisins and dried figs

II.3.3.1 PCR amplifications

PCR represents a powerful tool for the identification of the main Ochratoxigenic producing *Aspergillus* species in grapes. In order to discriminate between *A. niger* and *A. carbonarius* among the 103 selected black Aspergilli isolates, two species-specific primers for *A. carbonarius* and *A. niger* were used

An amount of 19 isolates were molecularly identified as *A. carbonarius* using specific primers CAR1/CAR2, a single fragment of about 420bp was only obtained when genomic DNA from *A. carbonarius* strains was used (Fig. II-12). While for *A. niger*, thirty-five isolates were molecularly identified as *A. niger* using the primer pair ITS1/NIG. A single fragment of about 420 bp was amplified only when genomic DNA from the *A. niger* aggregate strains was used (Fig. II-13).

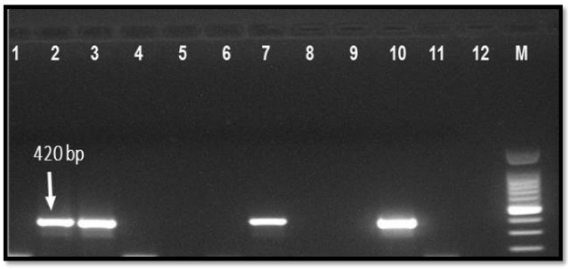


Figure II-12: Agarose gel electrophoresis of amplification products from genomic DNA of black Aspergillus isolates, obtained with the primers CAR1/CAR2.
Lane 2-3-7: positive samples *A. carbonarius*; Lane 10: positive control from *Aspergillus* collection (+) O11 O16; Lane 11: negative control A. niger (-) M1M5; Lane 12: water control; M: 100bp DNA ladder marker.

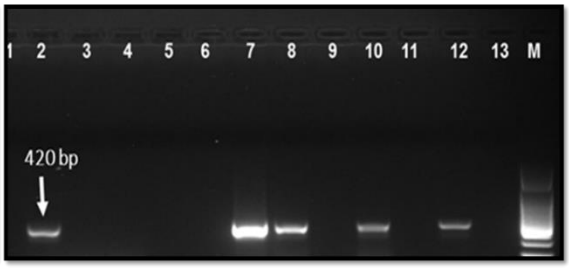


Figure II-13: Agarose gel electrophoresis of amplification products from genomic DNA of black Aspergillus isolates, obtained with the primers NIG/ITS1.
Lane 2-7-8 and 10: positive samples *A. niger*; Lane 12: positive control from *Aspergillus* collection (+) M1M5; Lane 13: negative control A. carbonarius (-) O11 O16; M: 100bp DNA ladder marker.

II.3.3.2 Real-time LAMP (loop-mediated isothermal amplification) for *Aspergillus carbonarius* OTA producing species.

Given that *A. carbonarius* has been found to be the most powerful OTA-producing species in grapes, we focused on this species. A LAMP assay was performed, based on the polyketide synthase genes (PKS) involved or putatively involved in ochratoxin A (OTA) biosynthesis in *A. carbonarius*, therefore it gives positive results only with OTA-producing *A. carbonarius* strains. We tested all isolates identified previously as *A. carbonarius* using PCR and the other black aspergilli, excluding only the ones identified as *A. niger* with PCR.

The results reported were found using LAMP primers AcPKS designed by Storari et al. (2013), but with the Real-time as the detection method. An amount of 18 isolates gave a positive signal and were therefore identified as OTA-producing *A. carbonarius* OTA (Fig. II-14), 2 of them were negative with the PCR, and 3 strains were found negative with the LAMP but positive with the PCR.

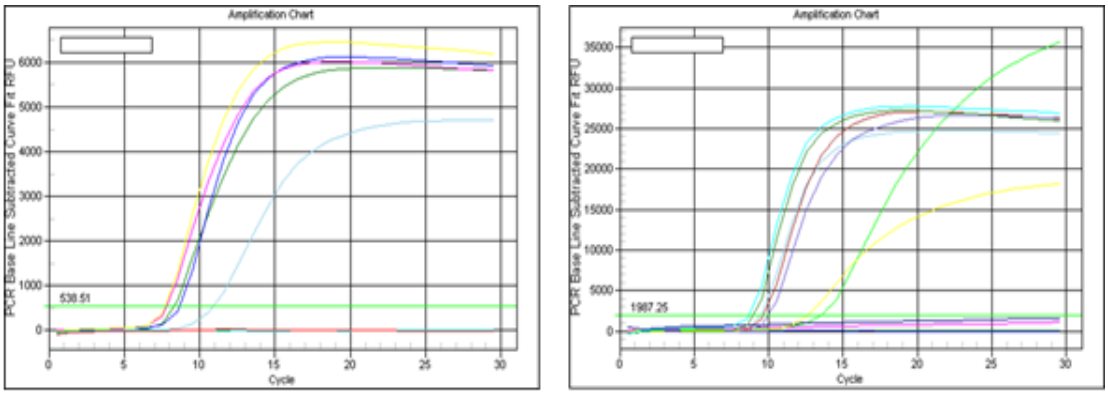


Figure II-14: Real-time LAMP amplifications using AcPKS primers for the identification of *A. carbonarius* OTA-producing strains.

II.3.4 Ochratoxin A and Aflatoxin B1 production by Aspergilli isolated from raisins and dried figs

II.3.4.1 Ochratoxin A

In order to evaluate OTA potential production by the isolated black Aspergilli, 103 strains were analysed, and OTA production was quantified by HPLC. Seventy-nine strains (76%) of the 103 strains of *Aspergillus* section *Nigri*, were OTA producers. The levels of toxin produced ranged from 0.8 to 1200 ppb. The highest percentage of ochratoxigenic strains was detected in *A. carbonarius* species, of which more than 90% were OTA producers, with levels ranging from 1.9 to 1200 ppb (average level 201.35 ppb). Whereas 65% of *A. niger* strains were OTA producers with levels ranging from 0.8 to 27.4ppb and an overall mean level of 10.240 ppb.

II.3.4.2 Aflatoxin B1

A number of 40 selected strains of A. *flavus* and *A. parasiticus* were identified by morphological means, of which 30 were from raisin samples, and 10 from dried figs, these strains were checked for their ability to synthesize Aflatoxin B1. After extraction from fungal cultures and HPLC analysis, 16 out of 30 strains found in raisins were identified as AfB1 producers, with a level varying from 0.3 to 84ppb. Whereas 6 out of 10 strains isolated from 3 dried figs samples were identified as AfB1 producing at levels ranging from 0.8 to 55ppb.

II.3.5 Contamination of raisins and dried figs by Ochratoxin A and Aflatoxin B1

II.3.5.1 Ochratoxin A

OTA was found in all the tested dried fig samples (12), at low levels (less than 0.58 ppb), whereas OTA was found in 6 out of 15 raisin samples, with a level ranging from 7

to 64 ppb. All tested raisin samples from Turkey were contaminated with OTA, whereas only one sample from Algeria was contaminated.

II.3.5.2 Aflatoxin B1

AfB1 was found in just 2 out of 12 dried fig samples at low levels (about 0.13ppb), whereas for raisin, 5 out of 15 samples were found contaminated with AfB1 at levels ranging from 2.3 to 147 ppb.

Table II-6: Dried figs contamination by Ochratoxin A and Aflatoxin B1

Dried figs	OTA (ppb)	AfB1 (ppb)
F1	0.19	ND
F2	0.4	ND
F3	ND	ND
F4	0.5	0.6
F5	0.6	ND
F6	0.5	ND
F7	0.13	traces
F8	ND	ND
F9	0.13	ND
F10	0.17	ND
F11	0.33	0.12
F12	0.58	ND

Table II-7: Raisins contamination by Ochratoxin A and Aflatoxin B1

Raisins	OTA (ppb)	AfB1 (ppb)
R1	ND	ND
R2	ND	ND
R3	ND	ND
R4	ND	2.46
R5	ND	2.3
R6	ND	ND
R7	ND	ND
R8	ND	ND
R9	7.25	ND
R10	ND	ND
T11	14	ND
T12	23.3	**147**
T13	63.75	ND
T14	12.5	5.25
T15	28.8	3.6

ND = not detected; traces = detected but below quantification level.

II.3.6 Detection of aflatoxin producers within *Aspergillus* section *Flavi* directly from dried figs and raisins

Samples of naturally contaminated dried figs and raisins were analysed with the *nor1* LAMP assay, after applying a simple protocol for cell disruption, by vortexing conidial suspensions with a mixture of glass beads of two different diameters using neutral red for visible signal detection, reactions were considered positive when the colour turns into a reddish pink, well distinguished from the yellow negative reactions. No single positive LAMP result was obtained with 15 samples of Algerian raisins (Fig. II-15), Whereas nearly 60 % of dried fig samples showed a positive LAMP result (Fig. II-16).

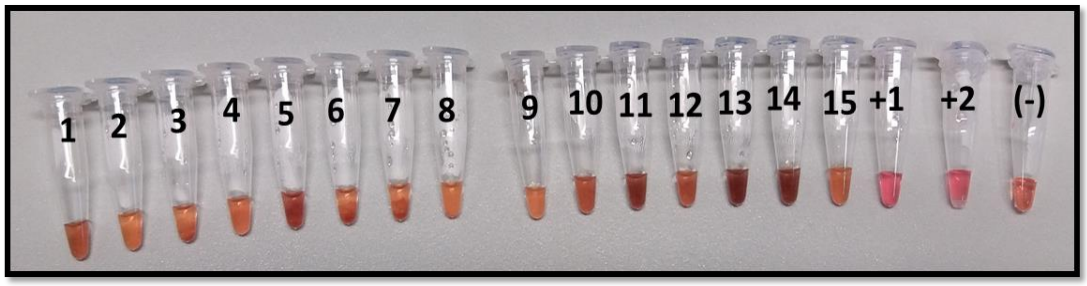


Figure II-15: *nor1* primers LAMP assay reactions, using a DNA from conidia extracted from raisin samples after mechanical treatment with glass beads and direct application of the supernatant as template.
From 1 to 10: raisin samples from Algeria. From 11 to 15: raisin samples from Turkey. +1: *A. flavus*. +2: *A. parasiticus.* (-): water.

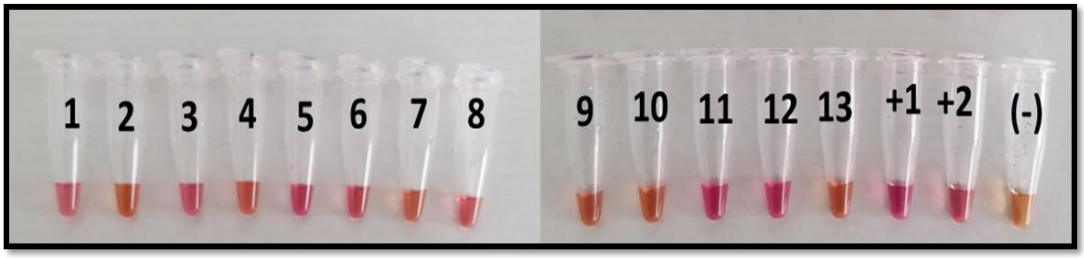


Figure II-16: *nor1* primers LAMP assay reactions, using a DNA from conidia extracted from dried fig samples after mechanical treatment with glass beads and direct application of the supernatant as template.
From 1 to 12: dried figs samples from Algeria. 13: dried fig sample from the market. (+1): *A. flavus*. (+2): *A. parasiticus.* (-): water.

With the levels of total aflatoxins having been determined previously, after extraction, purification and HPLC analysis of raisin and dried fig samples, the analysis of raisins resulted in no positive LAMP reaction, suggesting that no aflatoxin-producing fungi were present in any of the samples. However, the presence of measurable amounts of aflatoxins in 33% of the samples previously determined (Table II-8) showed that the reaction might rather be inhibited. Analysis of dried figs (one fruit per sample) revealed that the number of dried figs displaying a positive LAMP signal was higher than the anticipated number of aflatoxin containing samples (Table II-9). In addition, Table II-10 summarizes the percentages of positive and negative samples and percentages of false results for the dried fig and raisin samples.

Table II-8: Detection of Aflatoxin producing *Aspergillus* spp. in Algerian figs using nor1 LAMP and corresponding aflatoxin concentrations.

Sample ID	AfB1 (ppb)	LAMP result
F1	ND	+
F2	ND	-
F3	ND	+
F4	0,06	-
F5	ND	+
F6	ND	+
F7	traces	-
F8	ND	+
F9	ND	-
F10	ND	-
F11	0.1	+
F12	ND	+

Table II-9: Detection of Aflatoxin producing *Aspergillus* spp. in Algerian raisins using nor1 LAMP and corresponding aflatoxin concentrations.

Sample ID	AfB1 (ppb)	LAMP results
R1	ND	-
R2	ND	-
R3	ND	-
R4	2,46	-
R5	2,3	-
R6	ND	-
R7	ND	-
R8	ND	-
R9	ND	-
R10	ND	-
T11	ND	-
T12	147	-
T13	ND	-
T14	5,25	-
T15	3,6	-

ND = not detected; traces = detected but below quantification level.

Table II-10: Overview over samples analysed with the nor1 LAMP assay after minimal sample preparation.

Sample matrix	Samples tested (n)	Positive (%)	Negative (%)	false positive (%)	false negative (%)
Figs (single fruit)	12	58.0	42.0	50.0	17.0
Raisins	15	0.0	100.0	------	33.3

II.4 Discussion

In this work, in order to confirm previous research from Mediterranean areas concerning the high percentage of OTA-producing isolates belonging to *A. carbonarius* and to investigate the presence of Aflatoxin B1 in Algeria, we studied the presence of Aflatoxin B1 and Ochratoxin A as well as their producing fungi in raisins and dried figs collected from Algeria and Turkey.

From 2013 to 2015 Aspergilli isolates were systematically found on raisins from Algeria and Turkey. The *Aspergillus* genus was predominant, with species belonging to

the Section Nigri (Black Aspergilli) accounting for 87%. The other Aspergilli, including the green ones and *Penicillium* spp. represented only 12% of the isolates. Among black Aspergilli, the predominant species isolated was *A. niger* with 45%, whereas *A. carbonarius* accounted for 14%, the rest were other black Aspergilli species such as *A. awamori* and *A. foetidus*.

Generally, *A. carbonarius* accounts for less than 20% of the black Aspergilli in grapes. Battilani et al. (2006) reported an incidence of 8% of *A. carbonarius* among black Aspergilli in table grapes. Similarly, Chulze et al. (2006) found that *A. niger* was prevalent on *A. carbonarius* in dried vine fruits sampled in Argentina. On the other hand, Abarca et al. (2003) reported the opposite, he found that *A. carbonariu*s accounts for 58% of total black Aspergilli in raisins sampled in Spain. In addition, Fodil et al. (2015) found that in raisins, *A. carbonarius* was predominant with 50% of the total black Aspergilli, while *A. niger* accounted for 30%.

Concerning dried fig samples, only in 3 dried figs out of 12 samples, were found around 25 isolates of filamentous fungi. These consisted of green and yellow Aspergilli (*A. flavus* and *A. parasiticus*) and *Penicillium* spp. No black Aspergilli species were found. This result coincides with previous research on dried figs, where contamination with green Aspergilli and Penicilli species is more typical (Oktay et al., 2011; Buchanan et al., 1975).

As regards OTA production by the strains isolated from raisins and dried figs, 76% of the 103 selected strains of *Aspergillus* section *Nigri* were OTA producers. The highest percentage of ochratoxigenic strains was detected in *A. carbonarius* species, with more than 90% of strains OTA producers, the average concentration of OTA produced was 201.35 ppb and the most toxigenic strain was capable of producing 1,200ppb. This percentage is consistent with previous research by other authors. In particular, Abarca et al. (2003) reported a percentage between 80% and 97% for OTA-producing *A. carbonarius* strains isolated from raisins in Spain, and Magnoli et al. (2004) found 82% of OTA-producing *A. carbonarius* strains from raisins in Argentina.

Concerning *A. niger*, 65% of the isolated strains were able to synthesize OTA, with an average concentration of 10.24ppb. This percentage was much higher than the one found in various prior studies by Magnoli et al. (2004); Perrone et al. (2006), and Serra et al. (2003).

With regard to Aflatoxin B1 contamination, 16 out of 30 strains isolated from raisins and identified morphologically to be *A. flavus* and/or *A. parasiticus* were AfB1 producers, with a level varying from 0.3 to 84ppb. There is not much available information on the occurrence of *A. flavus* and AfB1 on grapes and grape products in the Mediterranean region, and the few studies conducted reported a very low occurrence of *A. flavus* in the vineyards, since *A. flavus* is considered a non-habitual member of the natural grape mycobiota (Martinez-Culebras and Ramon 2007; Medina et al., 2005; Fredj et al., 2007).

Concerning dried fig samples, none of the black *Aspergillus* group were found. furthermore, also the incidence of *A. flavus* and *A. parasiticus* was low, and only in 3 out of 12 samples were a few strains found, and 6 out of the 10 strains of *A. flavus* and/or *A. parasiticus* were able to produce AfB1 at a low level ranging from 0.8 to 55ppb. These results were different from those previously reported, where *A. flavus* and Aflatoxin contamination were frequently isolated from dried figs (Boyacioglu and Gonul, 1990; Iamanaka et al., 2007; Steiner et al., 1988). However, Özay et al. (1995) reported a low incidence of such contamination in a study conducted on dried figs from Turkey in which the presence of aflatoxin B1 (30 ppb) was detected in only one of 32 samples analysed, probably due to the use of manual harvesting and tunnel drying which were effective methods to reduce aflatoxin contamination. Therefore, the incidence of such contamination in dried figs could be decreased by means of good agricultural and manufacturing practices.

Contamination by Ochratoxin A and Aflatoxin B1 was assessed also directly from raisin and dried fig samples. OTA was detected in all raisin samples coming from Turkey at levels ranging from 12 to 64 ppb, and in one sample from Algeria. However, for dried figs, OTA contamination was found at very low levels (less than 0.6ppb) in almost all samples. Concerning Aflatoxin contamination, it was found in only 2 of the 12 dried fig samples at a very low level (less than 0.1 ppb). While for raisins, aflatoxin was found in 2 Algerian samples (out of 10) with a mean level of 2.4 ppb, and in 3 Turkish raisin samples (out of 5), with the highest recorded level of 147 ppb in one sample from Turkey. These results were peculiar because OTA contamination is usually more frequent in grapes and grape products, whereas Aflatoxin contamination is more associated to dried figs than to grapes (Battilani et al., 2003; Blesa et al., 2004; Bircan et al., 2008; Meyvaci et al., 2005; Özay et al., 1991; Steiner et al., 1988).

Nevertheless, numerous researchers reported in previous surveys that more than one mycotoxin can occur in the same commodity, which means that both Aflatoxin and Ochratoxin A can be found in both raisins and in dried figs (Drusch and Aumann 2005; Khoury et al., 2008; Senyuva et al., 2005; Trucksess and Scott, 2008).

The highest number of isolates were found in grape samples coming from Mostaganem region, represented by the Supernova seedless and Sultanine varieties. The difference between the number of *Aspergillus* strains found in these samples compared to the number of *Aspergillus* strains found in other samples, and in particular compared to those isolated from Sultanine samples from Biskra region, could be explained considering the climatic differences between the sampling regions. Biskra is located in a semi-arid region far from the sea (360 km), whereas Mostaganem, is situated along the Algerian coast and characterized by Mediterranean climate, with wide differences in temperature and humidity between day and night. In addition to the effect of the environmental factors, the characteristics of the varieties themselves could have an effect, since Centennial variety coming from Mascara region had the lowest number of contamination with *Aspergillus* species, whereas Sublima seedless and Sultanine varieties from the same region and year, had higher number of isolates (450 and 200 respectively). Also, we noticed differences in the number of isolates between the two years 2013 and 2014. For Sultanine grape variety coming from Mascara region 200 *Aspergillus* were isolated in 2013 but only 60 in 2014. The highest number of isolates found in Turkish samples came from the samples collected in Alaşehir, in Manisa region, that reached 9,300 isolates in one sample, which was much higher compared to isolates from samples coming from Sarıgöl and Ahmetli (that are also in Manisa regions). This could be due to different agricultural practices since the three studied regions are close to each other and also the grape variety was the same, Therefore, the presence and the composition of ochratoxigenic *Aspergillus* species isolates can vary depending on both environmental and pomological factors. as reported in previous research (Bellí et al., 2005; Blesa et al., 2006; Häggblom, 1982; Marquardt and Frohlich, 1992).

The key in mycotoxigenic fungi disease management is the early detection of the pathogens. The LAMP method was adopted to identify the most important Ochratoxigenic species in grape and grape products, *A. carbonarius*, by using primers designed by Storari et al. (2013) that used the Hydroxynaphthol blue (HNB) as a colorimetric indicator for DNA amplification. In this work we used the same primers but with a different LAMP products detection that was a with Real-time measurement.

AcPKS primers were designed based on the polyketide synthase genes involved OTA biosynthesis in *A. carbonarius*; therefore, they gave a signal only when the tested species was an OTA-producing *A. carbonarius*. Our results showed that 18 strains gave a positive signal with the AcPKS LAMP primers, 3 of them were negative with the conventional PCR primers (CAR1/CAR2) for the detection of *A. carbonarius*, probably because the LAMP was proven to be more sensitive compared to PCR assays. This is because LAMP uses four primers targeting six specific regions and it provides rapid amplification, greater yield of amplification products and lower detection limits than PCR assays (Niessen and Vogel, 2010; Wang et al., 2008; Xu et al., 2012). We found also 3 strains that were negative with the LAMP and positive with the PCR, 2 of which were not OTA-producing and the third of which had traces of OTA. Therefore, we confirmed that the LAMP primers detect only strains able to produce OTA, since the primers were designed based on the polyketide synthase genes (PKS) involved in OTA biosynthesis in *A. carbonarius,* thus the absence of this gene will result in the lack of signal with the LAMP assay.

Aimed to differentiate between aflatoxin-producing and non-aflatoxin-producing isolates within *A. flavus* and *A. parasiticus* species, Niessen et al. (2017) designed LAMP primers based on *aflD, aflO,* and *aflP* genes. Scherm et al. (2005) found that these genes are strictly correlated with the production or non-production of aflatoxins in cultures of all analysed strains. This primer-based detection system targets not only one species but a group of species sharing the common property of aflatoxin production. In this work, using the same framework of the detection of aflatoxin producing with the *nor1* LAMP assay created by Niessen et al. (2017), we set a simple protocol for cell disruption of conidia for direct use as template in the isothermal detection LAMP assays, to analyse raisins and dried figs sample for the presence of aflatoxin producers. For raisins the protocol was based on the detection of spores present on the surface of the samples, therefore whole raisins were used without cutting or chopping. On the contrary, dried figs were chopped into small pieces, to improve the extraction of fungal contaminations occurring in the interior cavity of the fruit (ostiole) (Doster at al., 1996).

In the 15 samples of raisins, after LAMP reaction no single positive LAMP was found. Although this commodity has been more associated with the occurrence of OTA rather than aflatoxins (Trucksess and Scott, 2008), in previously analyses of raisins samples we found that 5 samples (one third of the samples) had detectable concentrations of AfB1. Thus, the number of undetected samples (false negatives) was 33 % for the nor1 LAMP assay. The false negative results in the LAMP assay can be due to different factors:

a strong degradation of fungal material including its DNA can occur since the LAMP assay was carried out after aflatoxin analysis, in some cases, if only few conidia are present in the sample, there is a low chance to obtain their disruption by the used protocol, furthermore, if a non-contaminated bunch of grapes is in contact with a contaminated one, mycotoxin present in the contaminated sample can pass to the non-contaminated one (Ricelli, 2002), where only the mycotoxin will be detected but not the genes responsible for mycotoxin production.

The analysis of dried figs showed an opposite situation, with 7 out of the 12 tested dried figs (nearly 60 % of samples) having a positive result in the *nor1* LAMP assay, whereas we found only few samples (2 of 12) contaminated with low concentrations of aflatoxins. This can be due to the fact that *nor1* LAMP assay is a gene specific primer that targets the responsible gene for Aflatoxin production. Therefore, it is possible that this gene is present in some strains, albeit inactive. This can result in a positive signal with the LAMP and negative results for aflatoxin contamination. We also found 17% of false negatives with LAMP assay on dried figs, with 2 samples contaminated with aflatoxin, and a negative result with the LAMP assay. The false negative found in dried figs maybe be due to the same reasons mentioned for raisins, and in addition, it might be due also to the fact that a single dried fig was analysed by LAMP, but aflatoxin analysis was done on a bulk sample of several figs combined.

II.5 References

Abarca, M.L., Accensi, F., Bragulat, M.R., Castella, G. and Cabanes, F.J., 2003. *Aspergillus carbonarius* as the main source of ochratoxin A contamination in dried vine fruits from the Spanish market. *Journal of Food Protection*, 66(3), pp.504-506.

Akdeniz, A.S., Ozden, S. and Alpertunga, B., 2013. Ochratoxin A in dried grapes and grape-derived products in Turkey. *Food Additives and Contaminants*: Part B, 6(4), pp.265-269.

Aksoy, U., Eltem, R., Meyvaci, K.B., Altindisli, A. and Karabat, S., 2007. Five-year survey of ochratoxin A in processed sultanas from Turkey. *Food Additives and Contaminants*, 24(3), pp.292-296.

Azzoune, N., Mokrane, S., Riba, A., Bouras, N., Verheecke, C., Sabaou, N. and Mathieu, F., 2016. Contamination of common spices by aflatoxigenic fungi and aflatoxin B1 in Algeria. *Quality Assurance and Safety of Crops & Foods*, 8(1), pp.137-144.

Battilani, P., Giorni, P., Bertuzzi, T., Formenti, S. and Pietri, A., 2006. Black Aspergilli and ochratoxin A in grapes in Italy. *International Journal of Food Microbiology*, 111, pp.S53-S60.

Battilani, P., Pietri, A., Bertuzzi, T., Languasco, L., Giorni, P. and Kozakiewicz, Z., 2003. Occurrence of ochratoxin A–producing fungi in grapes grown in Italy. *Journal of Food Protection*, 66(4), pp.633-636.

Bellí, N., Ramos, A.J., Coronas, I., Sanchis, V. and Marín, S., 2005. *Aspergillus carbonarius* growth and ochratoxin A production on a synthetic grape medium in relation to environmental factors. *Journal of Applied Microbiology*, 98(4), pp.839-844.

Bennett, J.W. and Papa, K.E., 1988. The aflatoxigenic *Aspergillus* spp. *Advances in Plant Pathology: genetics of plant pathogenic fungi*, *6*, pp.263-80.

Bircan, C., Barringer, S.A., Ulken, U. and Pehlivan, R., 2008. Increased aflatoxin contamination of dried figs in a drought year. *food Additives and Contaminants*, 25(11), pp.1400-1408.

Blesa, J., Soriano, J.M., Moltó, J.C. and Manes, J., 2004. Concentration of ochratoxin A in wines from supermarkets and stores of Valencian Community (Spain). *Journal of Chromatography A*, 1054(1), pp.397-401.

Blesa, J., Soriano, J.M., Moltó, J.C. and Manes, J., 2006. Factors affecting the presence of ochratoxin A in wines. *Critical Reviews in Food Science and Nutrition*, 46(6), pp.473-478.

Boyacioglu, D. and Gönül, M., 1990. Survey of aflatoxin contamination of dried figs grown in Turkey in 1986. *Food Additives & Contaminants*, 7(2), pp.235-237.

Buchanan, J.R., Sommer, N.F. and Fortlage, R.J., 1975. *Aspergillus flavus* infection and aflatoxin production in fig fruits. *Applied Microbiology*, 30(2), pp.238-241.

Cabañes, F.J., Accensi, F., Bragulat, M.R., Abarca, M.L., Castellá, G., Minguez, S. and Pons, A., 2002. What is the source of ochratoxin A in wine? *International Journal Of Food Microbiology*, 79(3), pp.213-215.

Chulze, S.N., Magnoli C.E. and Dalcero A.M. 2006. Occurrence of ochratoxin A in wine and ochratoxigenic mycoflora in grapes and dried vine fruits in South America. *International Journal of Food Microbiology* 111: S5-S9.

Doster, M.A., Michailides, T.J. and Morgan, D.P., 1996. *Aspergillus* species and mycotoxins in figs from California orchards. *Plant Disease*, 80(5), pp.484-489.

Drusch, S. and Aumann, J., 2005. Mycotoxins in fruits: Microbiology, occurrence, and changes during fruit processing. *Advances in Food and Nutrition Research*, 50, pp.33-78.

FAO, Food and Agriculture Organization of the United Nations, 2004. Worldwide regulations for mycotoxins in food and feed in 2003. *Food and Nutrition Paper* No. 81. Rome.

Fernane, F., Sanchis, V., Marin, S. and Ramos, A.J., 2010. First report on mould and mycotoxin contamination of pistachios sampled in Algeria. *Mycopathologia*, 170(6), pp.423-429.

Fodil, S., Yaseen, T., D'Onghia, A.M., Varvaro, L. and Ricelli, A., 2015. Use of ozone to control conidial germination in ochratoxin A producing black Aspergilli isolated in raisins from Algeria. In III International Symposium on Postharvest Pathology*: Using Science to Increase Food Availability* 1144 (pp. 163-168).

Fredj, S.M.B., Chebil, S., Lebrihi, A., Lasram, S., Ghorbel, A. and Mliki, A., 2007. Occurrence of pathogenic fungal species in Tunisian vineyards. *International Journal of Food Microbiology*, 113(3), pp.245-250.

Gómez, C., Bragulat, M.R., Abarca, M.L., Mínguez, S., Cabañes, F.J., 2006. Ochratoxin A producing fungi from grapes intended for liqueur wine production. *Food Microbiology* 23, 541–545.

González-Salgado, A., Patiño, B., Vázquez, C. and González-Jaén, M.T., 2005. Discrimination of *Aspergillus niger* and other *Aspergillus* species belonging to section *Nigri* by PCR assays. *FEMS Microbiology Letters*, 245(2), pp.353-361.

Gourama, H. and Bullerman, L.B., 1995. *Aspergillus flavus* and *Aspergillus parasiticus*: aflatoxigenic fungi of concern in foods and feeds: a review. *Journal of Food Protection*, 58(12), pp.1395-1404.

Häggblom, P., 1982. Production of ochratoxin A in barley by *Aspergillus ochraceus* and *Penicillium viridicatum*: effect of fungal growth, time, temperature, and inoculum size. *Applied and Environmental Microbiology*, 43(5), pp.1205-1207.

Iamanaka, B.T., de Menezes, H.C., Vicente, E., Leite, R.S. and Taniwaki, M.H., 2007. Aflatoxigenic fungi and aflatoxins occurrence in sultanas and dried figs commercialized in Brazil. *Food Control*, 18(5), pp.454-457.

Imperato, R., Campone, L., Piccinelli, A.L., Veneziano, A. and Rastrelli, L., 2011. Survey of aflatoxins and ochratoxin a contamination in food products imported in Italy. *Food Control*, 22(12), pp.1905-1910.

Khalef, A., Zidane, C., Charef, A., Gharbi, A., Tadjerouna, M., Betbeder, A.M., Creppy, E.E., 1993. Ochratoxicose humaine en Algérie. In: Creppy, E.E., Castegnaro, M., Dirheimer, G. (Eds.), *Human ochratoxicosis and its pathologies*. *INSERM vol 231*. *John Libbey Eurotext Ltd, Paris*, pp. 123-127.

Khoury, A.E., Rizk, T., Lteif, R., Azouri, H., Delia, M.L. and Lebrihi, A., 2008. Fungal contamination and Aflatoxin B1 and Ochratoxin A in Lebanese wine–grapes and musts. *Food and Chemical Toxicology*, 46(6), pp.2244-2250.

Klich, M.A., 2007. Environmental and developmental factors influencing aflatoxin production by *Aspergillus flavus* and *Aspergillus parasiticus*. *Mycoscience*, 48(2), pp.71-80.

Lucchetta, G., Bazzo, I., Dal Cortivo, G., Stringher, L., Bellotto, D., Borgo, M. and Angelini, E., 2010. Occurrence of black aspergilli and ochratoxin A on grapes in Italy. *Toxins*, 2(4), pp.840-855.

Magan, N. and Aldred, D., 2005. Conditions of formation of ochratoxin A in drying, transport and in different commodities. *Food Additives and Contaminants*, 22(s1), pp.10-16.

Magnoli, C., Astoreca, A., Ponsone, L., Combina, M., Palacio, G., Rosa, C.A.R. and Dalcero, A.M., 2004. Survey of mycoflora and ochratoxin A in dried vine fruits from Argentina markets. *Letters in Applied Microbiology*, 39(4), pp.326-331.

Marquardt, R.R. and Frohlich, A.A., 1992. A review of recent advances in understanding ochratoxicosis. *Journal of Animal Science*, 70(12), pp.3968-3988.

Martinez-Culebras, P.V. and Ramon, D., 2007. An ITS-RFLP method to identify black *Aspergillus* isolates responsible for OTA contamination in grapes and wine. *International Journal of Food Microbiology*, 113(2), pp.147-153.

Medina, A., Mateo, R., López-Ocaña, L., Valle-Algarra, F.M. and Jiménez, M., 2005. Study of Spanish grape mycobiota and ochratoxin A production by isolates of *Aspergillus tubingensis* and other members of *Aspergillus* section *Nigri*. *Applied and Environmental Microbiology*, 71(8), pp.4696-4702.

Meyvaci, K.B., Altindisli, A., Aksoy, U., Eltem, R., Turgut, H., Arasiler, Z. and Kartal, N., 2005. Ochratoxin A in sultanas from Turkey I: Survey of unprocessed sultanas from vineyards and packing-houses. *Food Additives and Contaminants*, 22(11), pp.1138-1143.

Niessen, L. and Vogel, R.F., 2010. Detection of Fusarium graminearum DNA using a loop-mediated isothermal amplification (LAMP) assay. *International Journal of Food Microbiology*, *140*(2), pp.183-191.

Niessen, L., Bechtner, J., Fodil, S., Taniwaki, M.H. and Vogel, R.F., 2017. LAMP-based group specific detection of aflatoxin producers within *Aspergillus* section *Flavi* in food raw materials, spices, and dried fruit using neutral red for visible-light signal detection. *International Journal of Food Microbiology*, 266, pp.241-250.

Oktay, H.I., Heperkan, D., Yelboga, E. and Karaguler, N.G., 2011. *Aspergillus flavus*-primary causative agent of aflatoxins in dried figs. Mycotaxon, 115(1), pp.425-433.

Özay, G. and Alperden, I., 1991. Aflatoxin and OchratoxinA contamination of dried figs (*Ficus carica* L) from the 1988 crop. *Mycotoxin Research*, 7(2), pp.85-91.

Özay, G., Aran, N. and Pala, M., 1995. Influence of harvesting and drying techniques on microflora and mycotoxin contamination of figs. *Molecular Nutrition & Food Research*, 39(2), pp.156-165.

Patiño, B., González-Salgado, A., González-Jaén, M.T. and Vázquez, C., 2005. PCR detection assays for the ochratoxin-producing *Aspergillus carbonarius* and *Aspergillus ochraceus* species. *International Journal of Food Microbiology*, 104(2), pp.207-214.

Perrone, G., Mulè G., Susca A., Battilani P., Pietri A. and Logrieco A. 2006. Ochratoxin A production and amplified fragment length polymorphism analysis of *Aspergillus carbonarius*, *Aspergillus tubingensis*, and *Aspergillus niger* strains isolated from grapes in Italy. *Applied and Environmental Microbiology* 72:680-685.

Pitt, J.I. and Hocking, A.D., 1997. Fungi and Food Spoilage Blackie Academic & Professional. *New South Wales, Australia*.

Raper, K.B. and Fennell, D.I., 1965. The genus *Aspergillus*. *Williams and Wilkins, Baltimore*.

Riba, A., Bouras, N., Mokrane, S., Mathieu, F., Lebrihi, A. and Sabaou, N., 2010. *Aspergillus* section *Flavi* and aflatoxins in Algerian wheat and derived products. *Food and Chemical Toxicology*, 48(10), pp.2772-2777.

Riba, A., Mokrane, S., Mathieu, F., Lebrihi, A. and Sabaou, N., 2008. Mycoflora and ochratoxin A producing strains of *Aspergillus* in Algerian wheat. *International Journal of Food Microbiology*, 122(1), pp.85-92.

Ricelli A. 2002. *Prevenzione della contaminazione da ocratossina A in uve da tavola mediante lotta biologica* (PhD thesis in Scienze Botaniche). Università degli Studi di Roma "Sapienza". Italy.

Richard, J.L., 2008. Discovery of aflatoxins and significant historical features. *Toxin Reviews*, 27(3-4), pp.171-201.

Samson, R.A., Hoekstra, E.S. and Frisvad, J.C., 2004. Introduction to food-and airborne fungi (No. Ed. 7). *Centraalbureau voor Schimmelcultures* (CBS).

Scherm, B., Palomba, M., Serra, D., Marcello, A. and Migheli, Q., 2005. Detection of transcripts of the aflatoxin genes *aflD*, *aflO*, and *aflP* by reverse transcription–polymerase chain reaction allows differentiation of aflatoxin-producing and non-producing isolates of *Aspergillus flavus* and *Aspergillus parasiticus*. *International Journal of Food Microbiology*, 98(2), pp.201-210.

Senyuva, H.Z., Gilbert, J., Ozcan, S. and Ulken, U., 2005. Survey for co-occurrence of ochratoxin A and aflatoxin B1 in dried figs in Turkey by using a single laboratory-validated alkaline extraction method for ochratoxin A. *Journal of Food Protection*, 68(7), pp.1512-1515.

Serra, R., Kozakiewicz, Z. and Venâncio, A., 2003. Black *Aspergillus* species as ochratoxin A producers in Portuguese wine grapes. *International Journal of Food Microbiology*, 88(1), pp.63-68.

Stefanaki, I., Foufa, E., Tsatsou-Dritsa, A. and Dais, P., 2003. Ochratoxin A concentrations in Greek domestic wines and dried vine fruits. *Food Additives & Contaminants*, 20(1), pp.74-83.

Steiner, W.E., Rieker, R.H. and Battaglia, R., 1988. Aflatoxin contamination in dried figs: distribution and association with fluorescence. *Journal of Agricultural and Food Chemistry*, 36(1), pp.88-91.

Storari, M., Rohr, R.V., Pertot, I., Gessler, C. and Broggini, G.A., 2013. Identification of ochratoxin A producing *Aspergillus carbonarius* and *A. niger* clade isolated from grapes using the loop-mediated isothermal amplification (LAMP) reaction. *Journal of Applied Microbiology*, 114(4), pp.1193-1200.

Tjamos, S.E., Antoniou, P.P., Kazantzidou, A., Antonopoulos, D.F., Papageorgiou, I. and 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. *Journal of Phytopathology*, 152(4), pp.250-255.

Trucksess, M.W. and Scott, P.M., 2008. Mycotoxins in botanicals and dried fruits: a review. *Food Additives and Contaminants*, 25(2), pp.181-192.

Valero, A., Marín, S., Rarnos, A.J., Sanchis, V., 2005. Ochratoxin A-producing species in grapes and sun-dried grapes and their relation to ecophysiological factors. *Letters in Applied Microbiology* 41, 196–201.

Wang, L., Shi, L., Alam, M.J., Geng, Y. and Li, L., 2008. Specific and rapid detection of foodborne Salmonella by loop-mediated isothermal amplification method. *Food Research International*, 41(1), pp.69-74.

Xu, Z., Li, L., Chu, J., Peters, B.M., Harris, M.L., Li, B., Shi, L. and Shirtliff, M.E., 2012. Development and application of loop-mediated isothermal amplification assays on rapid detection of various types of staphylococci strains. *Food Research International*, 47(2), pp.166-173.

Chapter III: Effect of Potassium sorbate (E-202) and PgAFP protein from *Penicillium chrysogenum* on *Aspergillus carbonarius* growth and ochratoxin A production in raisins.

This chapter of the research project was performed under the supervision of Dr. Alicia Rodríguez and Dr. Josué Delgado. The experiments were carried out in the Food Hygiene and Safety, Meat and Meat Products Research Institute, Faculty of Veterinary Science, University of Extremadura, Avda. de las Ciencias, s/n. 10003-Cáceres, Spain, in the context of a doctoral internship abroad as intended in the PhD program.

III.1 Introduction

Very high levels of OTA contamination have been reported on raisins worldwide, (Meyvaci et al., 2005; Aksoy et al., 2007; Palumbo et al., 2011; Battilani et al., 2006). Ochratoxin A (OTA) in raisins is mainly related to the presence of black *Aspergillus species*. Among them, *A. carbonarius* has been reported to be the major producer, with most isolates able to produce OTA in synthetic media (Heenan et al., 1998; Pitt, 2002; Cabañes et al., 2002; Serra et al., 2006; Abarca et al., 2003). A previous research showed that after sun drying the occurrence of *A. carbonarius* was higher than that recorded at harvesting time (Valero et al., 2005). During the drying period grapes are subjected to dehydration until they acquire the desired sugar content and the a_w drops from 0.95 to 0.75. In these conditions, only few micro-organisms such as black aspergilli and in particular *A. carbonarius* are capable to resist because they are less susceptible than other species to the germicidal UV rays and the strong sunlight heating due to their thick, heavily melanised walled pigmented spores (Hocking et al., 2007; Rotem and Aust 1991; Valero et al., 2005; Covarelli et al., 2012; Magan and Aldred, 2005). The increase of the load of black *Aspergillus* during the drying period and the presence of OTA producer strains may explain the higher levels of OTA in dehydrated grapes.

Given the healthful connotations of raisins, which is also recommended in the diet of children, the presence of a toxic metabolite is particularly serious. Prevention and/or the detoxification of OTA production is then of prime importance in protecting consumer health. Several strategies are available for the detoxification of this mycotoxin. They can be classified as physical, chemical and microbiological approaches with varying degrees of success. (Sinha, 1998; Bejaoui et al., 2006; Quintela et al., 2013). Pre-harvest strategies

are aimed to reduce fungal infection by applying good agricultural practices. During harvest, the use of clean farming equipment, mechanical damage prevention and overripe or fermented fruits discard are convenient practices. In the post-harvest, storage is the most critical phase. Environmental conditions, in particular moisture and temperature, have to be well controlled in this stage (Amézqueta et al., 2009). Fungicides are effective but must be applied with care since some of them, have been found to reduce fungal microbiota, but to stimulate OTA production (Medina et al., 2007). Such as potassium sorbate (E-202), an antimicrobial preservative in various foods. Regulatory agencies such as the European Food Safety Authority (EFSA) and Food and Agriculture Organization of the United Nations (FAO), have determined that potassium sorbate is "generally regarded as safe," (GRAS), and had set a temporary acceptable daily intake of 3 mg per kg of body weight per day (EFSA, 2015). However, some researchers believe that potassium sorbate can cause a wide range of long-term health problems and side effects on human health. It seems to cause mutagenicity and DNA-damaging activity, a genotoxicity to the human peripheral blood lymphocytes *in vitro*, as well as weak genotoxic effect in Chinese Hamster cells. (Abe and Sasaki, 1977; Hasegawa et al., 1984 **;**Kitano et al., 2002; Mamur et al., 2010; Mpountoukas et al., 2008). Therefore, strategies for reducing potassium sorbate quantities from human diet should be considered.

Microbiological decontamination may be a good alternative to OTA detoxification, it is the most promising methods nowadays (Quintela et al., 2013). The antifungal proteins produced by molds that may serve to combat unwanted molds, like PgAFP protein from *Penicillium chrysogenum* that inhibits growth of some toxigenic molds. (Acosta et al., 2009; Delgado et al., 2015). Given that *P. chrysogenum* is regarded as safe mold commonly found in foods, PgAFP may be useful to prevent in a safe way the growth of toxigenic molds in food and agricultural products.

The aim of this study was to examine and to compare at eco-physiological level; the efficacy of potassium sorbate and PgAFP protein against OTA accumulation by *A. carbonarius*, on a synthetic medium that simulate raisin composition, and in two different low activity water, to have similar condition of drying phase of grapes which is a critical stage. As well as at a transcriptional level, where the expression of the *pks* and *Rho1* gene, involved in OTA biosynthesis and in cell wall integrity pathway (CWI), respectively. This can give us information about the mechanism of action of PgAFP or E-202 to control OTA production by *A. carbonarius*.

III.2 Material and methods

III.2.1 Fungal strains

Two *Aspergillus carbonarius* strains: A157 and A171 OTA producer were used in this study. These strains were isolated from Algerian raisins and deposited in the Mediterranean Agronomic Institute of Bari (MAIB, Italy). PgAFP-producing *P. chrysogenum* strain RP42C (CECT 20922) from the Spanish Type Culture Collection (CECT) was also used.

III.2.2 Inoculum preparation

The two *Aspergillus carbonarius* strains were initially inoculated on Malt Extract Agar (MEA; Scharlab S.L.) and incubated at 25° C for 7 days. The spores were collected using 3 mL of phosphate-buffered saline (PBS) and rubbing the surface with a scarpel in order to remove conidia. The spore suspensions were counted using a Thoma counting chamber Blaubrand® (Brand, Wertheim, Germany), and adjusted to 10^6 spores/mL to be used as inoculum.

III.2.3 Medium preparation

Synthetic nutrient medium (SNM) that simulate raisins composition between veraison and ripeness (Delfini, 1982) was used in this study, and was modified to adjust it to two a_w values. To prepare SNM medium at 0.93 a_w, 196.0 g of D(+)glucose, 204.0 g of D(-)fructose and 288.5 of glycerol were added before adjusting to 1L of final volume with deionised water. For the 0.95 a_w medium, 135.2 g of glucose and 140.8 g of fructose were mixed for a total volume of 1L (Valero et al., 2005).

III.2.4 PgAFP purification

The PgAFP-producing *P. chrysogenum* CECT 20922 was inoculated into malt extract broth (MEB, 20 g/L malt extract, 20 g/L glucose, and 1 g/L peptone; MEB), pH was adjusted at 4.5, and incubated up to 21 days at 25°C. PgAFP was obtained from the cell free medium by fast protein liquid chromatography (FPLC) with a cationic exchange column HiTrap SP HP (Amersham Biosciences, Uppsala, Sweden) and further purified and desalted through a HiLoad 26/60 Superdex 75 gel filtration column for FPLC (Amersham Biosciences) (Acosta et al., 2009; Rodríguez-Martín et al., 2010).

The concentration of the pooled PgAFP stock solution was measured by Lowry method (Lowry et al. 1951), sterilized through 0.22 µm acetate cellulose filters (Jet Bio-Filtration Co., China)), and stored at -20°C until use.

III.2.5 Effect of PgAFP on *A. carbonarius* growth

In order to test the *A. carbonarius* strains sensitivity against PgAFP, a microplate with seven PgAFP concentrations (1.2-75 µg/mL) was prepared using potato dextrose broth (PDB, Scharlab S.L., Spain) as culture medium. Each well was inoculated with 10^5 conidia and was incubated at 25° C for 96h and measured at 595 nm. The assay was run in sextuplicate wells and repeated once.

III.2.6 Experimental settings

In order to investigate the effect of potassium sorbate (E-202) and PgAFP on the growth, the sporulation and OTA production of the two strains of *Aspergillus carbonarius* OTA producers, two concentrations of food-grade E-202 (Droguería El Barco, Spain) were tested: 60 and 500 ppm. They were added directly to the media which was then sterilized by autoclaving. While the PgAFP protein was added after autoclaving, when the media were cooled at 50°C, three repetitions were used for each treatment. Once the media were poured into 53 mm-Petri plates, inoculation then was performed by adding 2µl from 10^6 conidia/mL spore suspension in the centre of the plate, then kept at 25 °C.

III.2.7 Lag phase prior to growth and growth assessment

The diameter of the colonies was measured daily in two directions at right angles to each other in order to determine the growth rates of strains. Primary modelling was carried out on the temporal radial colony expansion data. Data plots showed, after a lag phase, a linear trend with time. Data was fitted using a linear model obtained by plotting the results against time. Only the linear parts were used. From this primary model the growth rates (radius mm/day) were obtained as the slope of the curve (García et al., 2009). Lag times (in days) were calculated by equalling the regression line formula to the original inoculum size (radius mm).

III.2.8 Gene expression studies

In order to know the effect of E-202 and PgAFP on *Aspergillus carbonarius* at transcriptomic level, gene expression of three genes was assessed. Two sampling days were chosen: at the final of the exponential phase and in the beginning of the stationary

phase. Sampling days were different for each treatment, given that the growth rates were different. At a_w 0.93 the period for the first sampling was within 6 days and for the second one within 7 days, while at a_w 0.95 the period for every sampling was within 24 h. The biomass was harvested and quickly frozen in liquid nitrogen, and stored at -80 C until RNA extraction procedure (Peromingo et al., 2017).

III.2.8.1 RNA extraction

RNA was isolated using the the commercial kit Spectrum Plant Total RNA (Sigma-Aldrich, USA). Samples of 250- 500 mg of mycelium biomass were ground to fine powder in a pre-frozen mortar with a pestle and liquid nitrogen. The resulting powder was resuspended in 500 mL of a Lysis Solution/2-mercaptoethanol mixture provided by the kit. The samples were incubated at 56 °C for 3 min. All further procedures were followed as manufacturer´s instructions (Protocol A). To remove genomic DNA contamination, samples were treated with DNase, RNase-free (Fermentas/Thermo Fisher Scientific, Germany) according to the manufacturer's instructions. The RNA concentration and purity (A260/A280 ratio) were spectrophotometrically determined using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, USA).

III.2.8.2 Reverse transcription real-time PCR assays

III.2.8.2.1 Primers.

Three primer pairs were used in this study (Table III -1) for assessing respectively the transcription of: polyketide synthase (*pks*) gene involved in OTA biosynthesis (Spadaro et al. 2011), *Rho1* gene related to cell wall stress (da Cruz Cabral et al., 2017) and *β-tubulin* gene (Castellá et al., 2015) as endogenous control.

Table III-1: Oligonucleotide sequences of primers used in this study.

Gene	Primer pairs	Nucleotide sequences (5'-3')	References
pks	acpks-F1	AGCATCTATGCTGGCCAATC	(Spadaro et al., 2011)
	acpks-R1	AATGTACTCTCGCGGGCTAA	
Rho1	Rho1-F1	CTTTCCCCGAGGTCTACGTC	(Da Cruz-Cabral et al., 2017)
	Rho1-R2	TCGTAATCCTCCTGACCAGC	
B-tubulin	ASPBTUBF	GGGTACCCTCCTGATCTCCAA	Castellá et al., 2015
	ASPBTUBR	AGAGTGGCGTTGTAAGGCTCAA	

III.2.8.2.2 cDNA synthesis.

cDNA was synthesised using around 500 ng of total RNA according to the instructions of the PrimeScript™ RT Reagent kit protocol (Takara Bio Inc., Japan) and subsequently used for qPCR.

III.2.8.2.3 Relative gene expression using real-time PCR reactions

The primer pairs acpks, *Rho1* and aspbtub were first evaluated in a SYBR Green protocol. To optimise the amount of primers and template DNA (or cDNA for *Rho1* primer pair) in the reactions, *A. carbonarius* A157 was used. The tested concentrations ranged from 600 to 150 nM for primers and from 10 ng to 0.001 ng for template DNA.

The qPCR assays were performed in the Applied Biosystems Fast 7500 Real-Time PCR System (Applied Biosystems, USA). They were prepared in triplicate of 12.5 µL reaction mixtures in MicroAmp optical 96-well reaction plates and sealed with optical adhesive covers (Applied Biosystems). A template-free negative control was also included in every run. Every reaction mixture consisted of 6.25 µL of SYBR® *Premix Ex Taq*™ (Takara Bio Inc.), 0.125 µl of 50x ROX™ Reference Dye (Takara Bio Inc.), different concentrations of each primer (Table 1) and 2.5 µL of cDNA template in a final volume of 12.5 µL. For acpks primer pair an activation step of 2 min at 50°C followed by 10 min at 95°C, all subsequent 40 cycles were performed according to the following temperature regime: 95°C for 30 s, 55°C for 50 s and 60°C for 50 s. For *Rho1* primer pair an activation step of 10 min at 95°C, all subsequent 40 cycles were performed according to the following temperature regime: 95°C for 15 s, and 55°C for 60 s. Finally, for aspbtub primer pair an activation step of 2 min at 50°C followed by 10 min at 95°C, all subsequent 40 cycles were performed according to the following temperature regime: 95°C for 15 s, 60°C for 60 s. At the end of every PCR, melting curve analysis of the PCR products was performed by heating to 72–95°C and continuous measurement of the fluorescence to verify the PCR product. Quantification cycle (Cq) values were automatically calculated by the instrument using default parameters.

Relative quantification of the expression of the *pks* and *Rho1* genes were performed using the housekeeping *β-tubulin* gene as an endogenous control to normalise the quantification of the mRNA target for differences in the amount of total cDNA added to each reaction. The expression ratio was calculated as previously described (Livak and Schmittgen, 2001). Before using this method, it was tested that experimental treatments

did not influence expression of the endogenous control, and the amplification efficiencies of the target and reference genes were ≤ 10% (91.29% for *pks*, 93.8% for *Rho1* and 100.92% for *β-tubulin* genes, respectively) in order to comply with the method assumptions. This method allows calculation of the expression ratio of a target gene between a tested sample and its relative calibrator ("control" sample). For the temporal relative, *pks* and *Rho1* gene expression, control samples were used as controls.

III.2.9 Extraction and quantification of OTA

III.2.9.1 Sample preparation

One day after the second sampling for the RNA extraction, sampling for OTA analysis was carried out. Three agar plugs weighing around 0.75 g were removed from the fungal cultures and placed in previously weighed 2 mL volume Safe-Lock tube (Eppendorf, Germany). Three replicates per treatment were collected, weighed, immediately frozen at -20 °C and stored until extraction.

III.2.9.2 OTA Extraction

OTA was essentially extracted following a QuEChERS-based procedure described previously by Kamala et al. (2015). The OTA-extracts were evaporated to dryness under a gentle stream of nitrogen and kept at -20 °C.

After analysis the samples were resuspended in Acetonitrile (Brand). The resulting solution was filtered through 0.45 µm acetate cellulose filters (Jet Bio-Filtration Co.) previous analysis.

III.2.9.3 Quantification of OTA by LC/MS

OTA was analysed by an uHPLC system, Thermo Scientific (United Kingdom) Dionex UltiMate 3000 coupled to an Ion Trap Mass Spectrometer System amaZon SL (Bruker Daltonics Inc., Germany). The mobile phase consisted of 0.1% formic acid-10 mM ammonium formate (solvent A) and acetonitrile (solvent B). Analysis was done in a gradient mode from 35 to 98% B. The stationary phase consisted of a C18 column (100 mm x 2.1 mm, 2 µm; YMC Agilent Technologies, USA). The injection volume was 10 µL and flow rate was set at 0.2 mL/min. MS detection of OTA was performed using the precursor ion 404, and the quantitation ion 358. The run time was 15 min, being the OTA detected at 8.7 ± 0.2 min. Signals were processed by Hystar 3.2 software (Bruker Daltonics Inc.).

Standard solutions of OTA (Methanol) were used (from 1 to 100 ng/mL) to build a calibration curve by uHPLC-MS. This curve revealed a linear relationship ($R^2 \geq 0.998$) between detector response and amount of OTA standard (Sigma Aldrich Química S.A., Spain). Limits of detection and quantification (LD and LQ) were determined as described previously by Currie, 1999; Long and Winefordner, 1983 and were 1.32 µg/kg and 4.04 µg/kg, respectively.

III.2.10 Statistical analysis

Statistical analysis was performed using the Statistica software (ver. 7.0, StatSoft). Data sets of lag time, relative growth rates, toxin production and gene expression were tested for normality using the Shapiro–Wilk test. All data sets failed the normality test. Therefore, non-parametric data analysis was performed using the Kruskal–Wallis rank sum test. After that, the Duncan's comparison was carried out to determine significant differences, test was applied to compare the median values obtained. The statistical significance was set at $p \leq 0.05$.

III.3 Results

III.3.1 *Aspergillus carbonarius* sensitivity against PgAFP

To study the sensitivity of *A. carbonarius* to the antifungal protein PgAFP both *A. carbonarius* strains were tested against different PgAFP concentrations (1.17-75 µg/mL) in microtiter plates. In general, both toxigenic strains showed the same behaviour against this antifungal protein. At 72 and 96h, A157 showed significant growth reduction ($p \leq 0.05$) from 2.34 µg/mL of PgAFP, reaching over 70% of inhibition at 72 h with 9.38 µg/mL. A171 showed significant growth reduction from 4.69 and 2.34 µg/mL at 72 and 96 h, respectively. The inhibition achieved by 9.38 µg/mL on antifungal protein was over 80% in comparison with non-PgAFP added controls at 72h. The maximum growth inhibition was achieved with the highest PgAFP concentration tested (75 µg/mL) reaching 84 and 88% in A157 and A171 respectively, at 96h (Fig. III-1).

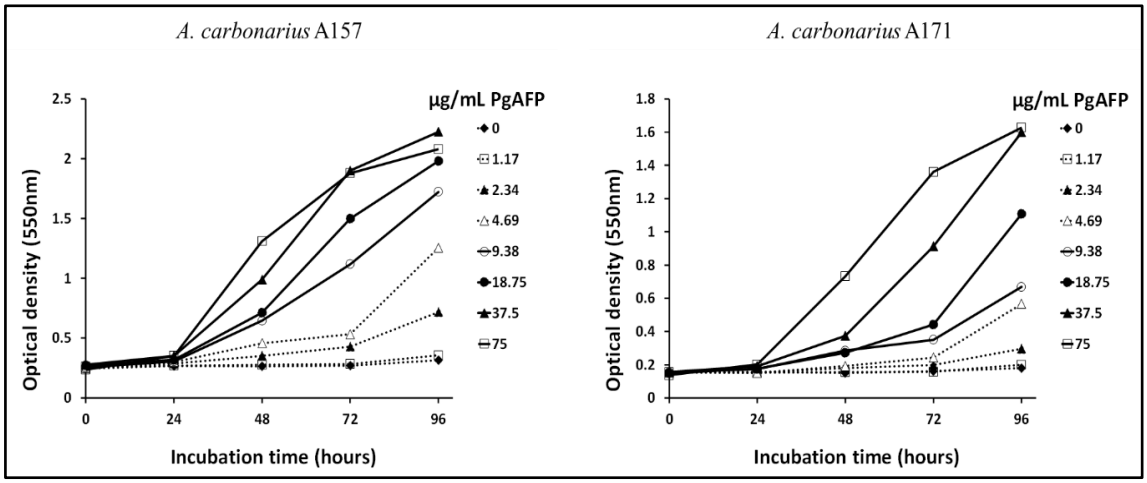


Figure III-1: Sensitivity of two strains of *A. carbonarius* A157 and A171 against different PgAFP concentrations.

III.3.2 Lag phases prior to growth and growth rates

A concentration of 500ppm of E-202 was also investigated, the results were not shown because no growth was noticed at any of the two strains.

III.3.2.1 Effect of E-202 and PgAFP × ionic water activity on lag times of *Aspergillus carbonarius* strains

Figure III-2 shows the effect of E-202 or PgAFP in two raisins-processing typical conditions of a_w on the lag times prior to growth for the two strains of *Aspergillus carbonarius:* tested. The two strains behave similarly for the different treatments and a_w values. The treatment with 60ppm of E-202 at 0.93 a_w had the longest lag phase for both strains, being almost 5 days, followed by the antifungal protein PgAFP (almost 3 days) compared with the control that had the shortest lag phase (around 2 days) ($p \leq 0.05$).

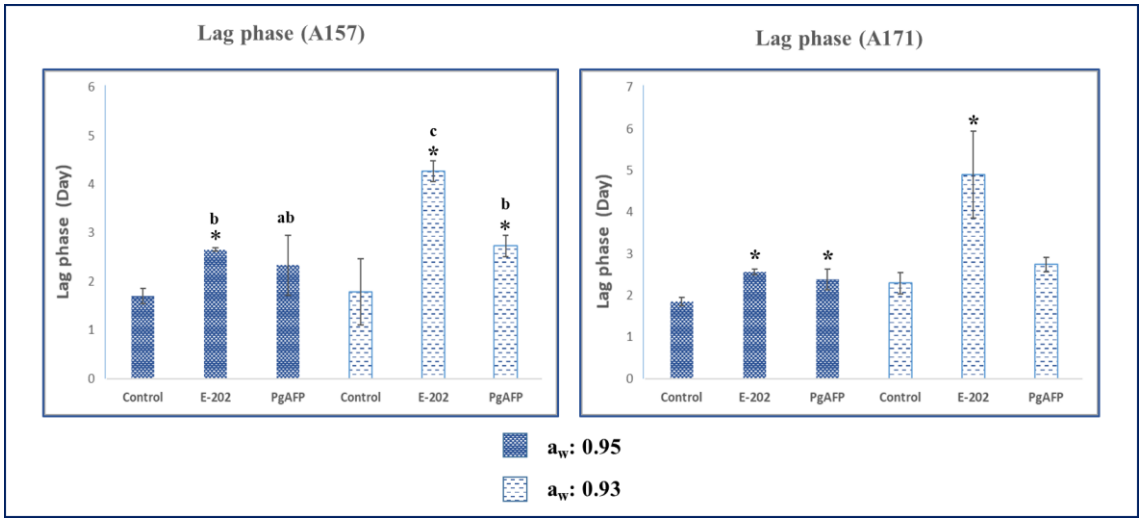


Figure III-2: Effect of E-202 and PgAFP on lag phase of *A. carbonarius* A157 and A171 on SNM at 25°C and a_w 0.93 and 0.95.

III.3.2.2 Effect of E-202 and PgAFP × ionic water activity on the growth rate of *Aspergillus carbonarius* strains

The effect of E-202 and PgAFP on the mean growth rates for the two *Aspergillus carbonarius* strains on the synthetic nutrient medium (SNM) that simulates raisins composition is shown in Figure III-3. At 0.95 a_w maximum growth rate occurred in PgAFP-treated batches for both strains, while no significant difference was noted between E-202 and the control. At 0.93 a_w, similar results were observed, the maximum growth rate was in PgAFP-treated samples, followed by the control and the slowest growth was noticed for both strains when treated with 60ppm of E-202. Therefore, the E-202 addition was the most effective treatment for delaying the growth rate at lower a_w. Generally, in all conditions both strains grew faster at 0.95 a_w compared to 0.93 a_w. No significant difference between the two *Aspergillus carbonarius* strains in all tested conditions was found. Statistical analysis showed that a_w, treatment and their interactions significantly affected relative radial growth rates ($p \leq 0.05$), while no significant effect of the strains on the growth rates were found.

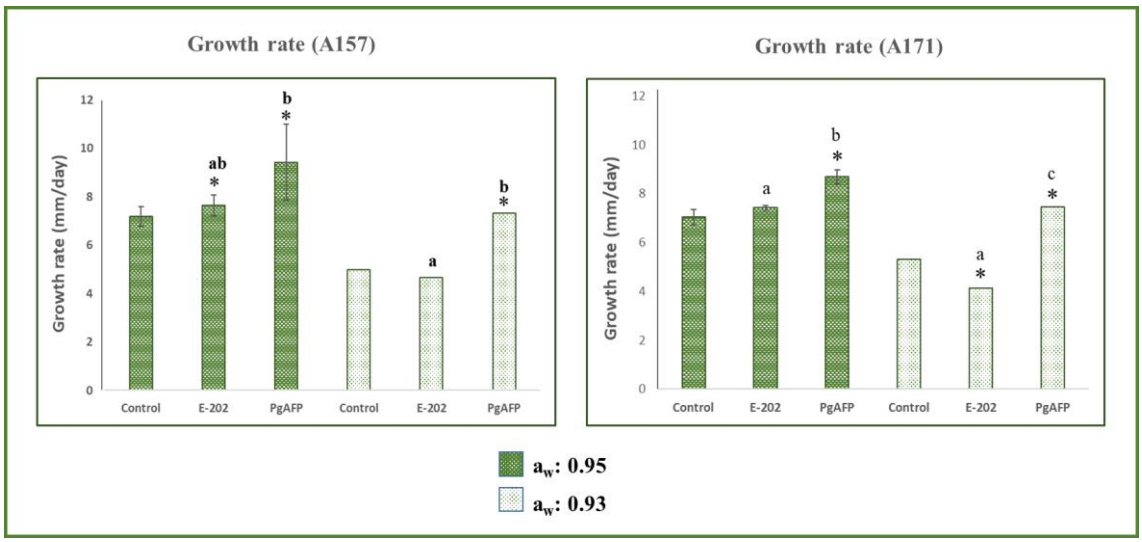


Figure III-3: Effect of E-202 and PgAFP on growth rate of *A. carbonarius* A157 and A171 on SNM at 25°C and a_w 0.93 and 0.95.

III.3.3 Gene expression studies

The relative gene expression of *otapks* and *Rho1* were evaluated in both *A. carbonarius* treated with 10 µg/g of PgAFP or 60 ppm of E-202 at two sampling days. Both strains showed similar behaviour for *otapks* relative gene expression at every sampling day (Fig. III-4). For the first sampling day, there was underexpression of the *otapks* gene for PgAFP and E-202 treated samples at 0.95 a_w ($p \leq 0.05$), and similar results

were observed for samples grown at 0.93 a_w except for the E-202 treatment in A171 where this treatment provoked an overexpression. In the second sampling, only two treatments provoked significative differences in the transcriptomic profile with regard to the control batches: In A157 at 0.93 a_w the E-202 overexpressed *otapks* gene and in A171 at 0.95 a_w PgAFP under-expressed this gene.

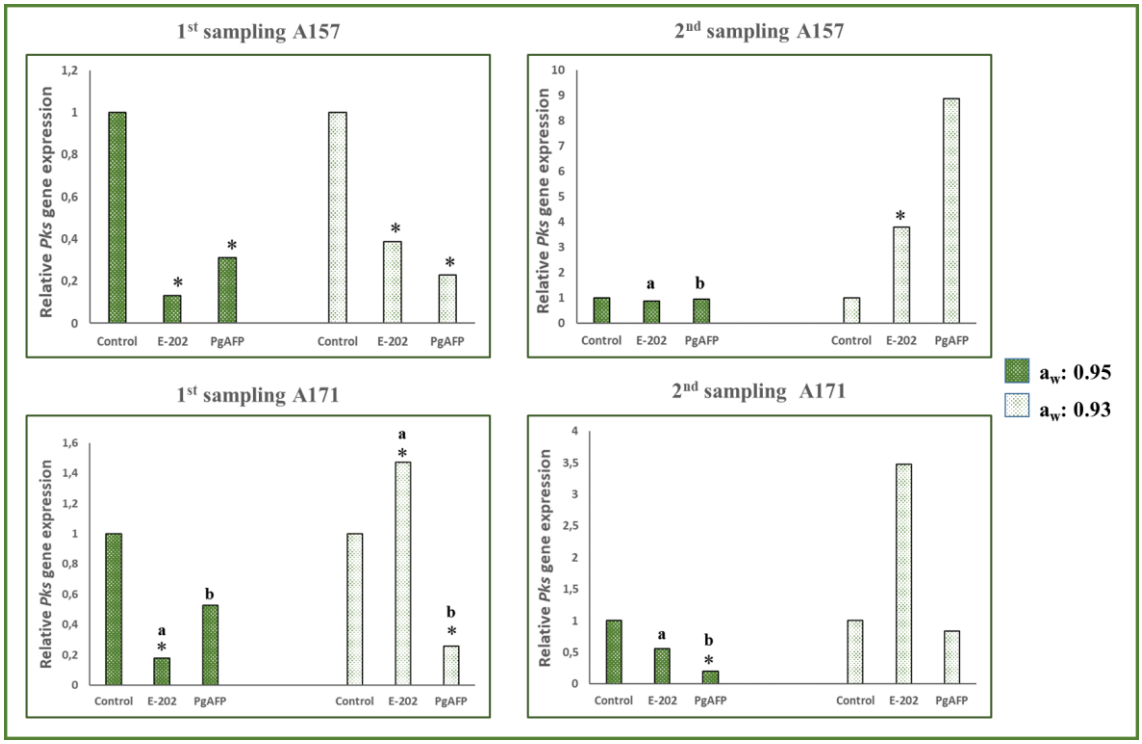


Figure III-4: *Otapks* gene expression of *A. carbonarius* A157 and A171 treated with 10 µg/g of PgAFP or 60 ppm of E-202 at two sampling days.

The *Rho1* transcriptomic profile showed different tendency in both strains at 0.95 a_w but similar behaviour at 0.93 a_w (Fig. III-5). In the first sampling, at 0.95 a_w only AC46 showed changes in the relative expression with overexpression provoked by PgAFP and E-202 treatments ($p \leq 0.05$). At 0.93 a_w only PgAFP treatment affected to *Rho1* gene expression by reducing the expression in both strains, and the E-202 treatment provoked overexpression with regard to PgAFP treatment ($p \leq 0.05$). In the second sampling no differences ($p \geq 0.05$) were found among treatments for A157 and only an overexpression was observed for A171 at 0.95 a_w provoked by PgAFP and E-202 treatments.

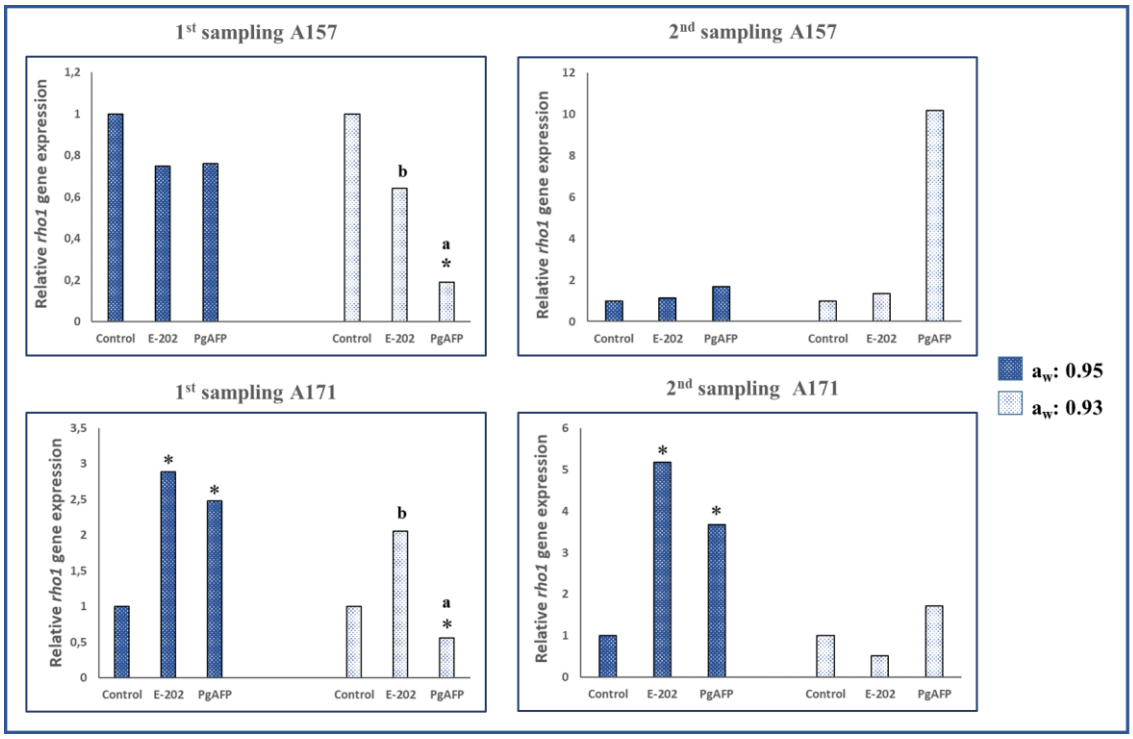


Figure III-5: *Rho1* gene expression of *A. carbonarius* A157 and A171 treated with 10 µg/g of PgAFP or 60 ppm of E-202 at two sampling days.

The correlation between *otapks* and *Rho1* gene expression was significative ($p \leq 0.001$) with a $\rho = 0.674$. This correlation was also significative for *otapks* gene expression in the first sampling and OTA amounts produced ($p \leq 0.05$) and $\rho = 0.374$, no statistical significance was found for the *otapks* gene expression in the second sampling and the OTA amounts. Neither in the first nor the second sampling the *Rho1* gene expression was correlated with OTA amounts produced ($p \geq 0.05$).

III.3.4 Effect of E-202 and PgAFP × ionic water activity on OTA production by *Aspergillus carbonarius*

Figure III-6 shows the effect of E-202 or PgAFP at 0.93 and 0.95 a_w on OTA production by the two strains of *Aspergillus carbonarius:* A157 and A171, which was evaluated immediately after the beginning of the stationary phase, from 10 to 18 days of incubation (depending on the growth rate). Similar OTA production profiles between both treatment and the control at two a_w for both strains were noticed. The OTA levels were lower at 0.93 a_w than those produced at 0.95 a_w which is a better condition for *Aspergillus carbonarius* growth and OTA production. The maximum OTA production at 0.93 a_w was found in the treated batch with E-202: 4.9 and 4.4 ng/g for A157 and A171 respectively. Control and PgAFP-treated samples produced lower OTA amounts but similar amounts of OTA between them. At 0.95 a_w, E-202 provoked an overproduction of OTA, more than

77ppb for both strains, compared to the control and PgAFP that were only 7.8 and 4.6 ppb for A157 as well as 25.2 and 6ppb for A171, respectively. PgAFP was able to control the production of OTA, which was lower than the control for both strains but only significant differences ($p \leq 0.05$) were found for the strain A171 at 0.95 a_w. The treatment with E-202 provoked higher OTA production by *A. carbonarius* strains in comparison to PgAFP-treated samples, in every condition tested ($p \leq 0.05$). Statistical analysis showed that treatment, a_w and their interactions significantly affected OTA production.

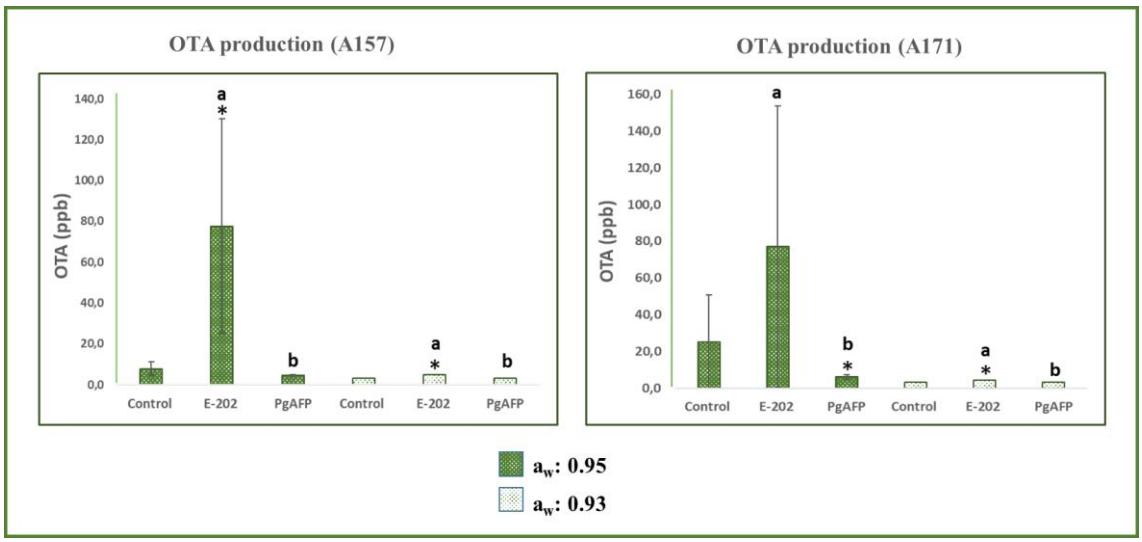


Figure III-6: Effect of E-202 and PgAFP on OTA production at the beginning of the stationary phase in *A. carbonarius* A157 and A171 cultured on SNM at 25°C, a_w 0.93 and 0.95.

III.4 Discussion

In this study, the effect of Potassium sorbate and the antifungal protein PgAFP ata_w values found in the drying raisings, 0.93 and 0.95, on two OTA producing strains of *A. carbonarius*, isolated from raisins were evaluated. This experiment was performed on a synthetic nutrient medium (SNM) that simulate raisins composition (Delfini, 1982). Raisins are at greater risk of OTA contamination because of the rate of *A. carbonarius* that increases during drying (Gómez et al., 2006; Valero et al., 2005; Magan and Aldred, 2005) and nearly all strains of this species produce OTA. *A. carbonarius* has been identified as the key species responsible for OTA contamination of grapes and raisins (Cabañes et al., 2002; Battilani et al., 2003). Therefore, the aim of choosing two usual a_w values found in the drying stage of raisins, was to understand the effect of PgAFP and E-202 at a critical point, the drying phase, for both growth of *A. carbonarius* and OTA production. This can be useful for targeting control strategies in a critical stage to prevent

OTA contamination within the HACCP framework in raisins production, and to minimize consumer exposure to such natural toxins.

The results of this study showed that *A. carbonarius* starts growing earlier and faster at higher a_w (0.95), while at lower a_w (0.93) the fungus seems to be stressed therefore needs more time before start growing, it was previously reported that *A. carbonarius* grew faster at a_w: 0.98-0.995, whereas slower growth rates were reported at 0.93 and 0.95 a_w (Bellí et al., 2004). When *A. carbonarius* strains were treated with 60ppm of potassium sorbate, a significant difference between the control and potassium sorbate were found for both a_w and both strains, and a deeper effect on lag phase was displayed at 0.93 a_w (lag phase: 4 to 5 days) compared to 0.95 a_w (lag phase: 2 days), it has been noticed that potassium sorbate is more effective on delaying the growth of *A. carbonarius* strains at lower a_w. Therefore, the fungus was stressed not only because of the lower water availability, but also because of the presence of E-202, that is widely used as food preservative, being reported in previous researches that this preservative can delay or prevent spore germination, initiation of growth, and decrease the rate of growth of *P. patulum, A. parasiticus, A. flavus* and *P. expansum* (Bullerman, 1983 and 1984; Lennox and Mcelroy, 1984). Also, the dose of PgAFP tested delayed the growth slightly compared to the control. Attending to the dose-response provoked by increasing PgAFP concentrations on *A. carbonarius*, a higher concentration of this antifungal peptide would delay in a greater extent the growth of these strains.

Concerning growth rate, the treatment with E-202 did not show any significant difference compared to the control, only the strain A171 grown at 0.93 a_w displayed slower growth. While PgAFP treated fungus showed a higher growth rate compared to the control and to the E-202 for both a_w and both strains, contrary to the few previous reports about PgAFP, that were found active against some toxigenic species of both *Penicillium* and *Aspergillus* by Acosta et al. (2009), also Delgado et al. (2015) found that PgAFP efficiently reduced counts of *A. flavus* and *P. restrictum* inoculated on a dry fermented sausage. This fact can be explained for the less dense mycelium found in PgAFP-treated samples when grown in solid culture medium, compared to the non-treated samples where the mycelium was denser (data not shown). This means that the biomass was lower in the treated samples than in the control ones. This is corroborated by the great inhibition achieved by PgAFP on *A. carbonarius* strains, observed in PDB in microtiter plates, showing a substantial biomass reduction by the action of PgAFP (Fig. III-7).

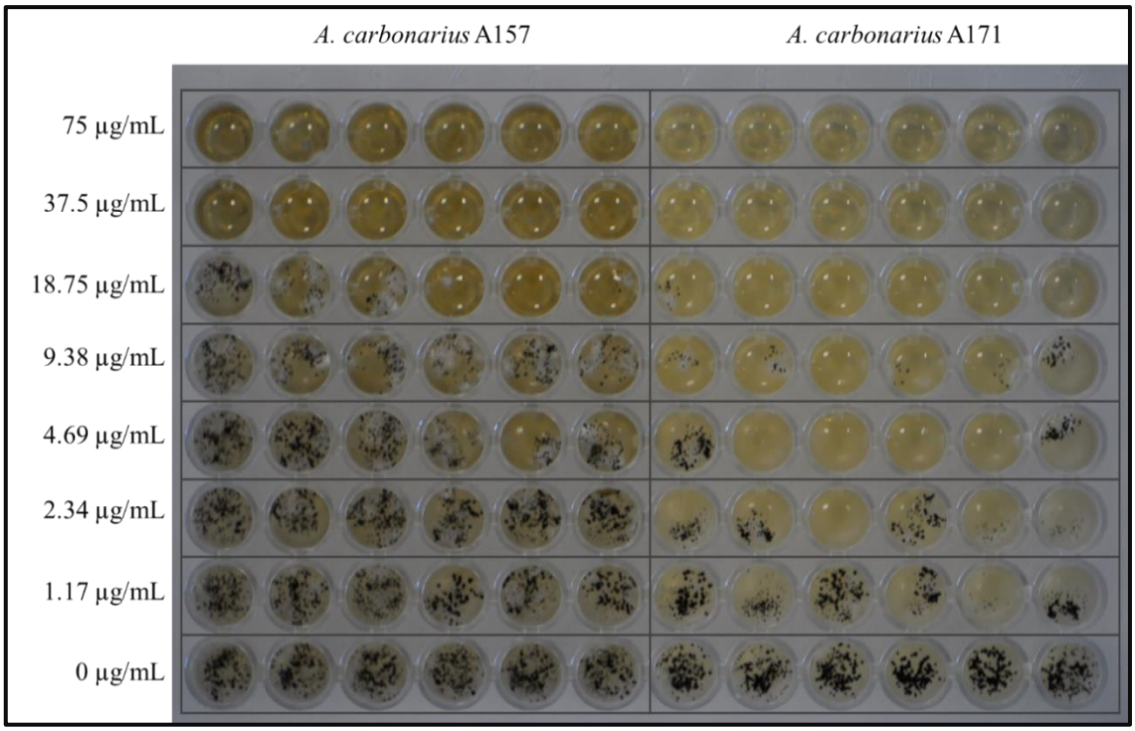


Figure III-7: Inhibition of *A. carbonarius* (A157 and A171) mycelium growth by different PgAFP concentrations on PDB microtiter plates, at 25°C for 96h.

The effect of PgAFP on the cell wall integrity (CWI) pathway, particularly on the *Rho1* protein levels has been used to explain its antifungal mechanism of action in *A. flavus*, A reduction in the level of this protein was linked to the sensitivity of *A. flavus* while an increment of *Rho1* in *Penicillium nordicum* led to overcome to the antifungal activity of PgAFP protein (Delgado et al., 2016). However, in general no evidence of lower expression of *Rho1* gene expression was found in the PgAFP-treated samples. Only at 0.93 a_w was found a similar under-expression pattern in both strains for the first sampling. However, this result does not seem to be related to the lag-phase prior to growth or growth rate from these samples.

In another hand, the present study also suggests that PgAFP is able to control the production of OTA, given that was lower than the control for the strain A171 at 0.95 a_w, the most critical condition of the two tested. While E-202 provoked a substantial increment of OTA production. specially at the highest a_w value tested. Bellí et al. (2005) reported that the optimum a_w level for fungus growth seems to correspond with those optimum for OTA production. However, other authors have demonstrated that the optimum conditions for OTA production can be very different from those for growth (Mitchell et al., 2004), particularly for a_w values which provokes two OTA production peaks, under optimal and suboptimal growth conditions (Schmidt-Heydt et al., 2008). The

results yielded by the present work confirmed that the higher OTA production is not always related with the optimal growth conditions. Previous researches showed that Potassium sorbate greatly reduced or prevented the production of Aflatoxin BI by *A. parasiticus* and *A. flavus* (Bullerman, 1983) and patulin by *P. patulum (*Bullerman, 1984; Lennox and Mcelroy, 1984), one of the few work done on ochratoxin by Bullerman (1985) showed that sorbate reduced OTA synthesis by *Penicillium* sp, but on another hand what was interesting is that he found that *A. ochraceus* appeared to produce greater amounts of ochratoxin in the presence of sorbate, when incubated at 25°C. Also, Yousef and Marth, (1981) reported that *A. parasiticus* and *A. flavus*, respectively, produced equal or greater amounts of aflatoxins in the presence of sub-inhibitory levels of sorbate.

The OTA production was also monitored with the *pks* gene expression. The results obtained from both sampling days yielded different worth information related to OTA production. The *pks* gene expression from the first one was related with the OTA production at the final of the incubation ($p \leq 0.05$) but the gene expression from the second sampling day was not related with the final OTA production. Similar results were found in *A. carbonarius* by Gallo et al. (2009; 2014), when the transcriptomic profile of *pks* gene was correlated with the OTA production. In addition, these authors highlight the fact that *pks* transcription preceded phenotypic OTA production as it is observed in the present work for the first sampling day. However, after the maximum expression and OTA production peak it is observed a reduction on the gene transcription, which would explain the lack of relation between the transcriptomic profile observed in the second sampling day and the OTA quantity one day later. This lack of correlation was also found by Spadaro et al. (2011), who analysed the *pks* gene expression and the OTA production, both in the same sampling date (9 days). The *Rho1* gene expression did not correlate with OTA amounts in any of the sampling days. Therefore, this gene seems to have limitations as temporal relative expression to predict OTA production in this particular species and substrate.

With regard to the use of the E-202, the European Food Safety Authority (EFSA) and Food and Agriculture Organization of the United Nations (FAO), have determined that potassium sorbate is generally recognised as safe (GRAS) and had set a temporary acceptable daily intake of 3 mg/kg of body weight per day (EFSA, 201531). Nevertheless, this substance was reported in some previous researches as a genotoxic and mutagenic compound (Abe and Sasaki, 1977**;** Hasegawa et al., 1984; Kitano et al., 2002; Mamur et al., 2010; Mpountoukas et al., 2008). A reduction in the added quantity from this

preservative, set the maximum level of 1 mg/kg in dried fruit (CR EU, 2011), could be regarded as strategy to not exceed the EFSA recommendation. However, this strategy can be a risk for human health, given its ability of increasing mycotoxins production when use at sub-inhibitory concentrations, as stated in this work. In addition, consumers demand natural and minimally processed foods, free of chemically synthetized preservatives. Therefore, it is required a different strategy to prevent OTA contamination during the drying process. *P. chrysogenum* is used to obtain GRAS compounds. Then, the protein PgAFP as a tool for controlling OTA, may be implemented through HACCP procedures for reducing the health hazard due to this mycotoxin in raisins. To that end, future studies should be focused on setting the appropriate level of PgAFP to maximize the OTA inhibition, as well as studies in drying grapes as previous step to use it on the grapes.

III.5 References

Abarca, M. L., Accensi, F., Bragulat, M. R., Castella, G., and Cabañes, F. J. (2003). *Aspergillus carbonarius* as the main source of ochratoxin A contamination in dried vine fruits from the Spanish market. *Journal of Food Protection*, 66(3), 504-506.

Abe, S. and Sasaki, M., 1977. Chromosome Aberrations and Sister Chromatid Exchanges in Chinese Hamster Cells Exposed to Various Chemicals 2. *Journal of the National Cancer Institute*, 58(6), pp.1635-1641.

Acosta, R., Rodríguez-Martín, A., Martín, A., Núñez, F. and Asensio, M.A., 2009. Selection of antifungal protein-producing molds from dry-cured meat products. *International Journal of Food Microbiology*, 135(1), pp.39-46.

Aksoy, U., Eltem, R., Meyvaci, K.B., Altindisli, A. and Karabat, S., 2007. Five-year survey of ochratoxin A in processed sultanas from Turkey. *Food Additives and Contaminants*, 24(3), pp.292-296.

Amézqueta, S., González-Peñas, E., Murillo-Arbizu, M. and de Cerain, A.L., 2009. Ochratoxin A decontamination: A review. *Food Control*, 20(4), pp.326-333.

Battilani, P., Giorni, P., Bertuzzi, T., Formenti, S. and Pietri, A., 2006. Black aspergilli and ochratoxin A in grapes in Italy. *International Journal of Food Microbiology*, 111, pp.S53-S60.

Battilani, P., Pietri, A., Bertuzzi, T., Languasco, L., Giorni, P. and Kozakiewicz, Z., 2003. Occurrence of ochratoxin A–producing fungi in grapes grown in Italy. *Journal of Food Protection*, 66(4), pp.633-636.

Bejaoui, H., Mathieu, F., Taillandier, P. and 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 Microbiology Letters*, 255(2), pp.203-208.

Bellí, N., Marın, S., Sanchis, V. and Ramos, A.J., 2004. Influence of water activity and temperature on growth of isolates of *Aspergillus* section *Nigri* obtained from grapes. *International Journal of Food Microbiology*, 96(1), pp.19-27.

Bellí, N., Ramos, A.J., Coronas, I., Sanchis, V. and Marín, S., 2005. *Aspergillus carbonarius* growth and ochratoxin A production on a synthetic grape medium in relation to environmental factors. *Journal of Applied Microbiology*, 98(4), pp.839-844.

Bullerman, L.B., 1983. Effects of potassium sorbate on growth and aflatoxin production by *Aspergillus parasiticus* and *Aspergillus flavus*. *Journal of Food Protection*, 46(11), pp.940-942.

Bullerman, L.B., 1984. Effects of potassium sorbate on growth and patulin production by *Penicillium patulum* and *Penicillium roqueforti*. *Journal of Food Protection*, 47(4), pp.312-315.

Bullerman, L.B., 1985. Effects of Potassium Sorbate on Growth and Ochratoxin Production by *Aspergillus ochraceus* and *Penicillium* Species 1, 2. *Journal of Food Protection*, 48(2), pp.162-165.

Cabañes, F.J., Accensi, F., Bragulat, M.R., Abarca, M.L., Castellá, G., Minguez, S. and Pons, A., 2002. What is the source of ochratoxin A in wine?. *International Journal of Food Microbiology*, 79(3), pp.213-215.

Castellá, G., Alborch, L., Bragulat, M.R. and Cabañes, F.J., 2015. Real time quantitative expression study of a polyketide synthase gene related to ochratoxin a biosynthesis in *Aspergillus niger*. *Food Control*, 53, pp.147-150.

Covarelli, L., Beccari, G., Marini, A. and Tosi, L., 2012. A review on the occurrence and control of ochratoxigenic fungal species and ochratoxin A in dehydrated grapes, non-fortified dessert wines and dried vine fruit in the Mediterranean area. *Food Control*, 26(2), pp.347-356.

CR EU, 2011. Commission Regulation (EU) No. 1129/2011 of 11 November 2011 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council by establishing a Union list of food additives. *Official Journal of the European Union L*, 295(4), pp.12-11.

Currie, L.A., 1999. Detection and quantification limits: origins and historical overview1. *Analytica Chimica Acta*, 391(2), pp.127-134.

Da Cruz Cabral L., Delgado J., Andrade M. J., Rodríguez M., Rodríguez A., 2017. Changes in mould cell wall stress-related gene expression by a new real-time PCR method. *International Journal of Food Microbiology*. Submitted.

Delfini, C., 1982. Tecnica di Microbiologia Eonological, ed L. *Scicalpi*. Rome.

Delgado, J., Acosta, R., Rodríguez-Martín, A., Bermúdez, E., Núñez, F. and Asensio, M.A., 2015. Growth inhibition and stability of PgAFP from *Penicillium chrysogenum* against fungi common on dry-ripened meat products. *International Journal of Food Microbiology*, 205, pp.23-29.

Delgado, J., Owens, R.A., Doyle, S., Asensio, M.A. and Núñez, F., 2016. Increased chitin biosynthesis contributes to the resistance of Penicillium polonicum against the antifungal protein PgAFP. *Applied Microbiology and Biotechnology*, 100(1), pp.371-383.

EFSA (Eur. Food Saf. Auth.)., 2015. Scientific opinion on the re-evaluation of sorbic acid (E 200), potassium sorbate (E 202) and calcium sorbate (E 203) as food additives. *EFSA* J, 13, pp.4144-53.

Gallo, A., Knox, B.P., Bruno, K.S., Solfrizzo, M., Baker, S.E. and Perrone, G., 2014. Identification and characterization of the polyketide synthase involved in ochratoxin A biosynthesis in *Aspergillus carbonarius. International Journal of Food Microbiology*, 179, pp.10-17.

Gallo, A., Perrone, G., Solfrizzo, M., Epifani, F., Abbas, A., Dobson, A.D. and Mulè, G., 2009. Characterisation of a pks gene which is expressed during ochratoxin A production by *Aspergillus carbonarius. International Journal of Food Microbiology*, 129(1), pp.8-15.

García, D., Ramos, A.J., Sanchis, V. and Marín, S., 2009. Predicting mycotoxins in foods: a review. *Food Microbiology*, 26(8), pp.757-769.

Gómez, C., Bragulat, M.R., Abarca, M.L., Minguez, S. and Cabanes, F.J., 2006. Ochratoxin A-producing fungi from grapes intended for liqueur wine production. *Food Microbiology*, 23(6), pp.541-545.

Hasegawa, M.M., Nishi, Y., Ohkawa, Y. and Inui, N., 1984. Effects of sorbic acid and its salts on chromosome aberrations, sister chromatid exchanges and gene mutations in cultured Chinese hamster cells. *Food and Chemical Toxicology*, 22(7), pp.501-507.

Heenan, C.N., Shaw, K.J. and Pitt, J.I., 1998. Ochratoxin A production by *Aspergillus carbonarius* and *A. niger* isolates and detection using coconut cream agar. *Journal of Food Mycology*, 1, pp.67-72.

Hocking, A.D., Su-lin, L.L., Kazi, B.A., Emmett, R.W. and Scott, E.S., 2007. Fungi and mycotoxins in vineyards and grape products. *International journal of Food Microbiology*, 119(1), pp.84-88.

Kamala, A., Ortiz, J., Kimanya, M., Haesaert, G., Donoso, S., Tiisekwa, B. and De Meulenaer, B., 2015. Multiple mycotoxin co-occurrence in maize grown in three agro-ecological zones of Tanzania. *Food Control*, 54, pp.208-215.

Kitano, K., Fukukawa, T., Ohtsuji, Y., Masuda, T. and Yamaguchi, H., 2002. Mutagenicity and DNA-damaging activity caused by decomposed products of potassium sorbate reacting with ascorbic acid in the presence of Fe salt. *Food and Chemical Toxicology,* 40(11), pp.1589-1594.

Lennox, J.E. and Mcelroy, L.J., 1984. Inhibition of growth and patulin synthesis in *Penicillium expansum* by potassium sorbate and sodium propionate in culture. *Applied and Environmental Microbiology*, 48(5), pp.1031-1033.

Livak, K.J. and Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *Methods*, 25(4), pp.402-408.

Long, G.L. and Winefordner, J.D., 1983. Limit of detection. A closer look at the IUPAC definition. *Analytical Chemistry,* 55(7), pp.712A-724A.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry,* 193(1), pp.265-275.

Magan, N. and Aldred, D., 2005. Conditions of formation of ochratoxin A in drying, transport and in different commodities. *Food Additives and Contaminants*, 22(s1), pp.10-16.

Mamur, S., Yüzbaşıoğlu, D., Ünal, F. and Yılmaz, S., 2010. Does potassium sorbate induce genotoxic or mutagenic effects in lymphocytes?. *Toxicology in Vitro,* 24(3), pp.790-794.

Medina, Á., Mateo, R., Valle-Algarra, F.M., Mateo, E.M. and Jiménez, M., 2007. Effect of carbendazim and physicochemical factors on the growth and ochratoxin A production of *Aspergillus carbonarius* isolated from grapes. *International Journal of Food Microbiology,* 119(3), pp.230-235.

Meyvaci, K.B., Altindisli, A., Aksoy, U., Eltem, R., Turgut, H., Arasiler, Z. and Kartal, N., 2005. Ochratoxin A in sultanas from Turkey I: Survey of unprocessed sultanas from vineyards and packing-houses. *Food Additives and Contaminants*, 22(11), pp.1138-1143.

Mitchell, D., Parra, R., Aldred, D. and Magan, N., 2004. Water and temperature relations of growth and ochratoxin A production by *Aspergillus carbonarius* strains from grapes in Europe and Israel. *Journal of Applied Microbiology*, 97(2), pp.439-445.

Mpountoukas, P., Vantarakis, A., Sivridis, E. and Lialiaris, T., 2008. Cytogenetic study in cultured human lymphocytes treated with three commonly used preservatives. *Food and Chemical Toxicology*, 46(7), pp.2390-2393.

Palumbo, J.D., O'Keeffe, T.L., Vasquez, S.J. and Mahoney, N.E., 2011. Isolation and identification of ochratoxin A-producing *Aspergillus* section *Nigri* strains from California raisins. *Letters in Applied Microbiology*, 52(4), pp.330-336.

Peromingo, B., Rodríguez, M., Delgado, J., Andrade, M.J. and Rodríguez, A., 2017. Gene expression as a good indicator of aflatoxin contamination in dry-cured ham. *Food Microbiology,* 67, pp. 31–40

Pitt, J.I., 2000. Toxigenic fungi: which are important?. *Medical Mycology*, 38(sup1), pp.17-22.

Quintela, S., Villarán, M.C., de Armentia, I.L. and Elejalde, E., 2013. Ochratoxin A removal in wine: A review. *Food Control*, 30(2), pp.439-445.

Rodríguez-Martín, A., Acosta, R., Liddell, S., Núñez, F., Benito, M.J. and Asensio, M.A., 2010. Characterization of the novel antifungal protein PgAFP and the encoding gene of *Penicillium chrysogenum*. *Peptides*, 31(4), pp.541-547.

Rotem, J. and Aust, H.J., 1991. The effect of ultraviolet and solar radiation and temperature on survival of fungal propagules. *Journal of Phytopathology*, 133(1), pp.76-84.

Schmidt-Heydt, M., Magan, N. and Geisen, R., 2008. Stress induction of mycotoxin biosynthesis genes by abiotic factors. *FEMS Microbiology Letters*, 284(2), pp.142-149.

Serra, R., Mendonça, C. and Venâncio, A., 2006. Ochratoxin A occurrence and formation in Portuguese wine grapes at various stages of maturation. *International Journal of Food Microbiology,* 111, pp.S35-S39.

Sinha, K.K., 1998. Detoxification of mycotoxins and food safety. *Mycotoxins in Agriculture and Food Safety*, pp.381-405.

Spadaro, D., Patharajan, S., Kartikeyan, M., Lorè, A., Garibaldi, A. and Gullino, M.L., 2011. Specific PCR primers for the detection of isolates of *Aspergillus carbonarius* producing ochratoxin A on grapevine. *Annals of Microbiology*, 61(2), pp.267-272.

Valero, A., Marín, S., Ramos, A.J. and Sanchis, V., 2005. Ochratoxin A-producing species in grapes and sun-dried grapes and their relation to ecophysiological factors. *Letters in Applied Microbiology,* 41(2), pp.196-201.

Yousef, A.E. and Marth, E.H., 1981. Growth and synthesis of aflatoxin by *Aspergillus parasiticus* in the presence of sorbic acid. *Journal of Food Protection*, 44(10), pp.736-741.

Chapter IV: Ozone efficacy to control Ochratoxin A and Aflatoxin contamination and the viability of some toxigenic fungi

The experiments described in this chapter were carried out in the Mediterranean Agronomic Institute of Bari (Italy) and in the Institute of Molecular Biology and Pathology-CNR, P.le A. Moro 5, 00185-Rome, Italy. under the supervision of Dr. T. Yaseen and Dr. A. Ricelli.

IV.1 Introduction

Until now, despite the use of all available means of food protection and modern storage practices, fungal and mycotoxin contamination are still major problems in different parts of the world. Control of these contamination is conducted by using different synthetic chemicals that have contributed significantly as preservatives in pest control of stored commodities. Unfortunately, most of these chemicals exhibit significant side effects on human health and environment, such as the appearance of resistant pathogen strains (Brul and Coote, 1999; Leroux, 2007; Prakash et al., 2015). Consumers are concerned about the use of continuous application of synthetic chemicals and there is an increasing demand for novel products able to control contaminations without leaving toxic chemical residues in the food and feed chain. In the last decades, some alternative strategies for sustainable management of food commodities gained the attention of both the research community and the food industry. However, each one of them has some drawback, as an example, the biological methods can serve as an alternative to some chemicals but are expensive and difficult to be applied on a large scale.

Among the novel strategies studied to control OTA and Aflatoxin contamination as well as the viability of some toxigenic fungi during the post-harvest period, ozone seems to be promising. This molecule offers negligible loss of nutrients or sensory qualities in food (Cullen et al., 2009). It is used for the inactivation of various microorganisms in water and is able of degrading chemical contaminants. Ozone is a highly unstable tri-atomic oxygen molecule (O3) formed by the addition of an oxygen atom to a molecular diatomic oxygen (O2) that can be generated readily and economically for application to several commodities. Among the advantages, it is of particular interest the absence of detectable residues on treated products (Agriopoulou et al., 2016). In general, ozone is effective in inhibiting mycelium growth and the resulting nesting, as well as sporulation.

However, its effectiveness to slow or prevent decay is variable, depending on crop, cultivar, decay organism, and storage conditions (Miller et al., 2013).

For this reason, we carried out a study to investigate the ability of gaseous ozone treatment to control ochratoxin A and aflatoxin contamination and the viability of conidia from the toxigenic fungi.

IV.2 Materials and methods

IV.2.1 Samples

Raisin samples used in this study were obtained from the Technical Institute of Fruit Trees and Grapevine (ITAF), in Mascara region (Algeria). After harvesting and drying, samples were stored at 4 °C until use.

IV.2.2 Fungal strains

Two *A. carbonarius* strains were selected for this work, one was OTA-producing (A144) and the other one non-OTA-producing (A135). These strains were isolated from Algerian raisins, molecularly characterized and deposited in the microbial collection of the Mediterranean Agronomic Institute of Bari (MAIB, Italy).

IV.2.3 Aflatoxin B1 and Ochratoxin A

Aflatoxin B_1 and Ochratoxin A standard solutions were obtained from Sigma-Aldrich (Darmstadt, Germany)

IV.2.4 Ozone application on *Aspergillus carbonarius* conidia

Ozone (O3) was generated by a Corona discharge ozone generator (Air Met) MET srl. (Bologna). To investigate the effect of ozone gaseous treatment on the germinability of A. carbonarius conidia, OTA-producer and non-producer *A. carbonarius* strains were cultured on PDA (Sigma-Aldrich, Darmstadt, Germany) for 7 days at 24°C. After this period, huge amounts of conidia were produced, then sterile cover glasses were placed in contact with the colony of each strain, so that a large amount of conidia have adhered to the surface of the slides. The slides with conidia attached were put in a Petri dish and exposed to ozone treatment at 0.3 ppm for 14 days in storage chambers at 3°C. Three slides were sampled after 12 hours, 1, 2, 3, 6 and 14 days. The same number of slides with conidia were placed in a conventional cold storage chamber without ozone treatment, as control (Fig. IV-1).

Figure IV-1: Ozone treatment on *A. carbonarius* OTA-producer and on *A. carbonarius* isolate non-OTA-producing.

Determination of conidia germination

When the glass slides holding conidia were sampled after each period of ozone treatment, they were processed immediately by vortexing with 2ml of sterile distilled water containing tween 0.1 %. Then an amount of 200µl of the obtained suspension was plated on PDA plates as two drops of 100µl each, the drops were observed under microscope at 400x after 24 hours of incubation at 16°C. The ratio between the number of germinating and non-germinating conidia was calculated in each sample and normalized to compare the ozone-treated conidia with the untreated ones.

IV.2.5 Ozone application on Aflatoxin B1 and Ochratoxin A

Aflatoxin B1 and Ochratoxin A standards were treated with ozone to investigate its ability to degrade mycotoxins. Standards were dissolved in MeOH (Merck, Darmstadt, Germany), then distributed on glass slides in order to have 100, 200 and 500ng of Aflatoxin B1 and Ochratoxin A. After the evaporation of the solvent, samples were treated with 0, 0.1, 0.3 and 2ppm of ozone for a period of 5 and 10hours (Fig. IV-2). Three replicates were used for each combination.

Figure IV-2: AfB1 and OTA distributed on glass slides and treated with ozone at 0.1, 0.3 and 2ppm for 5 or 10hours. Three replicates were used for each combination.

Extraction and analysis

After ozone treatment, the slides were crushed and extracted using 10 ml of ethyl acetate acidified by means of 0.1% of acetic acid (Merck, Darmstadt, Germany). The extract was transferred in a 50ml bottom flask. The extraction was repeated three times. The collected extracts were evaporated *in vacuum* at 60°C, then dissolved in 1ml of MeOH and transferred in a vial. The solvent was evaporated by an air stream and the extract was dissolved in 200 ml and analyzed by HPLC.

IV.2.6 Ozone application on raisin artificially contaminated with Aflatoxin B1 and Ochratoxin A

The effect of the application of gaseous ozone to control mycotoxin contamination of raisins was investigated by blending 200g of raisins with 400ml distilled water in order to homogenize it, the obtained slurry was split into two parts in order to add AfB1 and OTA separately. The contamination was carried out at three different concentrations: 100, 200 and 500ppb. The contaminated slurry was distributed in Petri dishes, (6ml each) which were treated with different ozone concentrations: 0.1, 0.3 and 2ppm, during a period of 5hours, and three replicates were used for each application.

Figure IV-3: Making slurry from raisin to be spiked with AfB1 and OTA.

Extraction and purification

After ozone treatment, OTA was extracted from raisins slurry by using the same volume of a mixture constituted by methanol and 0.1M acetic acid 9:1 v/v (extraction mixture). The sample was shaken by an orbital shaker at 120 rpm/min for 30 minutes. Then, the sample was centrifuged at 4000 rpm for 10 minutes, the supernatant was recovered, and the pellet extracted again using the same volume of extraction mixture and the same procedure. The extracts were collected in a bottom flask and concentrated in *vacuum* at 45°C.

The concentrated extracts (3-5ml) were diluted with 10 ml of PBS (Phosphate buffered saline) buffer 0.1M and purified by Immuno-Affinity Columns (OchraStar, Rohmer, Getzersdorf, Austria). The sample was loaded to the IAC column, once it passed through, the IAC column was washed with PBS buffer (20ml), and eluted with 2ml of methanol acidified with 1% of 0.1M acetic acid. The extract was concentrated under an air flow and redissolved in 200µl of methanol. An aliquot of 20µl was analysed by HPLC.

The procedure for AfB1 extraction was the same, the only difference is that the raw extract from the raisins was passed through the IAC columns AflaStar Rohmer and the washing step after loading the sample in the IAC column was conducted using 20 ml of distilled water instead of 20ml of PBS buffer.

IV.2.7 HPLC measurement

The extracts were analysed by HPLC by using an Agilent 1260 Liquid Chromatograph equipped with a diode array detector. An isocratic mixture of acetonitrile: water: acetic acid 99:99:2 v/v/v was used as mobile phase, and an Agilent YMC-Triart C18 column (150x4,6mm; 3.0µm) equipped with a pre-column filter as stationary phase. The analyses were performed at a flow rate of 0.8ml/min.

The quantification of AfB1 and OTA was performed using the external standard method by comparing the peak values obtained by the pure standard with those having the same retention time obtained when analysing the samples.

IV.2.8 Statistical analysis

Each experiment was done in replicate for at least three times. Analyses were carried out in order to check whether there was a statistically significant difference between treatment with ozone compared to the control, as well as among different ozone concentrations and duration of treatment. The collected data were subjected to ANOVA test using Statistica software (ver. 7.0, StatSoft). A Duncan's comparison was also performed to determine significant differences, the results were considered significant only when the P value was less than or equal to 0.05.

IV.3 Results and discussion

IV.3.1 Ozone application on *Aspergillus carbonarius* conidia

After each period of treatment, conidia germinability was investigated after 24-48 hours of incubation on PDA at 15°C by microscopic observation (magnification x400). The germinability was tested by measuring the ratio between the number of germinating and non-germinating conidia in each sample. The results were normalized to compare the ozone-treated conidia with the untreated ones. In order to evaluate exclusively the fungi-toxic effect, the conidia that showed a delay in germination were considered among the germinating ones.

The results show that O_3 treatment causes a significant decrease in the germinating ability of the conidia of both OTA producing and non-OTA-producing *A. carbonarius*. The control of the germination ability starts after 1 day of treatment and increases until the end of the treatment period considered (14 days) (Fig. IV-4).

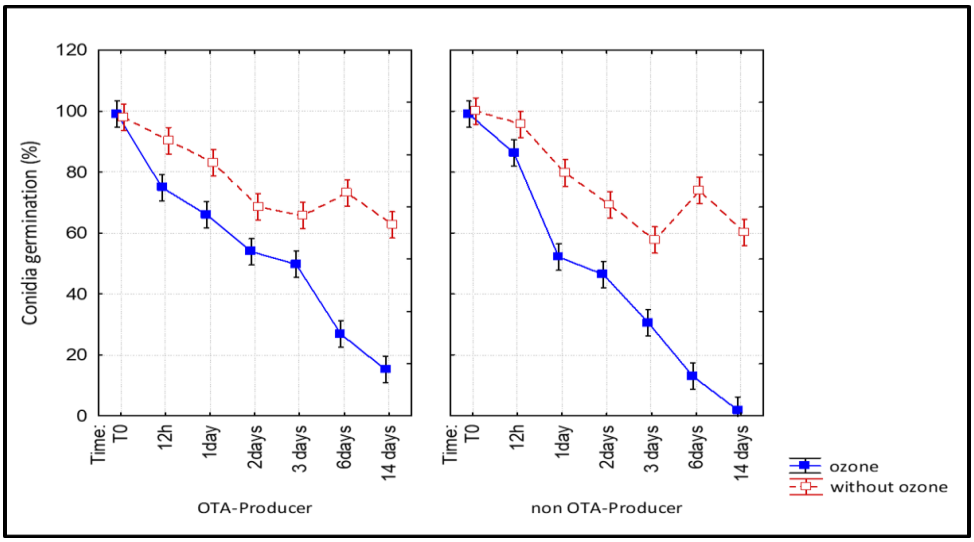


Figure IV-4: Effect of O3 treatment (0.3ppm) on conidia germination of *A. carbonarius* OTA-producing and non-OTA-producing strain (%) after different periods of treatment.

IV.3.2 Difference between ozone effect on conidia germination of *A. carbonarius* OTA-producing and non-OTA-producing strains

It is evident that O3 treatment is able to significantly control the germination of both OTA producing and non-OTA-producing strains. However, when comparing the two strains, we found that ozone was slightly more effective to reduce conidia germinability of the non-producing strain compared to the OTA-producing one (Fig. IV-5), this difference was statistically significant (P >0.05).

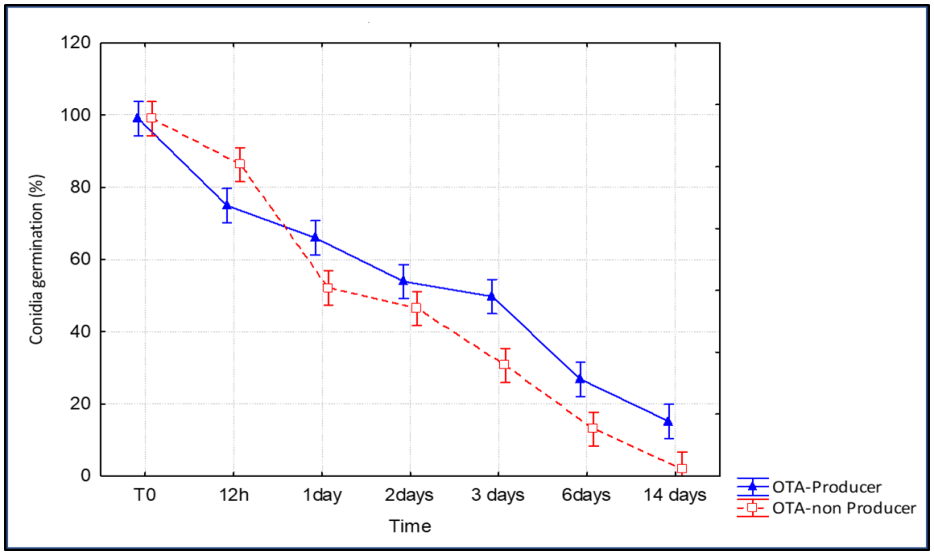


Figure IV-5: Comparison between the effect of ozone (0.3ppm) on conidia germination of *A. carbonarius* OTA-producing and non-OTA-producing, after different periods of treatment.

IV.3.3 Ozone application on Aflatoxin B1 and Ochratoxin A

To study the possible effect of ozone on the considered mycotoxins, 100µl of a methanolic solution containing different concentrations of OTA, AfB1 and their mixture (100, 200 and 500ng) were placed on different glass slides. After methanol evaporation, the slides were treated with ozone at different concentrations (0.1, 0.3 and 2 ppm) for 5 and 10 hours. The effect of O_3 was determined by HPLC measurement, the efficiency of the extraction was 77-91%, the results are presented in percentage, considering the untreated control as 100%.

IV.3.3.1 Ozone application on Aflatoxin B1

IV.3.3.1.1 Effect of ozone treatment

The decrease in the Aflatoxin B1 contamination as a function of ozone treatment and exposure time is shown in Figure IV-6. There is a significant difference between the control and the three ozone treatments for both 5h and 10h of treatment, this result was demonstrated after statistical analysis. In particular, AfB1 degradation was higher than 80% following the treatment with any of ozone concentrations when the amount of the toxin was 100ng (Fig. IV-6/A), when the AfB1's amount was 200ng its degradation was higher than 60% following the treatment with ozone at 0,1ppm and higher than 80% following the treatment with ozone at 0,3 and 2ppm (Fig. IV-6/B). When the AfB1's amount was 500ng, to obtain a degradation of AfB1 higher than 60% it is necessary to carry out ozone treatment at 2ppm for 5 hours. The same degradation level is obtained by or 0.3 and 2ppm for 10 hours (Fig. IV-6/C). When comparing among ozone treatment, there is a significant difference between 0.1 and 0.3ppm of O3.

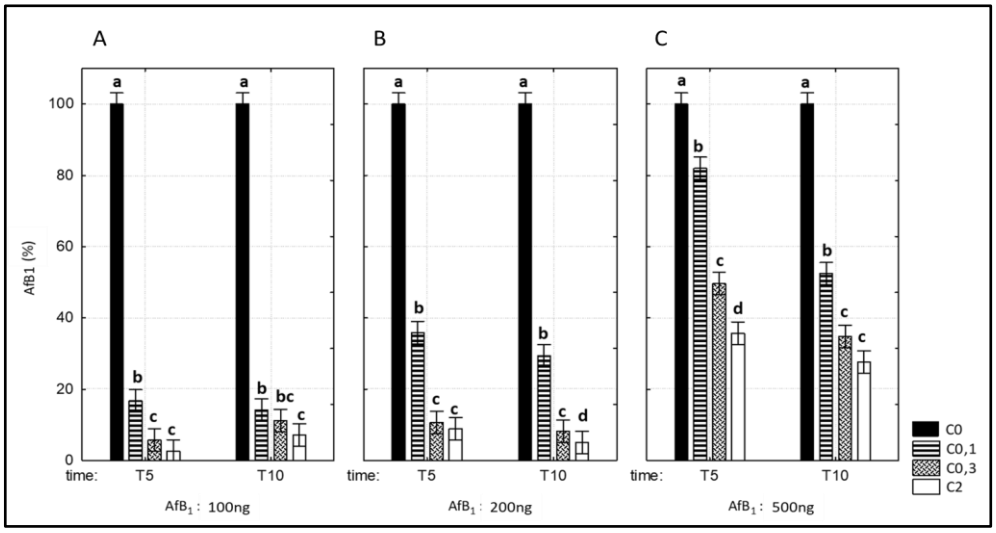


Figure IV-6: Effect of O_3 treatment at 0.1ppm (C0,1), 0.3ppm (C0,3), 2ppm (C2) and untreated as control (C0) for 5 (T5) and 10 (T10) hours on the degradation of 100, 200 and 500ng of AfB1. Columns with different letters indicate homogeneous groups according to Duncan's ANOVA honest significance test ($P \leq 0.05$). Statistical analyses were carried out separately for each AfB1 concentration and period of treatment.

IV.3.3.1.2 Effect of the duration of the treatment

The results showed that the longer period of ozone treatment (10 hours) led to a significantly higher degradation of the toxin in particular when AfB1 amount is 500ng and ozone treatment is applied at 0.1 or 0.3ppm (Fig. IV-7/C). At lower AfB1 amount (100 and 200ng) (Fig. IV-7/A, B) the decrease of aflatoxin presence following a 10hours ozone treatment is not significantly more pronounced compared to the 5hours ozone treatments and does not justify the longer treatment duration.

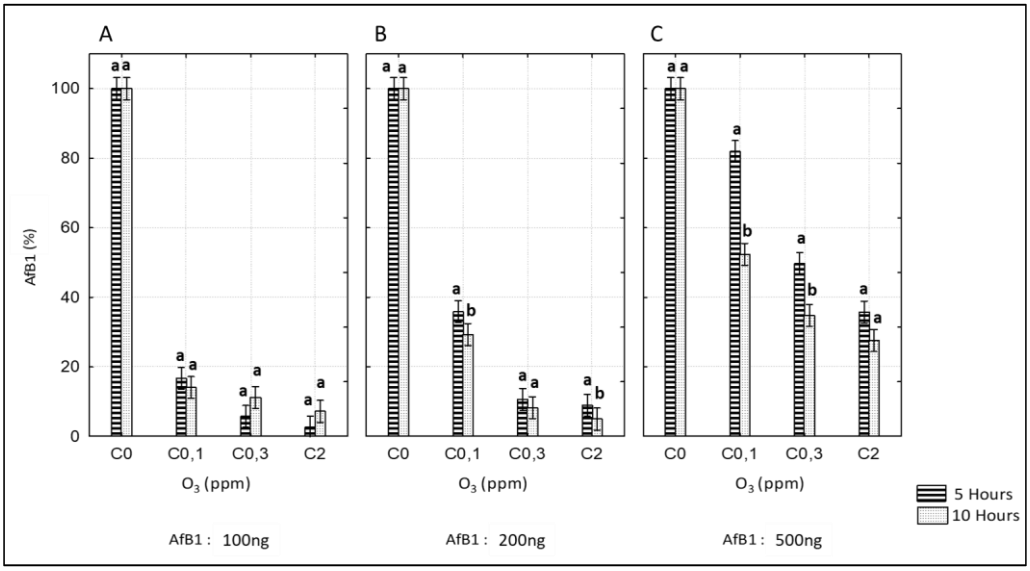


Figure IV-7: Effect of the duration (5 hours or 10 hours) of O_3 treatment at 0.1ppm (C0,1), 0.3ppm (C0,3), 2ppm (C2) and untreated as control (C0) on the degradation of 100, 200 and 500ng of AfB1.
Columns with different letters indicate homogeneous groups according to Duncan's ANOVA honest significance test ($P \leq 0.05$). Statistical analyses were carried out separately for each AfB1 concentration and concentration of O_3 treatment.

IV.3.3.2 Ozone application on Ochratoxin A

IV.3.3.2.1 Effect of ozone treatment

The results showed that O3 was also able to reduce OTA, this result was demonstrated after statistical analysis; which shows that there is a significant difference between the untreated samples and the ones treated with ozone at 0.1, 0.2 and 2 ppm for both 5h and 10h duration.

Also, when comparing among ozone treatments, the results show that a degradation of OTA higher than 60% is possible only using ozone treatment at 2ppm and if OTA contamination is 100ng or 200ng (Fig. IV-8/A, B). On 500ng of OTA, the highest degradation rate is between 50 and 60%, which was obtained applying ozone at 2ppm for 10hours (Fig. IV-8/C).

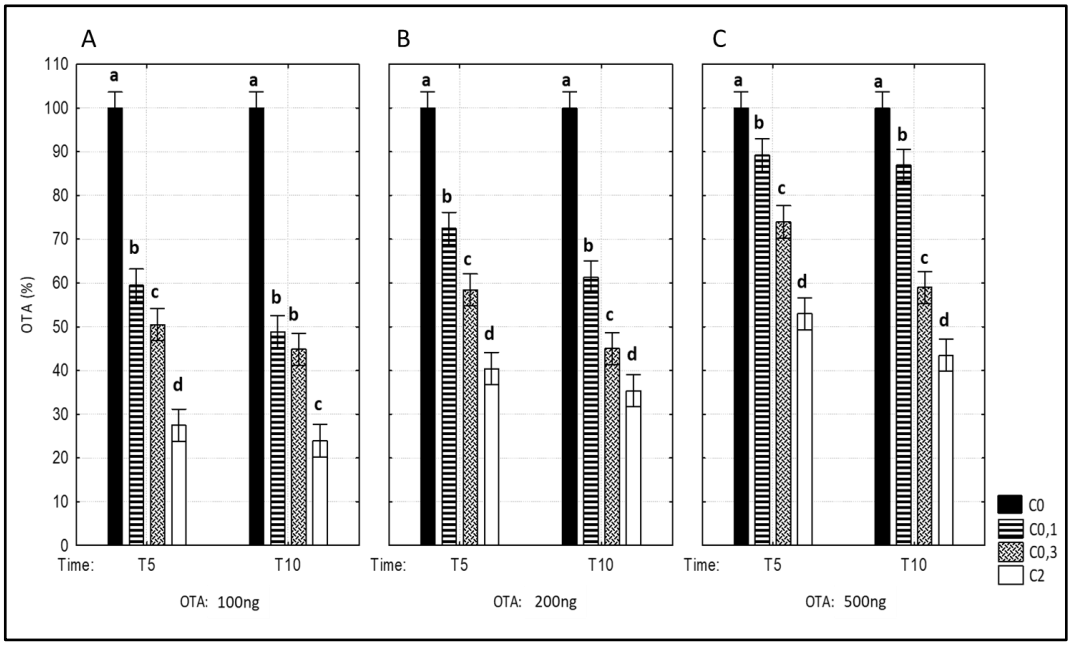


Figure IV-8: Effect of O_3 treatment at 0.1ppm (C0,1), 0.3ppm (C0,3), 2ppm (C2) and untreated as control (C0) for 5 (T5) and 10 (T10) hours on 100, 200 and 500ng of OTA.
Columns with different letters indicate homogeneous groups according to Duncan's ANOVA honest significance test ($P \leq 0.05$). Statistical analyses were carried out separately for each OTA concentration and period of treatment.

IV.3.3.2.2 Effect of the duration of the treatment

The results showed that when 200ng of AfB1 was treated with 0.1 and 0.3ppm of ozone (Fig. IV-9/B), and when 500ng of AfB1 was treated with 0.3 and 2ppm of ozone (Fig. IV-9/C), the 10hours treatment was significantly more effective than the 5hours one. While when the amount of OTA was 100ppb there was no significant difference in OTA degradation between 5 hours and 10 hours of ozone treatment (Fig. IV-9/A).

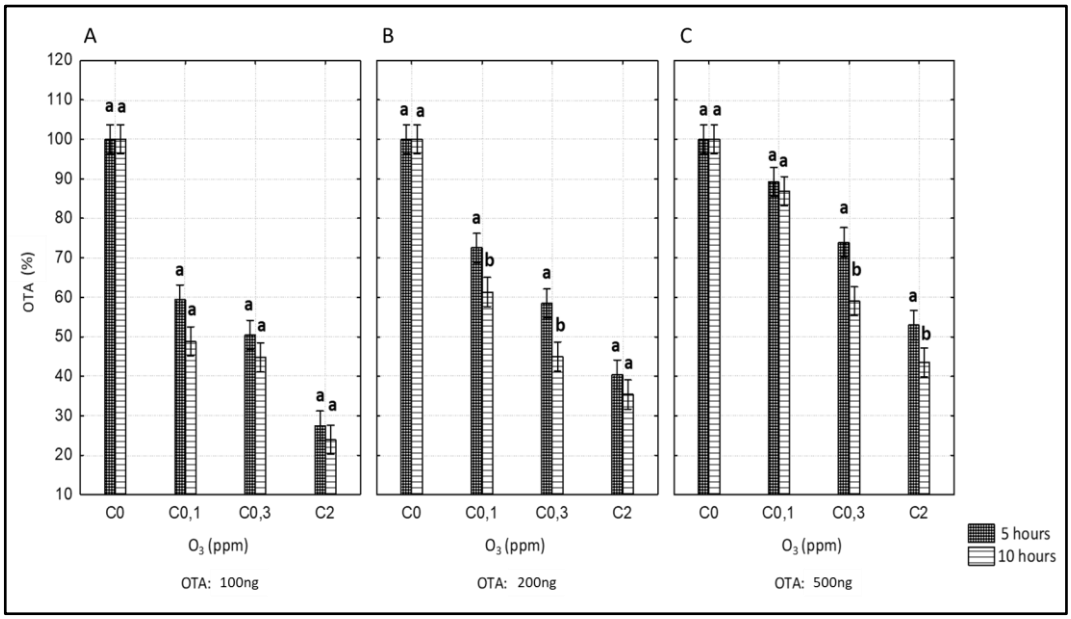


Figure IV-9: Effect of the duration (5 hours or 10 hours) of O_3 treatment at 0.1ppm (C0,1), 0.3ppm (C0,3), 2ppm (C2) and untreated control (C0) on the degradation of 100, 200 and 500ng of OTA.
Columns with different letters indicate homogeneous groups according to Duncan's ANOVA honest significance test ($P \leq 0.05$). Statistical analyses were carried out separately for each OTA concentration and concentration of O_3 treatment.

IV.3.3.3 Comparison between the effect of ozone treatment on OTA and AfB1

Results showed that ozone was more effective in decreasing AfB1 than OTA, at all the tested concentrations for both 5 and 10h of treatment (Fig. IV-10). These results were confirmed after statistical analysis, where a significant difference was found between AfB1 and OTA treated with ozone in all cases of ozone and OTA/AfB1 concentration.

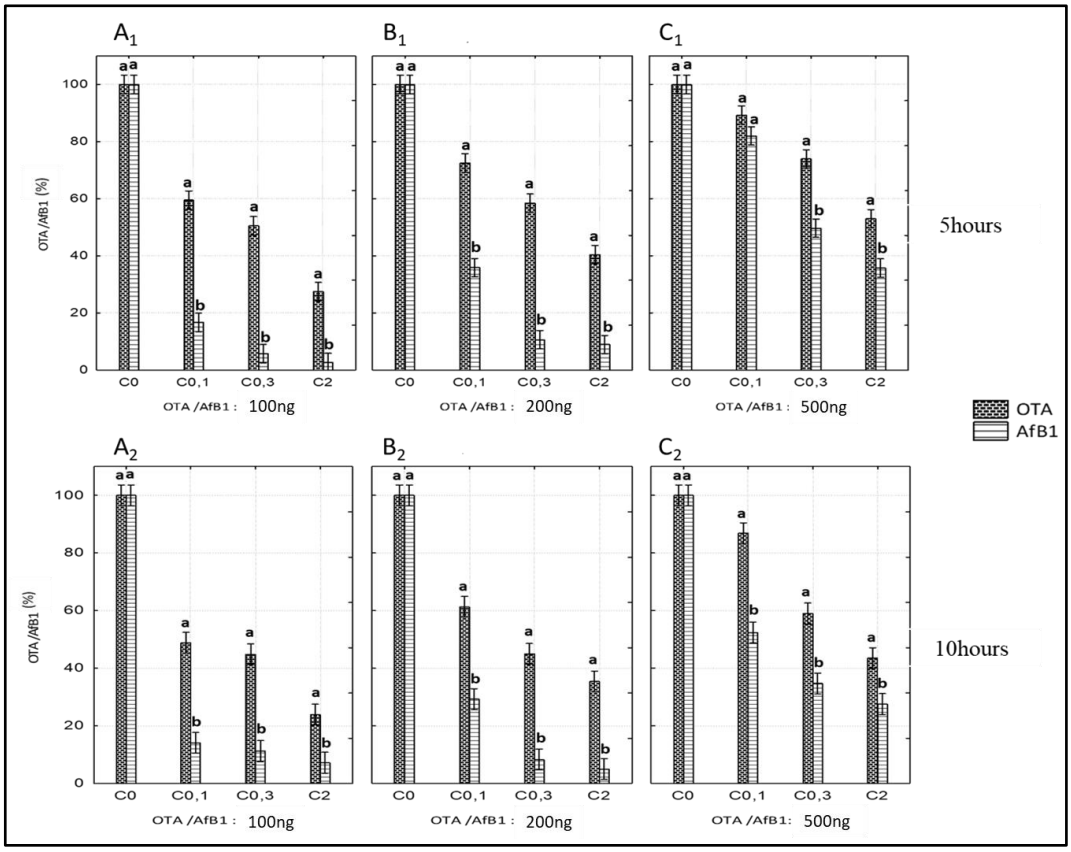


Figure IV-10: Comparison between the effect of O_3 treatment at 0.1ppm (C0,1), 0.3ppm (C0,3), 2ppm (C2) and untreated control (C0), following 5 hours (T5) and 10 hours (T10) treatment on the degradation of 100, 200 and 500ng of AfB1 and OTA.
Columns with different letters indicate homogeneous groups according to Duncan's ANOVA honest significance test ($P \leq 0.05$). Statistical analyses were carried out separately for each OTA/AfB1 concentration, ozone concentration and time of treatment.

IV.3.4 Ozone application on raisin artificially contaminated with Aflatoxin B1 and Ochratoxin A

Results of ozone application on raisin artificially contaminated by AfB1 and OTA after 5 hours of treatment, were elaborated after extraction, purification and HPLC analysis.

IV.3.4.1 Ozone application on raisin artificially contaminated with Aflatoxin B1

Ozone treatment significantly reduced AfB1 contamination in raisins contaminated at 100 and 200 and 500ppb; although no difference in the efficacy was found among ozone concentrations (Fig. IV-11). Ozone treatment at 0.1ppm is sufficient to obtain a significant reduction of AfB1 amount, either at low toxin level (100ppb) as well as at high toxin level (500ppb).

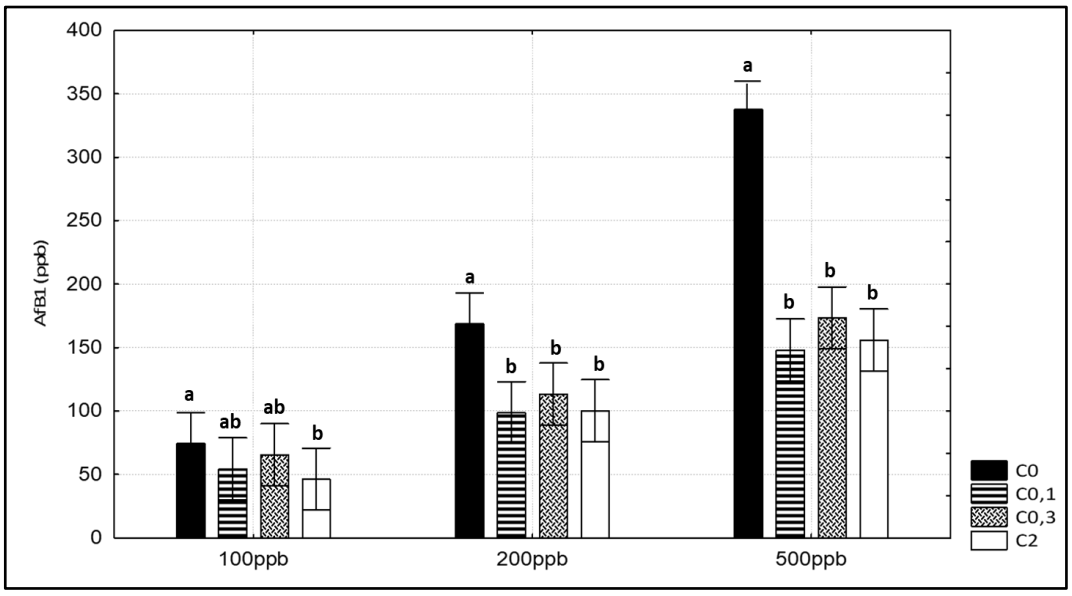


Figure IV-11: Effect of O_3 treatment at 0.1ppm (C0,1), 0.3ppm (C0,3), 2ppm (C2) and untreated as control 0 (C0), after 5 hours of application time on the degradation of 100, 200 and 500ppb of Aflatoxin B1 on raisins artificially contaminated.
Columns with different letters indicate homogeneous groups according to Duncan's ANOVA honest significance test ($P \leq 0.05$). Statistical analyses were carried out separately for each AfB1 concentration.

IV.3.4.2 Ozone application on raisin artificially contaminated with Ochratoxin A

After 5 hours of ozone treatment on raisin contaminated with OTA, the results showed that ozone was effective in decreasing the contamination at all the considered concentrations (100, 200 and 500ppb of OTA). These results were statistically analysed, where significant differences were found between the control and ozone treatment. No difference in the inhibition level was found among 0.1, 0.3 and 2ppm of ozone for 100 and 200ppb of OTA, whereas, in raisins contaminated at 500ppb of OTA, the treatment with ozone at 2ppm had a significantly higher efficacy in the decrease of OTA contamination compared with the treatment made at lower ozone concentrations (0.1 and 0.3ppm). In all cases no significant difference found between 0.1 and 0.3ppm of ozone (Fig. IV-12).

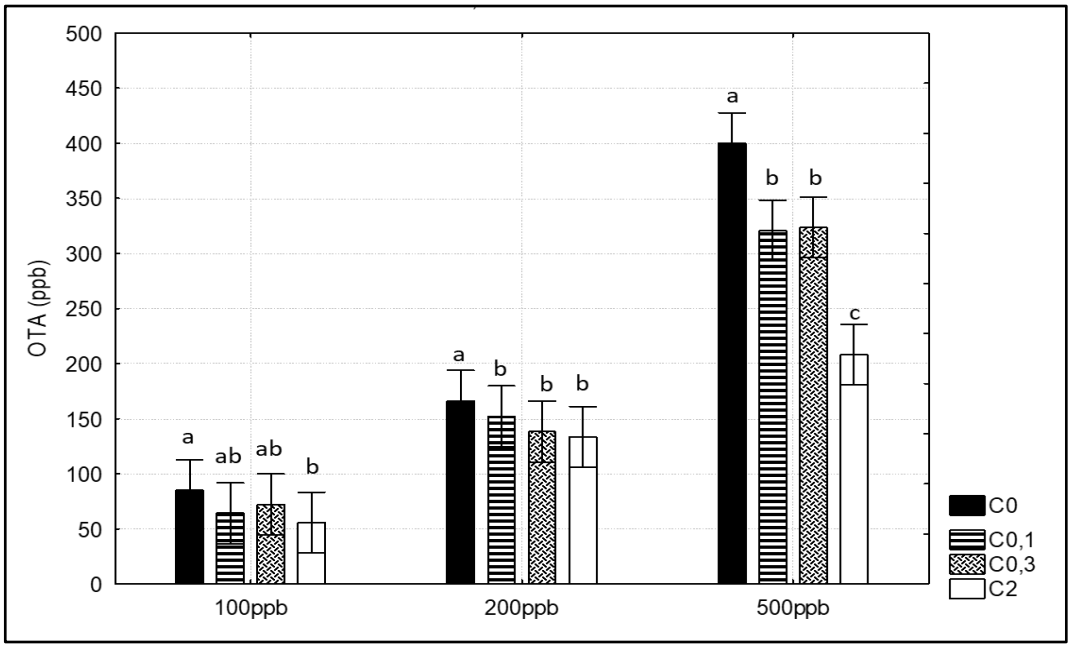


Figure IV-12: Effect of O_3 treatment at 0.1ppm (C0,1), 0.3ppm (C0,3), 2ppm (C2) and untreated as control 0 (C0), after 5 hours of application time on the degradation of 100, 200 and 500ppb of OTA on raisins artificially contaminated.
Columns with different letters indicate homogeneous groups according to Duncan's ANOVA honest significance test ($P \leq 0.05$). Statistical analyses were carried out separately for each OTA concentration.

IV.3.4.3 Comparison between the effect of ozone on OTA and AfB1 spiked on raisin

Comparing between the degradation of AfB1 and OTA by O_3 treatment on raisins, we found that at 100ppb of AfB1/OTA (Fig. IV-13/A), there is no significant difference in the reduction of the two mycotoxins concentration after 5h of ozone application at 0.1, 0.3 and 2ppb. At 200ppb AfB1/OTA contamination (Fig. IV-13/B), the effect of ozone was more visible in decreasing AfB1 compared to OTA, and this was statistically significant after treatment with 0.1ppm of O_3. When the level of mycotoxin contamination was 500ppb (Fig. IV-13/C), the reduction of AfB1 was significantly higher than the reduction of OTA when ozone treatment was performed at 0.1 and 0.3ppm. When ozone treatment was performed at 2ppm, the reduction of OTA and AfB1 was comparable.

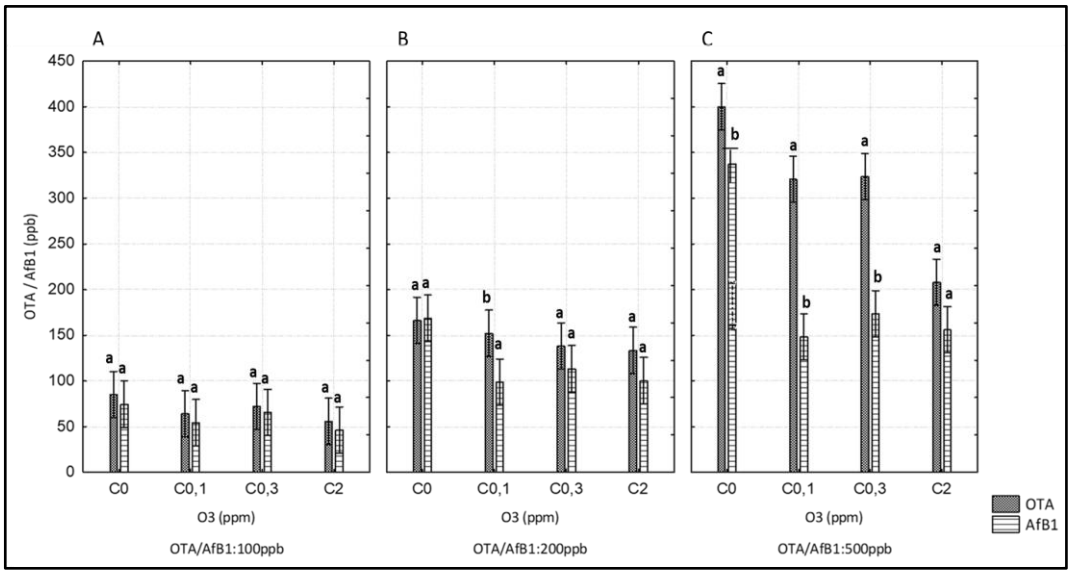


Figure IV-13: Comparison between the effect of O_3 treatment 0.1ppm (C0,1), 0.3ppm (C0,3), 2ppm (C2) and untreated as control (C0), after 5 hours of application time on the degradation of 100, 200 and 500ppb of AfB1 and OTA on raisins artificially contaminated.
Columns with different letters indicate homogeneous groups according to Duncan's ANOVA honest significance test ($P \leq 0.05$). Statistical analyses were carried out separately for each OTA/AfB1 concentrations, and each ozone concentrations.

IV.4 Discussion

In grape and grape products, A. carbonarius has been reported to be the major OTA producer (Heenan et al., 1998; Pitt, 2002; Cabañes et al. 2002; Téren et al. 1996; Abarca et al. 2003). For this reason, in the present study, we investigated if ozone treatment has a control effect on the viability of *Aspergillus carbonarius* conidia. In particular, we treated conidia of an OTA producing *A. carbonarius* strain and of a non-OTA-producing *A. carbonarius*. Ozone treatment at 0.3 ppm was able to significantly control conidia germination of both *A. carbonarius* strains starting from a 24 hours treatment (Fig. IV-4). We observed that the reduction of the germination, that was checked 24 hours after the end of the treatment, was in direct correlation with the duration of O_3 treatment. Since the inhibition of the germination lasted for a maximum of 10 days, and then fungal growth occurred, we can say that ozone has a fungi-static effect rather than a fungi-toxic effect. In previous research, Krause and Weidensaul (1978) reported that ozone at 0.3 ppm for two 6h periods significantly inhibited *Botrytis cinerea* sporulation and germination *in vivo* and *in vitro*. Also, Palou et al. (2001) found that gaseous ozone at 0.3ppm was able to control green and blue moulds on cold stored citrus, by inhibiting mycelial growth and sporulation. When comparing between the effect of ozone on the two strains of *A.*

carbonarius, we found that the non OTA-producing strain was more sensitive to ozone than the OTA-producing one (Fig. IV-5). Previous studies report that there is a difference among conidia viability in different black Aspergilli species after UV irradiation (García-Cela et al., 2016). In particular, this study reports that the observed behavior was due to the difference between the thickness of conidial walls and also to the variation of the surface/volume ratio. We can hypothesize that the difference in the results we found between conidia germination in the OTA-producing and in the non OTA-producing strains of *A. carbonarius* after ozone treatment, could be due to differences in the characteristics of condial walls.

Although the first study about ozone application focusing on mycotoxin degradation by Dwarakanath et al. (1968) reported that gaseous ozone reduced total aflatoxins in cottonseed meal and peanut meal, 30 years later, the use of ozone in mycotoxin degradation was again tested by McKenzie et al. (1997). Our results concerning the experiment performed in order to check the effect of ozone on AfB1 and OTA, showed that ozone effectively degraded both mycotoxins and the reduction reached more than 97% for AfB1 and 76% for OTA. These results are in agreement with some previous studies on mycotoxins degradation by ozone (Inan et al. 2007; Maeba et al., 1988; McKenzie et al., 1997; Qi et al., 2016). AfB1 is more sensitive to ozone compared to OTA.These differences in the degradation can be attributed to the difference between the chemical structure of the toxins (Agriopoulou et al., 2016; McKenzie et al., 1997). Concerning AfB1 ozone reacts across the 8, 9 double bond of the terminal furan ring through electrophilic attack, causing the formation of primary ozonides followed by rearrangement into monozonide derivatives such as aldehydes, ketones and organic acids (Inan et al., 2007). In the case of OTA, the degradation takes place via the hydrolysis of the OTA amide group and the subsequent release of the OTα and l-β-phenylalanine moieties (Abrunhosa et al. 2014).

For the time of treatment, we found that in most cases 5 hours could be enough to degrade AfB1 and OTA, however when the amount of mycotoxin was at the highest level (500ng), a significant difference between the two treatment duration occurred, and 10h were significantly more effective in reducing the level of AfB1 and OTA.

Concerning the experiment performed in order to check the effect of ozone treatment in controlling raisins artificially contaminated with AfB1 and OTA, we found ozone to be effective in reducing the level of these toxins on raisin in a similar way to the effect

observed when ozone was applied directly on AfB1 and OTA molecules. Therefore, even if for the experiment on pure molecules ozone was in direct contact with OTA and AfB1, while in the experiment on raisins, the probability of the contact between ozone and the toxins was reduced for the presence of different compounds in the samples, also in raisins the percentage of degradation was significant. For example, after 5h of treatment with ozone at 2ppm on 500ng of OTA and AfB1, the percentage of OTA and AfB1 degradation was 45% and 65% respectively. When the same ozone treatment was carried out on raisins artificially contaminated at 500ppb, the percentage of OTA and AfB1 degradation was 50% and 55% respectively.

Different ozone concentrations seem to have a significant effect only on raisins contaminated with OTA at 500ppb, where the results obtained by using the highest ozone concentration (2ppm) was significantly lower compared with the results obtained by using ozone at 0.1 and 0.3ppm (Fig. IV-12). Concerning AfB1, a significant decreasing effect was observed by the treatment with all the tested ozone concentrations. In particular, ozone at 0.1 was sufficient to significantly decrease AfB1 concentration also on the samples contaminated at 500ppb. (Fig. IV-11).

When comparing between the effect of ozone treatment on AfB1 and OTA in raisin (Fig. IV-13), it is possible to observe that AfB1 degradation is comparable to OTA degradation when the level of the toxins is 100 and 200ppb. When mycotoxin contamination is higher (500ppb), ozone treatment at 0.1 and 0.3ppm has a more pronounced effect on AfB1 compared to OTA. However, when ozone treatment is used at 2ppm, AfB1 and OTA degradation are comparable. This difference between the behaviour of the two mycotoxins confirmed what we found in the previous experiment on the pure molecules AfB1 and OTA.

IV.5 References

Abarca, M. L., Accensi, F., Bragulat, M. R., Castella, G., and Cabañes, F. J., 2003. *Aspergillus carbonarius* as the main source of ochratoxin A contamination in dried vine fruits from the Spanish market. *Journal of Food Protection*, 66(3), 504-506.

Abrunhosa, L., Inês, A., Rodrigues, A.I., Guimarães, A., Pereira, V.L., Parpot, P., Mendes-Faia, A. and Venâncio, A., 2014. Biodegradation of ochratoxin A by *Pediococcus parvulus* isolated from Douro wines. *International Journal of Food Microbiology*, 188, pp.45-52.

Agriopoulou, S., Koliadima, A., Karaiskakis, G. and Kapolos, J., 2016. Kinetic study of aflatoxins' degradation in the presence of ozone. *Food Control*, 61, pp.221-226.

Brul, S. and Coote, P., 1999. Preservative agents in foods: mode of action and microbial resistance mechanisms. *International Journal of Food Microbiology*, 50(1), pp.1-17.

Cabañes, F.J., Accensi, F., Bragulat, M.R., Abarca, M.L., Castellá, G., Minguez, S. and Pons, A., 2002. What is the source of ochratoxin A in wine?. *International Journal of Food Microbiology*, 79(3), pp.213-215.

Cullen, P.J., Tiwari, B.K., O'Donnell, C.P. and Muthukumarappan, K., 2009. Modelling approaches to ozone processing of liquid foods. *Trends in Food Science & Technology*, 20(3), pp.125-136.

Dwarakanath, C.T., Rayner, E.T., Mann, G.E. and Dollear, F.G., 1968. Reduction of aflatoxin levels in cottonseed and peanut meals by ozonization. *Journal of the American Oil Chemists' Society*, 45(2), pp.93-95

García-Cela, M.E., Marín, S., Reyes, M., Sanchis, V. and 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. *Journal of the Science of Food and Agriculture*, 96(6), pp.2249-2256.

Heenan, C. N., Shaw, K. J., and Pitt, J. I. 1998. Ochratoxin A production by *Aspergillus carbonarius* and *A. niger* isolates and detection using coconut cream agar. *Journal of Food Mycology*, 1, 67-72.

Inan, F., Pala, M. and Doymaz, I., 2007. Use of ozone in detoxification of aflatoxin B 1 in red pepper. *Journal of Stored Products Research*, 43(4), pp.425-429.

Krause, C.R. and Weidensaul, T.C., 1978. Effects of ozone on the sporulation, germination and pathogenicity of *Botrytis cinerea*. *Phytopathology*, 68(2), pp.195-198.

Leroux, P., 2007. Chemical control of *Botrytis* and its resistance to chemical fungicides. In *Botrytis*: Biology, pathology and control (pp. 195-222). *Springer Netherlands*.

Maeba, H., Takamoto, Y., Kamimura, M.I. And Miura, T.O., 1988. Destruction and detoxification of aflatoxins with ozone. *Journal of Food Science*, 53(2), pp.667-668.

McKenzie, K.S., Sarr, A.B., Mayura, K., Bailey, R.H., Miller, D.R., Rogers, T.D., Norred, W.P., Voss, K.A., Plattner, R.D., Kubena, L.F. and Phillips, T.D., 1997. Oxidative degradation and detoxification of mycotoxins using a novel source of ozone. *Food and Chemical Toxicology*, 35(8), pp.807-820.

Miller, F.A., Silva, C.L. and Brandão, T.R., 2013. A review on ozone-based treatments for fruit and vegetables preservation. *Food Engineering Reviews*, 5(2), pp.77-106.

Palou, L., Smilanick, J.L., Crisosto, C.H. and Mansour, M., 2001. Effect of gaseous ozone exposure on the development of green and blue molds on cold stored citrus fruit. *Plant Disease*, 85(6), pp.632-638.

Pitt, J.I., 2000. Toxigenic fungi: which are important?. *Medical Mycology*, 38(sup1), pp.17-22.

Prakash, B., Kedia, A., Mishra, P.K. and Dubey, N.K., 2015. Plant essential oils as food preservatives to control moulds, mycotoxin contamination and oxidative deterioration of agri-food commodities–Potentials and challenges. *Food Control*, 47, pp.381-391.

Qi, L., Li, Y., Luo, X., Wang, R., Zheng, R., Wang, L., Li, Y., Yang, D., Fang, W. and Chen, Z., 2016. Detoxification of zearalenone and ochratoxin A by ozone and quality evaluation of ozonised corn. *Food Additives & Contaminants:* Part A, 33(11), pp.1700-1710.

Téren J, Varga J, Hamari Z, Rinyu E and Kevei F., 1996. Immunochemical detection of ochratoxin A in black *Aspergillus* strains. *Mycopathologia* 134: 171–176.

Chapter V: General discussion and conclusion

V.1 General discussion

It is known that, concerning grapes and grape-products, the highest OTA concentration and incidence rate is reported in countries originating from southern and warmer regions of Europe and northern Africa (Mediterranean region). In particular the proximity to the sea appears to have a significant role.

Results of the current study revealed that in Algeria the situation concerning OTA presence in raisins appears to be similar to other Mediterranean regions. Samples coming from region in Algeria near the sea side (Mostaganem), with Mediterranean weather, had a higher contamination with OTA compared to samples that came from other region in Algeria (Biskra) with semi-arid weather. In regions near to the sea significant temperature fluctuations and rainfall patterns, extreme fluxes in drought/flooding, determine stress conditions which may result in an increased mycotoxin production in the strains able of synthesizing these metabolites from genetic and metabolic point of view. In this sense, the prospect of climate change is of concern.

Regarding the black Aspergilli species capable of producing OTA isolated in this study, although the highest amount of (90%) of toxigenic isolates were reported among *Aspergillus carbonarius* strains with the isolate able to produce the highest OTA concentration (1,200ppb), a high OTA producer strains percentage (65%) was reported among *A. niger* isolates. Such a percentage is very high compared to literature.

Aspergillus section *flavi* and AfB1 contamination were also reported in raisin samples coming from Algeria and Turkey, but at very low incidence. The highest recorded AfB1 level was 147 ppb from one strain isolated from Turkish sample. While with *nor1* LAMP assay all samples were negative.

Concerning the study on dried figs, no black Aspergilli were isolated, while Analyses directly from the fruit revealed that OTA was present in most of the samples at very low level less than 0.6ppb.

For AfB1 on dried figs, in few samples low incidence of *Aspergillus flavus* and *Aspergillus parasiticus* were reported, that produced AfB1 with a level ranging from 0.8 to 55ppb when the analyses were performed from isolated strains. When analyses were carried out from the fruit, AfB1 was found only in two samples at a very low level: less

than 0.1ppb. While nor1 LAMP assay revealed a higher incidence of 60% of the samples containing AfB1 contamination.

In the experiment carried out to investigate the effect of ozone treatment to control OTA and AfB1 contamination. 0.3 ppm of O_3 treatment was effective to control conidia germination of *A. carbonarius* strain, after 12hours of treatment by exercising a fungi-static effect rather than a fungi-toxic effect, and this was in direct correlation with the duration of O_3 treatment. In the experiment of ozone treatment on mycotoxins, ozone reduced AfB1 by 97% and OTA by 76%. AfB1 was more sensitive to ozone compared to OTA, and this can be attributed to the difference between the chemical structure of the toxins. This interesting effect of ozone application on AfB1 and OTA molecules was similar to its effect when raisins spiked with AfB1 and OTA were treated. Therefore, the different compounds present in raisins didn't reduce the probability to have the contact between ozone and the toxins and was comparable to its effect on AfB1 and OTA molecules, where ozone was in direct contact with these molecules.

Regarding the time, 5 hours of ozone treatment could be enough to degrade or even to eliminate OTA and AfB1. Also, the two ozone concentration: 0.1 and 0.3ppm had a similar effect and no significant differences were found between them, while the most effective ozone concentration was 2ppm.

Concerning the study about the effect of the antifungal protein PgAFP and potassium sorbate (E-202) on the growth of *Aspergillus carbonarius*, biosynthetic and stress-gene expression and its OTA production at two activity water (aw) levels: 0.95 and 0.97 and on a synthetic medium that simulate raisin composition. Results showed that the protein PgAFP controls OTA production in a significant way. However, although E-202 can slow *Aspergillus carbonarius* growth but might be dangerous by the fact that it provoked a considerable increment of OTA production. especially at the highest a_w value tested

V.2 Conclusion

This is the first report on the occurrence of OTA and AfB1 in dried figs and raisins in Algeria. The main motivation for this part of the study was the detection of Aflatoxin and Ochratoxin A and their producing Aspergilli in raisins and dried figs. This issue is particularly important in Algeria, where limits on the maximum tolerable levels of OTA contamination have never been established. Several OTA and AfB1 contaminations were encountered in dried figs and in raisins, as well as their *Aspergillus* producing, that were

isolated and had the ability to synthesize these toxins. The results we found in some cases where the level of OTA and AfB1 was above the EU maximum tolerable level underline the need to pass legislation to regulate the amount of maximum tolerable limit of OTA content in raisins and dried fruits in Algeria. (chapter II)

The nor1 LAMP assay was set up using a simple protocol directly from raisins and dried figs for the direct use as template in the isothermal detection. LAMP assay, constitutes an easy, fast and user-friendly tool for surveillance of any aflatoxin contamination in food commodities, and therefore for quality control in the food industry (chapter II).

The antifungal protein PgAFP was used to control the development of *A. carbonarius* and OTA production in raisins. Although PgAFP inhibition of fungal development was lower than that of potassium sorbate (E-202), E-202 caused a significant increase in the production of OTA at the beginning of the stationary phase, whereas PgAFP maintained its control effect on OTA biosynthesis. Thus, the antifungal protein is a biological product that can control OTA production more durably than potassium sorbate (E-202) (Chapter III).

Ozone treatment is potentially a good means of control of *Aspergillus carbonarius*, which is the key for ochratoxin-producing species, as well as for the degradation of OTA and AfB1 in raisins (Chapter IV).