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TOXIGENIC PROFILE OF FUNGI AND MULTI MYCOTOXINS ANALYSIS AS SUPPORTING TOOLS FOR A RISK EVALUATION AND MYCOTOXINS MINIMIZATION/DEGRADATION

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Content

Publications and conference contributions

Papers in Journal Citation Report (JCR):

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Abstract

Mycotoxins are secondary metabolites produced by certain filamentous fungi, which can contaminate crop plants or stored food and feed; among them the most important mycotoxigenic fungi involved in food contamination, belong to three genera: *Aspergillus*, *Fusarium* and *Penicillium.* More than 300 mycotoxins have been identified and these secondary metabolites can be harmful to human and animal health when ingested (Bennett and Klich, 2003). Main mycotoxins contaminant in food and feed are: aflatoxins, ochratoxins, fumonisins, trichothecenes and zearalenone. Aflatoxins represent the most important class of mycotoxins, commonly found in maize and other cereals, the main fungi responsible for their production are *Aspergillus flavus* and *A. parasiticus* (Shepard, 2008). Instead grains, coffee, cocoa, wine, beer, and foods from animal origin are often contaminated by ochratoxin A, that is mainly produced by *A. ochraceus* (Van der Merwe *et al*., 1965), *A. carbonarius*, *Penicillium verrucosum* and *P. nordicum. Fusarium verticillioides* and *F. proliferatum* produce fumonisins, which are often detected in maize and by-products (Dutton, 1996). Trichothecenes, which are often found in cereal grains, in particular in wheat and maize, are divided in four groups, the principal two groups are: type-A with T2 and HT-2 toxin, produced by *F. langhsetiae* and *F. sporotrichioides* (Van der Fels-Klerk and Stratakou, 2010); Type-B with deoxynivalenol (DON) and nivalenol (NIV) produced by *F. graminearum* and *F. culmorum* (Placinta *et al*., 1999; Turner, 2010). Moreover, DON contamination is frequently found in association with another mycotoxin produced by the same fungi, zearalenone (ZEA) (Logrieco *et al*., 2002a). These species are responsible for infections occurring both in the field and during postharvest storage, particularly when cereals are stored under inappropriate conditions (e.g. high temperatures and high humidity).

A large variety of toxic effects in animals and humans has been observed due to the ingestion of food contaminated with mycotoxins, such as: immunosuppression, carcinogenic, genotoxic, teratogenic or mutagenic effects (Peraica *et al*., 1999; Richard, 2007). Mycotoxin contamination became a public health concern with serious economical and ethical implications. Since it is not completely possible to prevent the synthesis of mycotoxins, national and

international authorities have adopted regulatory limits and guidelines to monitor mycotoxin levels in various food and feed products (EC 2006a and 2006b; Commission Recommendation 2013/165/UE).

Different physical, chemical and biological methods have been recommended for detoxification of food and feed contaminated by mycotoxins. Nevertheless, only a few of them have been accepted for practical use. A lot of specialists think that the best approach for mycotoxin decontamination should be the biological degradation, giving the possibility to remove mycotoxins under mild conditions, without using harmful chemicals and without significant losses in nutritive value and palatability of detoxified food or feed.

Depending on their mode of action, these feed additives may act either by binding mycotoxins to their surface (adsorption), or by degrading or transforming them into less toxic metabolites (biotransformation). The binder efficacy of these substances is based on the properties of both the binder and the mycotoxin. Biotransformation can be achieved by mycotoxin-degrading enzymes or by microorganisms (fungi and bacteria) producing such enzymes.

Various inorganic adsorbents, aluminosilicate and activated carbons, have been tested and used as mycotoxins binders (MB). An interesting alternative to inorganic adsorbents for the detoxification of mycotoxins is the use of organic binders, such as, cell wall components of yeast, lactic acid bacteria, conidia of *Aspergilli*. These MB are used to feed animal diet in order to reduce the absorption of mycotoxins from the gastrointestinal tract and their distribution to blood and target organs, thus preventing or reducing mycotoxicosis in livestock. Recently, the use of such substances as technological feed additives has been officially allowed in the European Union (Commission Regulation 2015/786).

Ligninolytic enzymes, such as laccase, from white-rot fungi, as *Pleurotus spp*. catalyzed the oxidation of a broad number of phenolic compounds and aromatic amines by using molecular oxygen as the electron acceptor, which is then reduced to water (Reinhammar and Malstrom, 1981). Adding the appropriate redox mediator to the reaction can extend the activity of the laccase enzymes to non-phenolic substrates, such as mycotoxins.

This PhD thesis is organized into six chapters and one annex, where the following tasks are described.

In Chapter 1, one hundred and seventy-five wheat samples were collected during the growing seasons: 2013-2014, 2014-2015 and 2015-2016 in different Italian regions. Trichothecenes (DON, NIV, HT-2 and T2 toxins) and ZEA levels were

monitored through the use of validated analytical methods, to provide an overview of the Italian distribution of mycotoxins in wheat. The *Fusarium* species isolated from the kernels were identified, based on their morphological characteristics.

In Chapter 2, the development of an innovative technology for the bioremediation of AfB1-contaminated maize and its bioconversion into high nutritional feed, was realized through the exploitation of the degradative capability of *Pleurotus eryngii*. For this purpose, the AfB1–degradative activity of a crude enzymatic extract from a spent substrate and the ability of the white-rot and edible fungus *P. eryngii* to degrade AfB1 both *in vitro* and in a laboratory-scale mushroom cultivation, were investigated.

In Chapter 3, the power of ground not-viable mycelium of *P. eryngii* (ITEM 13681) to absorb AfB1, was assessed. The influence of different parameters: pH (5, 7), AfB1 concentrations (50 and 1000 ng/mL), time (30 and 120 min), temperature (25 and 37°C), fungal mass (50 and 1000 mg), on the absorption capability of the mycelium of *P. eryngii*. were evaluated. Binding stability of AfB1-biosorbent and desorption studies were carried out varying, respectively, the pH to 7 and 3, for 24 hours of incubation at room temperature in the dark.

In Chapter 4, the degradation activity of two laccases from two edible fungi *(P. eryngii* and *P. pulmonarius*) towards AfB1, AfM1, FB1, ZEA and T2 toxin, were evaluated separately, adding to the reaction natural and artificial mediators. The effect of laccase-mediator systems (LMSs) were analyzed by liquid chromatography with specific detector, based on the chemical feature of each single toxin.

In Chapter 5, the aim pursued was to investigate the action of LMSs toward multiple toxins. For this purpose, several degradation assays were performed, screening the effect of different mediators, as acetosyringone (AS), syringaldehyde (SA), and synthetic mediator as 2,2,6,6-tetramethyl-piperidinyl-oxyl (TEMPO), on the activity of laccase from *Trametes versicolor* (EC 1.10.3.2) towards fusaric acid (FA) and mycotoxins, such as: DON, T2, FB_1, AfB1, OTA and ZEA. A multi mycotoxin method, was set up to simultaneously screen these seven toxins, by liquid chromatography/tandem mass spectrometry (LC-MS/MS).

In Chapter 6, the biodegrading activity of laccases enzymes towards ZEA has been further investigated. The degradation products were monitored by liquid chromatography–high resolution mass spectrometry (LC-HRMS). Data were

processed by MassHunter Workstation Software (Qualitative Analysis Navigator and Qualitative Analysis Workflow, version B.08.00), Mass Profile Professional (version 14.08) and MassHunter Molecular Structure Correlator (version B.08.00)) from Agilent Technologies, to allow their identification.
In Annex A, the Enniatins (A, A1, B and B1) and Beauvericin production from various *Fusarium spp.* were measured by ultra-performance liquid chromatography coupled with photodiode array and single quadrupole mass spectrometer (UPLC-PDA-QDa).

Key words: mycotoxins, fungi, biodegradation, biosorption.

Riassunto

Le micotossine sono metaboliti secondari prodotti da alcuni funghi filamentosi, che possono contaminare piante coltivate o alimenti e mangimi immagazzinati; tra questi i più importanti funghi micotossigeni coinvolti nella contaminazione alimentare, appartengono a tre generi: *Aspergillus*, *Fusarium* e *Penicillium*. Più di 300 micotossine sono state identificate e questi metaboliti secondari possono essere dannosi per la salute umana ed animale quando ingeriti (Bennett and Klich, 2003). Le principali micotossine contaminati di alimenti e mangimi sono: aflatossine, ocratossina, fumonisine, tricoteceni e zearalenone. Le aflatossine rappresentano la classe più importante di micotossine, comunemente presenti nel mais e in altri cereali, i principali funghi responsabili della loro produzione sono *Aspergillus flavus* e *A. parasiticus* (Shepard, 2008). Invece i cereali, il caffè, il cacao, il vino, la birra e gli alimenti di origine animale sono spesso contaminati dall'ocratossina A, prodotta principalmente da *A. ochraceus* (Van der Merwe *et al*., 1965), *A. carbonarius*, *Penicillium verrucosum* e *P. nordicum*. *Fusarium verticillioides* e *F. proliferatum* producono fumonisine, che sono spesso rilevate nel mais e nei sottoprodotti (Dutton, 1996). I trichoteceni, che si trovano spesso nei cereali, in particolare nel frumento e nel mais, sono divisi in quattro gruppi, i due principali sono: il tipo A con le tossine T2 e HT-2, prodotte da *F. langhsetiae* e *F. sporotrichioides* (Van der Fels-Klerk e Stratakou, 2010); il tipo B con il deossinivalenolo (DON) e il nivalenolo (NIV) prodotti da *F. graminearum* e *F. culmorum* (Placinta *et al*., 1999; Turner, 2010). Inoltre, la contaminazione da DON si trova frequentemente in associazione con un'altra micotossina prodotta dagli stessi funghi, lo zearalenone (ZEA) (Logrieco *et al*., 2002a). Queste specie sono responsabili di infezioni che si verificano sia nel campo che durante la conservazione post-raccolta, in particolare quando i cereali sono immagazzinati in condizioni inadeguate (ad esempio alte temperature e alta umidità).
Una grande varietà di effetti tossici negli animali e nell'uomo è stata osservata a causa dell'ingestione di cibo contaminato da micotossine, quali: immunosoppressione, effetti cancerogeni, genotossici, teratogeni o mutageni (Peraica *et al*., 1999; Richard, 2007). La contaminazione da micotossine è diventata una preoccupazione per la salute pubblica con gravi implicazioni economiche ed etiche. Dal momento che non è completamente possibile impedire

la produzione di micotossine, le autorità nazionali e internazionali hanno adottato limiti regolatori e linee guida per monitorare i livelli di micotossine in vari prodotti alimentari e nei mangimi (EC 2006a e 2006b; Commision Recommendation 2013/165/UE).

Diversi metodi fisici, chimici e biologici sono stati raccomandati per la detossificazione di alimenti e mangimi contaminati da micotossine. Tuttavia, solo alcuni di essi sono stati accettati per l'uso pratico. Molti specialisti pensano che l'approccio migliore per la decontaminazione da micotossine debba essere la degradazione biologica, dando la possibilità di rimuovere micotossine in blande condizioni, senza usare sostanze chimiche dannose e senza perdite significative nel valore nutritivo e nell'appetibilità di alimenti o mangimi detossificati.

A seconda della loro modalità di azione, questi additivi per mangimi possono agire legando micotossine alla loro superficie (adsorbimento) o degradandoli o trasformandoli in metaboliti meno tossici (biotrasformazione). L'efficacia del legante di queste sostanze si basa sulle proprietà sia del legante che della micotossina. La biotrasformazione può essere ottenuta da enzimi che degradano le micotossine o da microrganismi (funghi e batteri) che producono tali enzimi.

Vari adsorbenti inorganici, alluminosilicati e carboni attivi, sono stati testati e utilizzati come leganti micotossine (MB). Un'alternativa interessante agli adsorbenti inorganici per la detossificazione delle micotossine è l'uso di leganti organici, come i componenti della parete cellulare del lievito, i batteri dell'acido lattico, i conidi degli Aspergilli. Questi MB sono utilizzati l'alimentazione animale al fine di ridurre l'assorbimento delle micotossine dal tratto gastrointestinale e la loro distribuzione al sangue e agli organi bersaglio, prevenendo o riducendo le micotossicosi nel bestiame. Recentemente, l'uso di tali sostanze come additivi per mangimi tecnologici è stato ufficialmente autorizzato dall'Unione europea (Commission Regulation 2015/786).

Enzimi ligninolitici, come le laccasi, dai funghi del marciume bianco, come *Pleurotus spp.* che catalizzano l'ossidazione di un ampio spettro di composti fenolici e di ammine aromatiche utilizzando l'ossigeno molecolare come accettore di elettroni, che viene quindi ridotto ad acqua (Reinhammar e Malstrom, 1981). Aggiungendo alla reazione l'appropriato mediatore redox può estendere l'attività degli enzimi laccasi a substrati non fenolici, come le micotossine.

Questa tesi di dottorato è organizzata in sei capitoli e un allegato, in cui sono descritte le seguenti attività.

Nel Capitolo 1 sono stati raccolti centosettantacinque campioni di grano durante le stagioni agricole: 2013-2014, 2014-2015 e 2015-2016 in diverse regioni italiane. I tricoteceni (DON, NIV, tossine T2 e HT-2) e i livelli di ZEA sono stati monitorati attraverso l'uso di metodi analitici validati, per fornire una visione d'insieme della distribuzione italiana delle micotossine nel grano. Le specie di *Fusarium* isolate dai semi sono state identificate in base alle loro caratteristiche morfologiche.

Nel Capitolo 2, lo sviluppo di una tecnologia innovativa per la *bioremedation* del mais contaminato dall'AfB1 e della sua bioconversione in mangime ad un alto apporto nutrizionale, è stato realizzato attraverso lo sfruttamento della capacità degradativa del *Pleurotus eryngii*. A tale scopo, è stata studiata l'attività degradativa dei confronti dell'AfB1 da parte di un estratto enzimatico grezzo ottenuto da un substrato esausto e la capacità del fungo commestibile *P. eryngii* di degradare l'AfB1 sia *in vitro* che in una coltivazione dei funghi su scala di laboratorio.

Nel Capitolo 3, è stata valutata la capacità del micelio non vitale del *P. eryngii* (ITEM 13681) di assorbire l'AfB1. Sono stati valutati l'influenza di diversi parametri, quali: pH (5, 7), concentrazioni dell'AfB1 (da 50 a 1000 ng/mL), tempo (da 30 a 120 min), temperatura (25, 37 ° C), massa fungina (da 50 a 1000 mg), sulla capacità di assorbimento del micelio di *P. eryngii*. La stabilità del legame AfB1-bioassorbente e gli studi di desorbimento sono stati effettuati variando, rispettivamente, il pH a 7 e 3, per 24 ore di incubazione a temperatura ambiente e al buio.

Nel capitolo 4, l'attività di degradazione di due laccasi ottenute da due funghi commestibili (*P. eryngii* e *P. pulmonarius*) nei confronti di AfB1, AfM1, FB1, ZEA e tossina T2, sono state valutate singolarmente, aggiungendo alla reazione mediatori naturali ed artificiali. L'effetto dei sistemi laccasi-mediatore (LMSs) è stato analizzato mediante cromatografia liquida ad elevata prestazione accoppiata ad uno specifico rivelatore, scelto in base alle caratteristiche chimiche di ciascuna tossina.

Nel capitolo 5, l'obiettivo perseguito era quello di investigare l'azione dei sistemi laccase-mediatore (LMSS) verso più tossine, simultaneamente. A tal fine, sono stati eseguiti diversi test di degradazione, esaminando l'effetto dei diversi mediatori, come acetosyringone (AS), syringaldehyde (SA) e del mediatore sintetico 2,2,6,6-tetrametil-piperidinil-ossil (TEMPO), sull'attività della laccasi da *Trametes versicolor* (CE 1.10.3.2) verso l'acido fusarico (FA) e micotossine,

come: DON, T2, FB1, AfB1, OTA e ZEA. Un metodo multi micotossina, è stato sviluppato per determinare simultaneamente queste sette tossine, mediante cromatografia liquida con spettrometria di massa in tandem (LC-MS/MS).
Nel capitolo 6, l'attività di degradazione dell'enzima laccasi verso lo ZEA è stata ulteriormente investigata. I prodotti della biodegradazione sono stati monitorati mediante cromatografia liquida con spettrometria di massa ad elevata risoluzione (LC-HRMS). I dati sono stati elaborati dal software MassHunter Workstation (Qualitative Analysis Navigator e Workflow di analisi qualitativa, versione B.08.00), Mass Profile Professional (versione 14.08) e MassHunter Molecular Structure Correlator (versione B.08.00)) di Agilent Technologies, per consentirne l'identificazione.
Nell'Annesso A, la produzione di Enniatine (A, A1, B e B1) e Beauvericina da varie specie di *Fusarium* è stata valutata mediante cromatografia liquida ad alte prestazioni accoppiata con array di fotodiodi e spettrometro di massa a singolo quadrupolo (UPLC-PDA-QDa).

Parole chiave: micotossine, funghi, biodegradazione, bioassorbimento.

Aims of the thesis

Every year, worldwide, crop diseases caused by fungal infection lead to yield losses and poor food quality. Tons of highly valuable food and feed are wasted because of mycotoxin contamination that exceed the regulated limits. There is no sense in talking about food nutritional value, sensory or functional properties, if the food product itself it is not safe enough for human and animal consumption.
Research efforts to find out mitigation actions aimed to counteract mycotoxicological risk, progressively increased during the last decades.
In this scenario is placed my doctoral research, funded by the European Project called MycoKey "*Integrated and innovative key action for mycotoxin management in the food and feed chain"* Horizon 2020.
Evaluate mycotoxins production in food commodities is the first approach to have a view of the contamination rates. The levels vary according to the geographical areas and year of sampling, for each mycotoxin. For this reason, monitoring *Fusarium* mycotoxins and related species from durum wheat, collected in Italy, was been done during three growing seasons.
Based on presence and contamination of each mycotoxins in each foodstuff and/or feedstuff, is important to develop the best strategies, aimed to guarantee consumer's health. Different approach to reduce the contamination levels of mycotoxins in agricultural commodities were pursued.
The development of novel binding and/or degrading methods for decontamination and detoxification of agricultural commodities, could reduce food and feed waste, permitting benefits in terms of improved food and feed safety, consequently, with social and economic advantages.
The most promising strategies actually employed regard the use of biosorbent and biodegrading agents. The formers are capable to adsorb mycotoxins and other molecules at different levels, while the latter could decontaminate mycotoxins. The most promising biosorbents are derived from not viable microorganisms' cell wall, instead of using inorganic binders that could have some drawbacks, such as the negative impact on the nutritional quality. For these reasons, the absorption capability of ground not-viable mycelium of the fungus *P. eryngii* (ITEM 13681) towards mycotoxins was explored.
Regarding biodegrading agents, living microorganisms that demonstrate the mycotoxin detoxification activities, and the enzymes that catalyse the

biodegrading reactions are the continuously developed approaches, with the aim to replace the use of harmful chemicals. Moreover, these biological detoxification systems do not require additional, time consuming and expensive treatments, instead of the chemical detoxification methods.

The degradation capabilities of the edible fungus *P. eryngii*, during the growth of its basidiocarp, was investigated.

Ligninolytic enzymes extracted from spent mushroom substrates (*P. eryngii*), and different laccase enzymes extracted from *P. pulmonarius* (Lac2), purified from *P. eryngii* (Ery4) or commercial laccase purified from *Trametes versicolor*, were evaluated for their biodegrading activities towards main mycotoxins.

All these studies could bring newly and innovative technologies, to counteract the mycotoxins risk, into the feed chain.

General Introduction

Mycotoxin contamination is the result of the interaction between the toxigenic fungi and the agro-food matrix, depending on the suitable environmental conditions (Pitt, 2008). Physical factors such as humidity, temperature, physical integrity of the cereals or plant tissues; chemical factors, such as substrate composition and environmental factors like geographic area, processing and storing periods of the crops influence the growth of toxigenic fungi and mycotoxin accumulation (Magan and Olsen, 2004). Often times most factors are beyond human control (Hussein and Brasel, 2001).

The most relevant toxigenic fungi species belong to the genera *Aspergillus, Penicillium* and *Fusarium.* Some fungi are able to produce more than one mycotoxin and some mycotoxins are produced by more than one fungi species, and thus several mycotoxins are often simultaneously found in a single commodity.

Mycotoxins are produced as secondary metabolites by filamentous fungi, they are low-molecular-weight natural compound (i.e., small molecules). Although there are more than 300-400 mycotoxins known today (depending on classification; reviewed by Hussein and Brasel, 2001, or Bennet and Klich, 2003 for example), only a handful have received widespread attention.

The most important mycotoxins or mycotoxin groups are aflatoxins (AFs), ochratoxins (OTA), fumonisins (FBs) trichothecenes (DON, NIV, T2 and HT2) and zearalenone (ZEA) and among these aflatoxins are the most intensively studied (Binder, 2007) (Table I). Mycotoxins can enter the food chain directly via plant products such as cereal grains, coffee, oil seeds, spices, fruit juices and beverages (wine and beer), and indirectly from animal diets (pastures, feeds) contaminated with mycotoxins, which can leave residues in milk, meat, and derived products.

Direct economic losses are due to the presence of mycotoxins: human and animal exposure increase the costs of health care and veterinary care; production of cereals and livestock could be reduced, extra handling and processing became necessary steps; nutritional value of food and feed were affected; safely disposal of contaminated food and feed is costly and investments in research and applications to reduce mycotoxin problem were recommendable (Lee and Ryu, 2017).

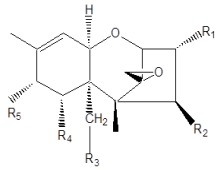
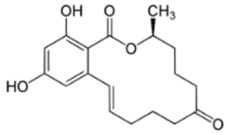
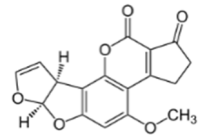
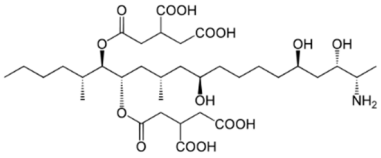
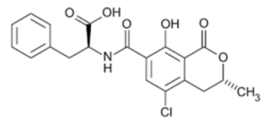
Type-A trichothecenes	Type-B trichothecenes
	
HT-2 toxin: R_1=R_2=OH, R_3=OAc, R_4=$OCOCH_2(CH_3)_2$	**Deoxynivalenol:** R_1=R_3=OH, R_2=H
T2 toxin: R_1=OH, R_2=R_3=OAc, R_4=$OCOCH_2(CH_3)_2$	**Nivalenol:** R_1= R2=R3=OH
Main producer: *F. sporotrichioides*, *F. poae*, *F. langsethiae*, *F. acuminatum*, *F. equiseti***.**	**Main producer***: F. graminearum, F. culmorum***.**
Main crops affected: maize, wheat, oats.	**Main crops affected:** maize, wheat.
Zearalenone	**Aflatoxin B_1**
	
Main producer: *F. graminearum, F. culmorum.*	**Main producer:** *A. flavus, A. parasiticus.*
Main crops affected: maize, wheat, barley, rye.	**Main crops affected:** maize, peanuts, tree nuts, rice, figs
Fumonisin B_1	**Ochratoxin A**
	
Main producer: *F. verticillioides, F. proliferatum.*	**Main producer:** *A. ochraceous, A. carbonarius, A. niger, Penicillium verrucosum.*
Main crops affected: maize, sorghum.	**Main crops affected:** cereals, coffee, cocoa, dried vine fruit, wine

Table I Overview of the main mycotoxins in foods, main producing fungal species and most susceptible crops of contamination.

These toxins account for millions of dollars annually in losses worldwide in human health, animal health, and condemned agricultural products. Factors contributing to the presence or production of mycotoxins in foods or feeds include, environmental, ecological conditions and storage.

In order to protect the health of consumers, many countries have established maximum levels and/or guidance values to regulate the presence of mycotoxins in food and feed. In Europe, harmonized maximum levels for mycotoxins (AFs, OTA, DON, FB and ZEA) in foodstuffs have been specified in the Commission Regulation 2006/1881/EC (EC, 2006a) that has been further amended. Very recently, the Recommendation 2013/165/EC has been issued setting maximum recommended levels for the total amount of T-2 and HT-2 toxins in cereals and cereal derived products (EC, 2013). In feedstuffs, maximum permitted levels of AfB1 was set in European Directive 2002/32 (EC, 2002 and 2003), while for DON, ZEA, OTA and FBs in European Commission 2006/576/EU (EC, 2006b). Conversely no guidelines or regulatory limits have been set for NIV, although this mycotoxin frequently contaminates cereals and commonly co-occurs with DON (Placinta, *et al* 1999).

Different physical, chemical and biological methods have been recommended for detoxification of mycotoxin-contaminated food and feed products Nevertheless, only a few of them have been accepted for practical use in animal feed.

I Main mycotoxins contaminant in food and feed

I.I Aflatoxins

The name aflatoxin has been derived from the combination of "a" for the *Aspergillus* genus and "fla" for the *flavus* species, and "toxin" meaning poison (Ellis *et al*., 1991).

Aflatoxins are difuranocoumarin derivatives produced by a polyketide pathway, highly toxic, mutagenic, teratogenic, and carcinogenic compounds that are produced as secondary metabolites by fungi belonging to several *Aspergillus* species, mainly *A. flavus* and *A. parasiticus* (Groopman *et al*., 1988; Massey *et al*., 1995; Romagnoli *et al*., 2007). Aflatoxins have a high presence in tropical and subtropical regions where humidity and temperature conditions are optimal for toxin production. Until now, nearly 18 different types of aflatoxins have been identified where in the major ones include aflatoxin B1, B2, G1, G2, and M1. Fungal species belonging to *Aspergillus flavus* typically produce AfB1 and AfB2,

whereas *A. parasiticus* produces AfG1 and AFG2 as well as AfB1 and AfB2. The 4 major aflatoxins (aflatoxin B1, B2, G1, and G2) are based on their fluorescence under blue or green light and their relative mobility during separation by thin-layer chromatography (TLC) (Stroka and Anklam 2000; Bennett and Klich 2003). In dairy cattle, another problem arises from the transformation of AfB1 and AfB2 into hydroxylated metabolites, aflatoxin M1 and M2 (AfM1 and AfM2), which are found in milk and milk products obtained from livestock that have ingested contaminated feed (Boudra *et al*., 2007).

These are unavoidable contaminants found in a variety of foods and feeds (CAST, 2003). Among these toxins, AfB1 is the most predominant and the most potent hepatocarcinogen (Busby and Wogan, 1984) and has been classified as a Group 1, known human carcinogen (IARC, 1993). AfB1 is also an immune suppressor (Bondy and Pestka, 2000; Jiang *et al*., 2005), inflammation promoter (Qian *et al*., 2013) and growth suppressor in animals and humans (Harvey *et al*., 1995; Marin *et al*., 2002).

I.II Fumonisins

Fumonisins are also known to be produced by *F. proliferatum* and other related species, especially on maize that has been previously infected during its pre-harvest stages. Reports are available on the presence of fumonisins in several agricultural products like corn, corn flour, dried milled maize fractions, dried figs, herbal tea, medicinal plants, bovine milk, and others (Omurtag and Yazicioglu 2004; Gazzotti *et al*.; 2009; Karbancioglu-Guler and Heperkan 2009; Pietri *et al*., 2009; Seo *et al*., 2009), indicating high risks to public health.

Seven different types of fumonisins (FA1, FA2, FB1, FB2, FB3, FB4 and FB6) have been reported, where in the “A” series is the amides and the “B” series possesses a free amine (Gelderblom *et al*., 1992). Even FC1 has also been reported in the “C” series.

Fumonisins (FBs) consist of similar analogs composed of a carbon-chain backbone with two tricarboxylic acid groups esterified at the C14 and C15 position (Gelderblom *et al*., 1988). The major producers, *F. verticilliodies* and *F. proliferatum* fungi, frequently contaminate maize and other cereal grains (Marasas, 1995) growing best at high temperatures in humid climates. Due the similarities in favorable fungal growth conditions, fumonisins often co-occur with AFs especially in corn (Kpodo *et al*., 2000; Kimanya *et al*., 2008; Sun *et al*., 2011). Fumonisin B1 (FB1), the most predominant and well-studied isoform, is

nephrotoxic and hepatotoxic in several species and has been classified as a Group 2B, possible human carcinogen (IARC, 2002). FB1 exposure has been associated with liver and esophageal cancers in high-risk populations (Alizadeh *et al*., 2012; Chu and Li, 1994). This toxin has also been reported to be immunosuppressive (WHO 2002). The International Agency for Research on Cancer (IARC, 1993) has classified fumonisins under group 2B (possibly carcinogenic to humans).

I.III Ochratoxins

The ochratoxins are a group of related pentaketide metabolites, comprised of a dihydroisocoumarin bonded to phenylalanine (Pohland *et al*., 1992). Ochratoxin A (OTA), is mainly produced by *Aspergillus ochraceus* (Van der Merwe *et al*., 1965) and *Penicillium verrucosum*, however other species of *Aspergillus* and *Penicillium* have been shown to produce the naturally-occurring ochratoxins A, B and C (Bennet and Klich, 2003). Ochratoxins have been isolated from foods all over the world, in both warm and cool climates, and are common contaminants of grains, coffee, cocoa, wine, beer, and foods from animal origin, particularly pork (Bayman and Baker, 2006). OTA is a potent nephrotoxin and based on animal evidence has been classified as a Group 2B, possible human carcinogen (IARC, 1993). It has been implicated in several nephropathies, including endemic porcine nephropathy (Jorgensen and Petersen, 2002), Balkan endemic nephropathy (BEN) (Krogh *et al*., 1977; Pfohl-Leszkowicz and Manderville, 2007) and urothelial tumors (Castegnaro *et al*., 1990). OTA has also been shown to be hepatotoxic, teratogenic, immunotoxic, and carcinogenic in experimental models (O'Brien and Dietrich, 2005). OTA is deemed to be nephrotoxic, immuno-suppressive, carcinogenic, and teratogenic.

I.IV Trichothecenes

Trichothecenes (TCT) are toxic metabolites majorly produced by *Fusarium spp*. (Cole *et al*., 2003). These toxins can be classified based on the substitution pattern of the tricyclic 12,13-epoxytri-chothec-9-ene (EPT), resulting in 4 groups, A, B, C and D. The EPT structure is shared by all trichothecenes and is considered essential for toxicity. Trichothecenes cause protein synthesis inhibition by affecting the 60S subunit of the ribosome interfering with the peptidyl transferase activity (Cundliffe *et al*., 1974) and are known to cause neurotoxicity, immunosuppression and renal toxicity (Richard, 2007).

Trichothecenes have strong impacts on the health of animals and humans due to their immunosuppressive effects. Group-A TCT are of major concern as they are more toxic than the type B TCT. Examples of type A TCT include T-2 and HT-2 toxin, while DON and NIV are some of the common naturally occurring type B TCT. Generally, TCT are found to accumulate in kernels of spikelets infected during the flowering period. The contamination is often associated with *Fusarium* head blight (FHB) of cereals, a disease rendering the grain unsuitable for human and animal consumption and responsible of a high economic loss, especially in wheat.

T-2 toxin is readily metabolized by the gut microflora of mammals into several other metabolites. HT-2 toxin is a primary metabolite in the gut and is absorbed into the blood after ingestion of T-2 toxin. *F. langsethiae* is the main T-2 and HT2 producer, but other species like *F. poae* and *F. sporotrichioides* can also synthesize them. The principal effects of perturbed protein synthesis from T-2 toxin are usually observed in the immune system.

Deoxynivalenol (DON) is the most important among the type B trichothecenes due to its natural occurrence in high levels. The fungal species responsible for DON contamination are *F. graminearum*, *F. culmorum*, *F. cerealis* (*F. croockwellense*), *F. sporotrichioides*, *F. poae*, *F. tricinctum*, and *F. acuminatum.* It is non-classifiable as carcinogen to humans (IARC, 1993), however it can cause deleterious health effects like anorexia, weight loss, malnutrition, endocrine dysfunction and immune alterations (Pestka, 2010). The gastrointestinal system is the target organ of this toxin (Alassane-Kpembi, *et al* 2015). Toxic symptoms of DON are food refusal, vomiting, and digestive disorders with losses of weight gain. Because of its effects in humans along with its resistance to food processing great efforts to control its presence in food have been done (Hassan, *et al* 2018).

Another type B trichothecenes is nivalenol (NIV), produced by *F. cerealis* (*F. croockwellense*), *F. poae, F. nivale, F. culmorum, and F. graminearum*. NIV toxic effects include bone marrow toxicity, erythropenia, leucopenia, hemorrhage, toxicity to lymphoid organs, diarrhea, and damage to the epithelial membranes of the intestine, the thymus and testis. NIV has often been reported in maize red ear rot throughout the European maize growing areas. It is a typical metabolite after dry and hot summers when harvest is performed earlier than usual (Nesic, *et al* 2014).

I.V Zearalenone

Zearalenone (ZEA) is an estrogenic resorcylic acid lactone mycotoxin produced by several species of *Fusarium.* Among the cereals in which ZEA can occur, maize has been shown to have the highest contamination levels (Goertz *et al.*, 2010; Marques *et al.*, 2008; SCOOP, 2003). The growth of ZEA producing fungi mainly occurs in temperate conditions and high levels of ZEA in cereals are mainly associated with wet temperate weather and improper storage in high moisture environments. Exposure to this mycotoxin has been linked to some cases of precocious puberty in girls (Massart *et al.*, 2008) likely due to the estrogenic activity exerted by ZEA and its metabolites upon interaction with the 17-b-estradiol receptors. Besides estrogenic effects, ZEA can also cause toxicity by production of reactive oxygen species (ROS) (El Golli Bennour *et al.*, 2009). Although this mycotoxin is also non classifiable as carcinogen to humans (IARC, 1993) is still a target of attention due to estrogenic activity along with anabolic effects.

II Strategies to reduce mycotoxins exposure

Methods of mycotoxin control can be classified into two categories:
- Prevention of fungi contamination and growth,
- Detoxification of contaminated products.

The prevention of fungi growth can be achieved either through pre- or post-harvesting strategies. Pre-harvest prevention strategies, such as good agricultural and manufacturing practices, (GAP and GMP) along with an effective Hazard Analysis and Critical Control Point (HACCP) approach, use of fertilizers, pest control and fungal-resistant crops and biocontrol agents are the best way to prevent mycotoxin formation at the field level.

The applied mycotoxins reduction procedure must effectively inactivate or remove the toxin, maintaining at the same time both nutritional and technological properties of the product and not generating reactive toxic products (López-García and Park, 1998; Pleadin *et al.*, 2014b). Prevention of mycotoxin contamination is not always possible; hence, many reduction or detoxification methods have been developed.

Methods of reduction can be divided into chemical, physical and biological. It must be underlined that EU regulation does not authorize any detoxification methods for those commodities, but only application is permitted in feed.

Commission Regulation (EU) 2015/786, May 19th, 2015 defined the criteria for detoxification processes that could be applied to products intended for animal feed (CR, 2015).

II.I Chemical agents

Numerous chemical agents are suitable for mycotoxin control involving bases (ammonia, hydrated oxide), oxidizing agents (hydrogen peroxide, ozone) organic acids (formic, propionic acid) and other agents.

Ammonization of grains not only reduces several mycotoxins (aflatoxins, fumonisins, OTA) to undetectable levels but also inhibits mycotoxigenic fungal growth. Recently, a mixture of glycerol and calcium hydroxide was shown to have a powerful detoxification effect for mycotoxins (Venter, 2014).

Among oxidizing agents, the ozone is an effective detoxification method, approved for use in food processing (FR, 2003). The ability of ozone to degrade aflatoxins was demonstrated (Agriopoulou *et al.*, 2016). A recent study indicated that, ozone concentration and exposure time influenced positively the reductions of DON (Piemontese *et al.,* 2018), aflatoxins and total fungal count (Trombete *et al*., 2017).

Several organic acids were reported to degrade OTA (Quintela, *et al* 2012).

The use of chemicals to inactivate or remove mycotoxins is almost impractical for food field, because of the toxic residues that alter the nutritional, sensory and functional properties of the foodstuffs. They are therefore considered as unsafe for humans. For these reasons, the chemical methods are mostly used for mycotoxins reduction in animal feed (Rustom, 1997). Chemical decontamination methods have already been accepted for use in industry; more novel detoxification methods must be developed and investigated for use in agricultural products while considering public concerns about animal feed and human food.

II.II Physical methods

Mycotoxin decontamination by physical methods includes various procedures such as sorting and separation, milling, immersing and washing, temperature, irradiation and adsorption.

Originally, at the end of the nineteenth century, sorting machines were based on particle weight and size (Mayer, 1898), then, since the 1960s till nowadays, optical sorting was established. An array of optical sensors detect grain differing in color and a jet of pressurized air removes the kernel from the stream (Fraenkel,

1962). Contemporary grain sorters have a throughput of dozens of tons grain per hour.

Through milling, small-grain cereals are processed in different fractions, high mycotoxin levels are found in bran, such as DON, while finished flour is contaminated to a much lower degree (Cheli *et al.,* 2013; Tibola *et al*., 2015). The efficiency of milling as a mycotoxin mitigation strategy is limited to commodity in which mycotoxins are enriched in fractions that can be removed from processing.

Immersing and washing technique is based on the lower density properties of contaminated grains. After immersion of grains in water, the floating contaminated fractions are easily discarded, thus, an amount of mycotoxins are removed.

The time/temperature combination undoubtedly remains one of the most important interventions by which industrial processing can affect the mycotoxin content in a finished food product. Most mycotoxins are chemically and thermally stable though. While conventional food preparation with temperatures up to 100 °C have little effect on most mycotoxins, higher temperatures used in frying, roasting, toasting, and extrusion might reduce mycotoxin contamination.

Irradiation may be an approach for removing mycotoxins on an industrial scale, providing in fact energy to both food constituents and contaminants: reactions occur and change the molecular structure of food constituents. Non-ionizing (solar, UV, microwave) and ionizing (gamma) radiations can reduce or eliminate pathogenic microorganisms, but partly also mycotoxins.

In the last several decades, various binders of different origins have been investigated for their efficacy and capacity to adsorb mycotoxins. The goal of adding adsorption agents (binders) to the feed of livestock and poultry is to alleviate harmful effects of mycotoxins by preventing their passage into the animal's blood and organs via a complex formation between the binders and the mycotoxins. A desirable mycotoxin adsorbent should possess the following properties: (a) binding ability against either a wide range of mycotoxins, especially mycotoxins with low hydrophobicity, or specific mycotoxins with higher efficacy;(b) high adsorption capacity in order to detoxify high load of contaminating mycotoxins; (c) reduced non-specific binding to nutrients such as minerals, vitamins, and amino acids in feeds; and (d) offer similar characteristics to feed compositions(e.g. vegetable fibers) to provide better tolerance in animals.

Simultaneously, the efficacy of developed binders shown *in vitro* studies must be verified through *in vivo* animal trials.

The first generation of binders, so called mineral adsorbents or inorganic adsorbents are mainly: aluminosilicate, (bentonite, montmorillonite, smectites, kaolinites, illites, zeolites); activated charcoal and hydrated sodium aluminosilicates (HSCAS).

Bentonite clay can bind and remove aflatoxin B1 from aqueous environments. In addition, it has been reported that 79% of aflatoxin M1 was removed from milk by adding bentonite clay (Magnoli *et al*., 2011).

Activated charcoal is used as a binder due to its porous structure with a high surface to mass ratio that allow to have an excellent adsorption capability in aqueous environments (Papaioannou *et al*., 2005; Jaynes *et al*., 2007; Dixon *et al*., 2008; Liu *et al*., 2011; Magnoli *et al*., 2011; Wan *et al*., 2013). Several studies have reported mycotoxins adsorption with activated charcoal, and it has been demonstrated to reduce aflatoxin residues, ZEA, DON and NIV due to its porous structure (Avantaggiato *et al*., 2003, 2004; Liu *et al*., 2011).

Additionally, a mixture of different adsorbents such as Q/FIS allies charcoal and HSCAS has the ability to adsorb various mycotoxins at the same time (Avantaggiato *et al*., 2007).

The binding efficacy of mineral adsorbents is related to the structures of both the binders and the mycotoxins. The charge distribution, surface area and pore size of the adsorbents and the charge distribution, polarity and shape of the mycotoxins significantly contribute to the overall binding compatibility (Galvano *et al*., 2001; Kabak *et al*., 2006).

In order to resolve this problem, mineral adsorbents have been modified with high molecular weight quaternary amines (quaternary long-chain alkyl/aryl amines). The modified mineral adsorbents have an increased hydrophobicity, hence, an improved adsorption of non-aflatoxin mycotoxins (Schall *et al.,* 2004; Dakovìca *et al*., 2007). However, such alterations do not always reduce observed toxicities. Among the other limitations for this approach are unwanted adsorption of vitamins, amino acids and minerals in feed as well as the potential risks of complexing chemicals to the mineral adsorbents (Huwig *et al*., 2001; Jouany, 2007). In an attempt to overcome some of these disadvantages, second generation adsorbents have been developed originating from the cell wall component of microorganisms. The main candidate microorganisms include yeast (Shetty *et al*., 2007), lactic acid bacteria (Gratz *et al*., 2005) and conidia of *Aspergilli* (Jard *et*

al., 2009). The mechanism of detoxification is still physical adsorption (ion-exchange) facilitated by inactivated cellular walls rather than the catabolism of mycotoxins by living microorganisms. Polysaccharide, protein, and lipid constituents of such walls provide numerous potential sites for mycotoxin attachment through hydrogen bonds, ionic, and hydrophobic interactions (Huwig *et al*., 2001; Ringot *et al*., 2007).

A new functional group of feed additives was defined by the Commission regulation (EC) No 386/2009 as ‘‘substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action’’ (EC, 2009). The additives are added to the diet of animals (mainly of swine, poultry and cattle) in order to reduce the absorption of mycotoxins from the gastrointestinal tract and their distribution to blood and target organs. These substances are known as detoxifying agents. Depending on their mode of action, these feed additives may act by reducing the bioavailability of the mycotoxins (adsorption), or by degrading them or transforming them into less toxic metabolites (biotransformation). Therefore, we can define at least two main categories: adsorbing agents (describes before) and bio-transforming agents (belong at biological methods).

II.III Biological methods

Biological methods consist of the use of living microorganisms, including bacteria and fungi, or enzymes, which are able to metabolize, degrade or inactivate mycotoxins into stable, less toxic, up to harmless compounds (Commission Regulation 2015/786/EU). These processes are widely recognized as efficient, specific and environmentally-friendly, without alteration of sensory characteristics such as colour and flavour.

Screening and isolating naturally existing microorganisms that show bio-transformation capabilities against specific mycotoxins has been a popular strategy. The possibility to use living microorganisms as whole cell biocatalysts for mycotoxins degradation has cost advantages. This represents a valid strategy, especially if multi step reactions are required, or if the microorganism is already implemented within industrial processes (Hassan *et al*., 2013).

Alternatively, direct applications of bioactive materials (e.g. enzymes, polypeptides, etc.) which are either commercially available or produced from related microorganisms with or without the use of recombinant DNA techniques

are also another possible practice. Enzymes guarantee reproducible and homogeneous performances in mycotoxins biodegradation, with specificity, ease-of-handling, no risks of contamination and no safety concerns for operators compared to the use of living microorganisms. Ligninolytic enzymes, such as laccase and peroxidase, from white-rot fungi, as *Pleurotus spp.*, have been proven to decontaminate the stable molecule of AfB1 (Motomurra, *et al* 2003).

II.III.I Pleurotus spp.

A number of fungal species have been shown to be capable of degrading mycotoxins, among them *Pleurotus spp*. is one of the most studied and it seems really promising. The genus *Pleurotus* (*Pleurotaceae*, higher Basidiomycetes) contain flavorful edible mushrooms found throughout the northern temperate zones. The common name "oyster mushroom" stems from the white shell-like appearance of the fruiting body. Various substrates that contain lignin and cellulose can be used for *Pleurotus* cultivation, such as wood chips, corn wheat, rice straw, cotton stalks, waste hulls and other agricultural wastes, some of which can be recycled and upgraded for use as animal feed or in the preparation of other products. The main advantage of using *Pleurotus spp* to upgrade lignocellulosic waste is their selective degradation of lignin and hemicelluloses, as a result of which the cellulose is exposed and can be utilized by ruminants. The ligninolytic system of *Pleurotus spp* has been extensively studied in recent years. Two ligninolytic enzyme families have been characterized: peroxidase and laccase (Sannia *et al*., 1991; Hatakka, 1994; Asada *et al*., 1995; Giardina *et al*., 2000; Camarero et al. 1999; Cohen *et al*., 2001). These enzymes can be used for various biotechnological and environmental applications. *Pleurotus* spp and their enzymes show promise in their ability to degrade and mineralize toxic chemicals, such as polycyclic aromatic hydrocarbons (PAHs), atrazine, organophosphorus and wastewaters (Pointing, 2001).

Figure I *Pleurotus eryngii* cultivated in a contaminated AfB1-mushroom medium (from Branà, *et al* 2017).

These enzymes proved to be promising in biological decontamination (Kim *et al.* 2017). For example, Motomura and other (2003) isolated an unidentified enzyme from *Pleurotus ostreatus* that reduced the fluorescence of AfB1 and attributed this to the disruption of its lactone ring, that plays an important role in carcinogenicity of this mycotoxins. Other author as Alberts *et al.* (2009) later, showed that supernatant from *P. ostreatus* degraded AfB1 by up to 76% and that degradation efficiency was strongly correlated with the activity level of pure laccase. Instead, Yehia (2014) used manganese peroxidases from *P. ostreatus*, to degrade AfB1. Finally, it is demonstrated that no translocation or "carry-over" of this toxin through the fungal thallus is present in mushrooms of *P. eryngii* grown in aflatoxin-contaminated substrates (Figure I; Branà, *et al* 2017).

II.III.II *Fungal laccases*

Laccases (EC 1.10.3.2), also named p-diphenol: dioxide oxidoreductases are blue multicopper oxidases (MCOs) that have the ability to catalyze the oxidation of a wide variety of organic aromatic compounds, concomitantly with the reduction of molecular oxygen to water (Ruiz-Duenas and Martìnez, 2009; Sakurai and Kataoka, 2007).

Laccases are considered “ideal green” catalysts because they employ O_2 as a co-substrate and generate H_2O as a byproduct.

$$AH_2 + \tfrac{1}{2}\,O_2 \xrightarrow{\textit{Laccase}} A + H_2O$$

In general terms, laccase substrate oxidation is a one-electron reaction generating a free radical. The initial product is typically unstable and may undergo a second oxidation by enzyme catalysis or from a non-enzymatic reaction as a hydration, polymerization or disproportionation (Kunamneni *et al.*, 2007).

Although most laccase substrates are phenolic compounds (ortho and para-diphenols, methoxysubstituted phenols, polyphenols, aromatic amines, benzenethiols, hydroxindols, 1-naphthol, syringaldazine), the number and type of substrates oxidized by laccase can be extended by a mechanism involving the participation of redox mediators. These mediators are low molecular weight compounds that can easily be oxidized by laccase, producing very reactive and unstable cationic radicals. However, at the same time these cationic radicals can oxidize complex compounds (not including phenolic substrates) before returning to their original state (Torres *et al.*, 2003). By this mechanism mediators act as diffusible electron transporters, allowing indirect oxidation of substrates, penetrating even to less accessible areas of its structure (Figure II).

Addition of mediators extend the use of laccases for industrial processes related to bioremediation; including delignification of lignocellulosics, color removal and detoxification of industrial dyes, bioremediation of xenobiotic compounds, pesticides, explosives, wastewater treatment, and treatment of other pollutants such as polycyclic aromatic hydrocarbons (Desai and Nityanand, 2011).

An ideal redox mediator must be a good laccase substrate; its oxidized and reduced forms must be stable but must not inhibit the enzymatic reaction. In addition, its redox conversion must be cyclic (Johannes and Majcherczyk, 2000).

At first, mediators were considered to mean low-molecular-weight laccase substrates whose enzymatic oxidation gave rise to stable high-potential intermediates. The latter took part in chemical (non-enzymatic) reactions with other compounds, not oxidizable by laccases alone, following the diffusion-controlled kinetics. The oxidized mediator was reduced to the initial form by the compound to be oxidized (Bourbonnais *et al.*, 1998; Fabbrini *et al.*, 2002), thereby closing the cycle:

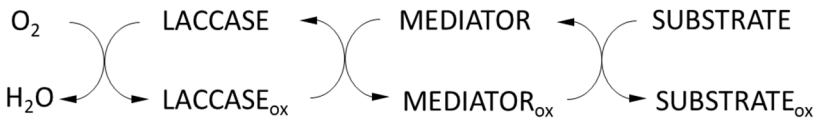


Figure II. Laccase Mediator Systems (LMSs).

More than 100 different mediators have been described, included ABTS (2,20-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)), TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxyl), syringaldehyde and acetosyringone.

Laccase-mediator system can be applied to industrial production, as well as bioremediation of toxic compounds, however, the elevated chemical mediator commercial costs, their high toxicity, and lack of studies on derivative effects, in addition to inactivation caused by their cationic radical exertion on laccases, makes the laccase-mediator system implementation still limited.

One interesting field of application of LMSs concern the mycotoxin detoxification and many articles and reviews on this topic have been published recently (Loi, *et al* 2016 and 2018).

Chapter 1

Occurrence of *Fusarium* mycotoxins and related species from durum wheat collected in Italy.

1.1 Abstract

Fusarium Head Blight (FHB) is a fungal disease affecting wheat and other small-grains in all cropping areas of the world. FHB of wheat is caused by a complex of species belonging mostly to *Fusarium* genus. Many of these species can produce a wide range of mycotoxins that can be accumulated in wheat kernels at maturity, among which the trichothecene, strong protein inhibitors, are the most common.

This survey had analysed 175 wheat samples from Northern, Central and Southern Italy during the 2013-2014, 2014-2015 and 2015-16 growing seasons, to understand the Italian distribution of *Fusarium* mycotoxins, in particular trichothecenes type-A (HT2 and T2 toxins), type-B (deoxynivalenol (DON) and nivalenol (NIV)) and zearalenone (ZEA). *Fusarium* species, isolated from infected kernels were identified on the base of their morphological features.

DON contamination levels exceeding the maximum regulated level (>1750 µg/kg) were found in one wheat sample from Northern Italy during the 2014-2015 growing season, and in two samples from Central Italy during 2013-2014 growing season, respectively. The contamination of ZEA, T-2 and HT-2 toxins occurred, in different years, in all the Italian regions considered. Only in Southern Italy, during the growing season 2013-2014, the sum of T-2 and HT-2 toxins exceeded the recommended levels in eighteen wheat samples. In the same year, ZEA contamination was above the maximum fixed level in three wheat samples from Central Italy. The NIV content was very low in the Northern and Central Italy. Moreover, DON and NIV were never found in Southern Italy.

Fusarium graminearum was the most occurring species in Northern Italy while in Central and Southern Italy *F. poae* was the principal species isolated from the kernels.

This study showed that a real mycotoxin risk related to *Fusarium* mycotoxins does exist along the whole Italy, but they vary according with the geographical areas and year of sampling.

1.2 Introduction

Mycotoxins are considered one of the most important groups of contaminants of foodstuffs and feedstuffs; cereals and cereal-based products are the principal sources of human exposure to these natural contaminants (Bennett and Klich, 2003). Fungal infection of crops is a worldwide problem due to economic damages caused by plant diseases leading to yield losses and poor food quality. In addition, the ability of certain fungal species to produce toxic compounds, known as mycotoxins, can impact food and feed safety. The species can vary according to the different geographical areas because they can be influenced by the changing environmental conditions.

Fusarium Head Blight (FHB) is a worldwide disease of wheat and other small-grain cereals, causing significant reduction of cereals yield and quality. This disease is caused by many species belonging to the *Fusarium* genus, that produces various toxic secondary metabolites, as trichothecenes and zearalenone. The primary etiological agent of FHB worldwide is the *Fusarium graminearum* species complex (FGSC) (Somma *et al.*, 2014), with *F. graminearum sensu stricto, F. culmorum* and *F. poae* as the main causal species.

During the infection process, *Fusarium* species are able to biosynthesize different mycotoxins and, among them, trichothecenes are considered to be the most important ones.

The term trichothecenes is derived from trichothecin, which was the one of the first members of the family identified. All trichothecenes contain a common 12,13-epoxytrichothene skeleton and an olefinic bond with various side chain substitutions. These toxins are classified into two main groups: type-A, including HT-2 toxin (HT2), T-2 toxin (T2) and type-B, including nivalenol (NIV), 4-acetyl-nivalenol, deoxynivalenol (DON), 3- and 15-acetyl-deoxynivalenol (3-Ac-DON and 15-Ac-DON) (Quarta *et al.*, 2006). Trichothecenes type-A are produced by *Fusarium langhsetiae, F. sporotrichioides, F. poae* and *F. acuminatum* (Van der Fels-Klerk and Stratakou, 2010). NIV is produced by *F. poae* and *F. croockwellense* (Logrieco and Visconti, 2004).

F. graminearum and *F. culmorum* produce both NIV and DON (Placinta *et al.*, 1999; Turner, 2010), depending on their chemotype, the latter is often found in cereal grains, in particular, on wheat and maize. DON is frequently found in association with another mycotoxin: zearalenone (ZEA) (Logrieco *et al.*, 2002) produced by *F. graminearum*, *F. culmorum* and *F. croockwellense* (IARC, 1993).

The toxic effects of mycotoxins have been extensively studied in animals, conversely, the toxicology of these mycotoxins remains largely unexplored in humans. They represent a pertinent problem due to their frequent occurrence in cereals and cereal based products, and for their impact on human health. Since it is not possible to completely prevent formation of mycotoxins, national and international authorities have adopted regulatory limits and guidelines to monitor mycotoxin levels in different food and feed products, including cereals and cereal-based products.

In order to protect the health of consumers, the European Commission has set DON maximum permitted levels in cereals and processed cereal-based food. In particular, the limit of 1750 µg/kg has been fixed for cereal flour and wheat bran as end product marketed for direct human consumption (EC, 2006a), whereas for zearalenone was of 100 µg/kg in unprocessed durum wheat, conversely, no guidelines or regulatory limits have been set for NIV, although this mycotoxin frequently contaminates cereals and commonly co-occurs with DON (Placinta, *et al* 1999). Regulatory limits are currently under discussion by the European Commission, considering the sum of T-2 and HT-2 in cereal and cereal products. The latest proposal at this regard is 100 µg/kg for unprocessed cereal (Commission Recommendation 2013/165/EU).

Under field conditions there are many factors that influence mycotoxin production as climate, agricultural practises, host plants and the presence of other microorganisms. For these reasons, it is very important to monitor the mycotoxins levels in cereals and to enforce the legal limits and thus increase the consumer safety.

The aims of this research were to monitor the occurrence of *Fusarium* species and related mycotoxins (NIV, DON, ZEA, T2 and HT2) on wheat in Italy, during different growing seasons (2013-2014, 2014-2015 and 2015-2016).

1.3 Materials and Methods

1.3.1 Materials

All solvents (HPLC grade) were purchased from VWR International Srl (Milan, Italy). DON, NIV, T2 and HT2 toxins, ZEA analytical standard grade (purity > 99%), sodium chloride (NaCl), Tween 20, Potato Dextrose Agar (PDA) medium, pentachloronitrobenzene (PCNB), neomycin and streptomycin were supplied by Sigma-Aldrich (Milan, Italy). Immunoaffinity columns were provided from

VICAM L.P. (Milford, MA, USA); glass microfiber filters and paper filters from Whatman (Maidstone, UK).

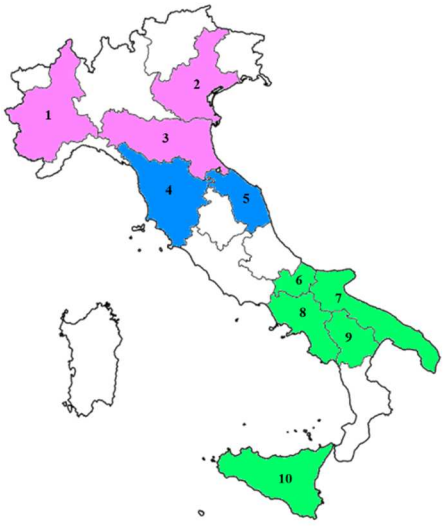
1.3.2 Collected wheat samples

Samples of durum wheat, from different regions of Italy (95, 52 and 31), were collected during the 2013-2014, 2014-2015 and 2015-2016 growing seasons, respectively. They were categorised into three distinct geographical area that parallel the climatological gradation along the Italian peninsula: Northern (Piemonte, Veneto and Emilia Romagna), Central (Marche and Toscana) and Southern (Molise, Puglia, Basilicata, Campania and Sicilia).

Kernels were milled and homogenised using an ultra-centrifugal mill (ZM 200, Retsch) equipped with a 500 µm sieve. After milling and homogenisation, an aliquot (0.5 kg) of the sample was stored at 4°C until the time of chemical analysis.

1.3.3 Morphological Identification

For each sample, 100 kernels were randomly selected, superficially sterilized and plated on Petri dishes containing PDA medium, added with 1% of PCNB, neomycin (50 mg/L) and streptomycin (100 mg/L). Fungal contamination for each sample was registered, Fusarium species were isolated and morphologically identified (Leslie and Summerel, 2006).



	Cereal samples (n= 178)		
Area	2013-14	2014-15	2015-16
1	3	3	-
2	3	-	-
3	6	3	-
4	31	15	13
5	28	20	18
6	2	-	-
7	6	1	-
8	6	3	-
9	5	1	-
10	5	6	-

Figure 1.1 Wheat samples collected in several areas of Italy during three growing seasons (2013-14, 2014-15 and 2015-16).

1.3.4 Analysis of T2 and HT2 toxins in wheat samples

Wheat samples were extracted with methanol:water (90:10, v/v) and diluted extracts were cleaned up through immunoaffinity columns. T2 and HT2 toxins were separated and quantified by Ultra Performance Liquid Chromatography (UPLC) with photodiode array (PDA) detector (λ=202 nm). The retention time of HT-2 and T-2 toxins were 1.97 and 4.9 min, respectively. The mycotoxins were quantified by comparing peak areas with a calibration curves obtained with standard solutions. The linearity of the analytical response was checked by analysing the calibration standards and using seven concentrations over the range 62.5–4000.0 ng/mL of T-2 and HT-2 toxins. The limit of detection (LOD) of the method was 8 µg/kg for both toxins (signal to noise 3: 1) (Pascale *et al*, 2011).

1.3.5 Analysis of DON and NIV in wheat samples

Wheat samples were extracted with water and the filtered extract were cleaned up through an immunoaffinity column containing a monoclonal antibody specific for DON and NIV. Toxins were separated and quantified by UPLC with PDA detector (λ=220 nm). The retention time of NIV and DON were 1.4 and 2.4 min, respectively. The linearity of the analytical response was checked by analysing the calibration standards and using eight concentrations over the range 10.0–5000.0 ng/mL of NIV and DON. The LOD of the method was 20 µg/kg for NIV and DON (signal to noise 3: 1) (Pascale *et al*, 2014).

1.3.6 Analysis of ZEA in wheat samples

Wheat samples were extracted with acetonitrile:water (90:10, v/v) and the extract was diluted with water (1:10, v/v) and applied to immunoaffinity column. The column was washed with water and ZEA was eluted with methanol and quantified by reversed-phase HPLC with fluorometric detection (λ_{ex} =274 nm, λ_{em}= 440 nm) using acetonitrile:water:methanol (46:46:8, v/v) as mobile phase. The retention time was 7.2 min. The linearity of the analytical response was checked by analysing the calibration standards and using six concentrations over the range 10.0–300.0 ng/mL of ZEA. The LOD of the method was 3 µg/kg for ZEA (signal to noise 3:1) (Visconti and Pascale, 1998).

1.4 Results and discussion

Samples from Northern Italy, during 2013-2014 growing season, were contaminated by DON, ZEA and T2+HT2, none of the samples shows contamination above the regulatory limits for DON and ZEA (Table 1.1), three samples were co-contaminated by both toxins and one of them with T2+HT2 too (Supplementary S1).
During 2014-2015 growing season in Northern Italy, the samples were contaminated by DON, NIV and ZEA (Table 1.1). One sample was co-contaminated by all of these toxins and DON level was up above the fixed limit (Supplementary S1).
The present of DON during the two years monitoring, showed a lower percentage of contaminated samples, with higher level, in the second year.

		Northern Italy			
		DON	**NIV**	**ZEA**	**T2+HT-2**
2013-2014 (12)	Number positive samples	7	0	5	2
	% positive samples	58	0	42	17
	Mean±SD	462±290	<LOD	9±8	11±3
	Median	506	<LOD	6	11
	Maximum	966	<LOD	23	13
2014-2015 (6)	Number positive samples	1	1	3	0
	% positive samples	17	17	50	0
	Mean±SD	2493±0	76±0	6±2	<LOD
	Median	2493	76	6	<LOD
	Maximum	2493	76	9	<LOD

Table 1.1 Describe statistic of trichothecene and zearalenone contamination (µg/kg) for wheat samples collected in the Northern Italy during 2013-2014 and 2014-2015 growing seasons.

Samples from Central Italy, during 2013-2014 growing season, were contaminated by DON, NIV, ZEA and T2+HT2, one sample for DON and one sample for ZEA show contamination above the regulatory limits (Table 1.2). Three were co-contaminated by DON and ZEA, two of them by NIV too. One sample was co-contaminated by DON and T2+HT2, one by NIV and T2+HT2, five by ZEA and T2+HT2 (Supplementary S2). The growing season 2014-2015 showed contamination in only one sample by DON, below the regulatory limit (Table 1.2).

During the 2015-2016 growing season DON was the only mycotoxins detected, five samples were contaminated but the levels were below the regulatory limit. In the first year considered, all mycotoxins were measured, while in the other two years only DON was detected (Table 1.2).

		Central Italy			
		DON	**NIV**	**ZEA**	**T2+HT-2**
2013-2014 (59)	Number positive samples	5	5	12	18
	% positive samples	8	8	20	31
	Mean±SD	2227±3472	55±12	35±38	28±20
	Median	469	50	31	18
	Maximum	9129	71	148	68
2014-2015 (35)	Number positive samples	1	0	0	0
	% positive samples	3	0	0	0
	Mean±SD	767±0	<LOD	<LOD	<LOD
	Median	767	<LOD	<LOD	<LOD
	Maximum	767	<LOD	<LOD	<LOD
2015-2016 (31)	Number positive samples	5	0	0	0
	% positive samples	16	0	0	0
	Mean±SD	507±129	<LOD	<LOD	<LOD
	Median	570	<LOD	<LOD	<LOD
	Maximum	610	<LOD	<LOD	<LOD

Table 1.2 Describe statistic of trichothecene and zearalenone contamination (µg/kg) for wheat samples collected in Central Italy during 2013-2014, 2014-2015 and 2015-2016 growing seasons.

Samples from Southern Italy, during 2013-2014 growing season, were contaminated by ZEA and T2+HT2, nineteen of them sample were co-contaminated for both and one of them above the limits (Supplementary S3). Three samples were contaminated by ZEA above the regulatory limits. Seventeen samples were contaminated by T2+HT2 above proposed limits (Table 1.3).
The growing season 2014-2015 showed contamination of ZEA and T2+HT2, below the regulatory and proposed limit (Table 1.3). Only one was co-contaminated by ZEA and T2+HT2 (Supplementary S3).
Both the growing seasons (2013-2014 and 2014-2015) were contaminated by same mycotoxins, but the first compared to the second, above the limits.

		Southern Italy			
		DON	**NIV**	**ZEA**	**T2+HT-2**
2013-2014 (24)	Number positive samples	0	0	20	20
	% positive samples	0	0	83	83
	Mean±SD	<LOD	<LOD	49±73	310±113
	Median	<LOD	<LOD	20	336
	Maximum	<LOD	<LOD	325	486
2014-2015 (11)	Number positive samples	0	0	4	5
	% positive samples	0	0	36	45
	Mean±SD	<LOD	<LOD	5	31
	Median	<LOD	<LOD	5±11	31±18
	Maximum	<LOD	<LOD	5	60

Table 1.3 Describe statistic of trichothecene and zearalenone contamination (µg/kg) for wheat samples collected in Southern Italy during 2013-2014 and 2014-2015 growing seasons.

The presence of *Fusarium* species in different Italian regions were evaluated (Figure 1.2) for the growing seasons considered (2013-2014, 2014-2015 and 2015-2016), and it was expressed as the incidence (%) of the strains of each species on the total identified isolates.

In Northern Italy, the main *Fusarium* species found in the growing seasons 2013-2014 were: *F. graminearum* (32%) and *F. poae* (32%), while in the growing season 2014-2015 were *F. graminearum* (19%), *F. poae* (19%) but an amount of *F. spp* (19%) remains unidentified at the species level (Figure 1.2 A, B).

In Central Italy, the main *Fusarium* species found in the growing seasons 2013-2014 were: *F. poae* (49%) and *F. graminearum* (14%), in the growing season 2014-2015 were: *F. poae* (62%) and *F. proliferatum* (14%), while in the growing season 2015-2016 were: *F. poae* (27%), *F. graminearum* (11%) but an amount of *F. spp* (12%) remains unidentified at the species level (Figure 1.2 C, D, E).

In Southern Italy, the main Fusarium species found during the growing season 2013-2014 were: *F. poae* (26%), *F. acuminatum* (17%) but an amount of *F. spp*. (17%) remains unidentified at the species level. While in the growing season 2014-2015 were *F. poae* (60%), *F. equiseti* (20%) and *F. culmorum* (20%) (Figure 1.2 F, G).

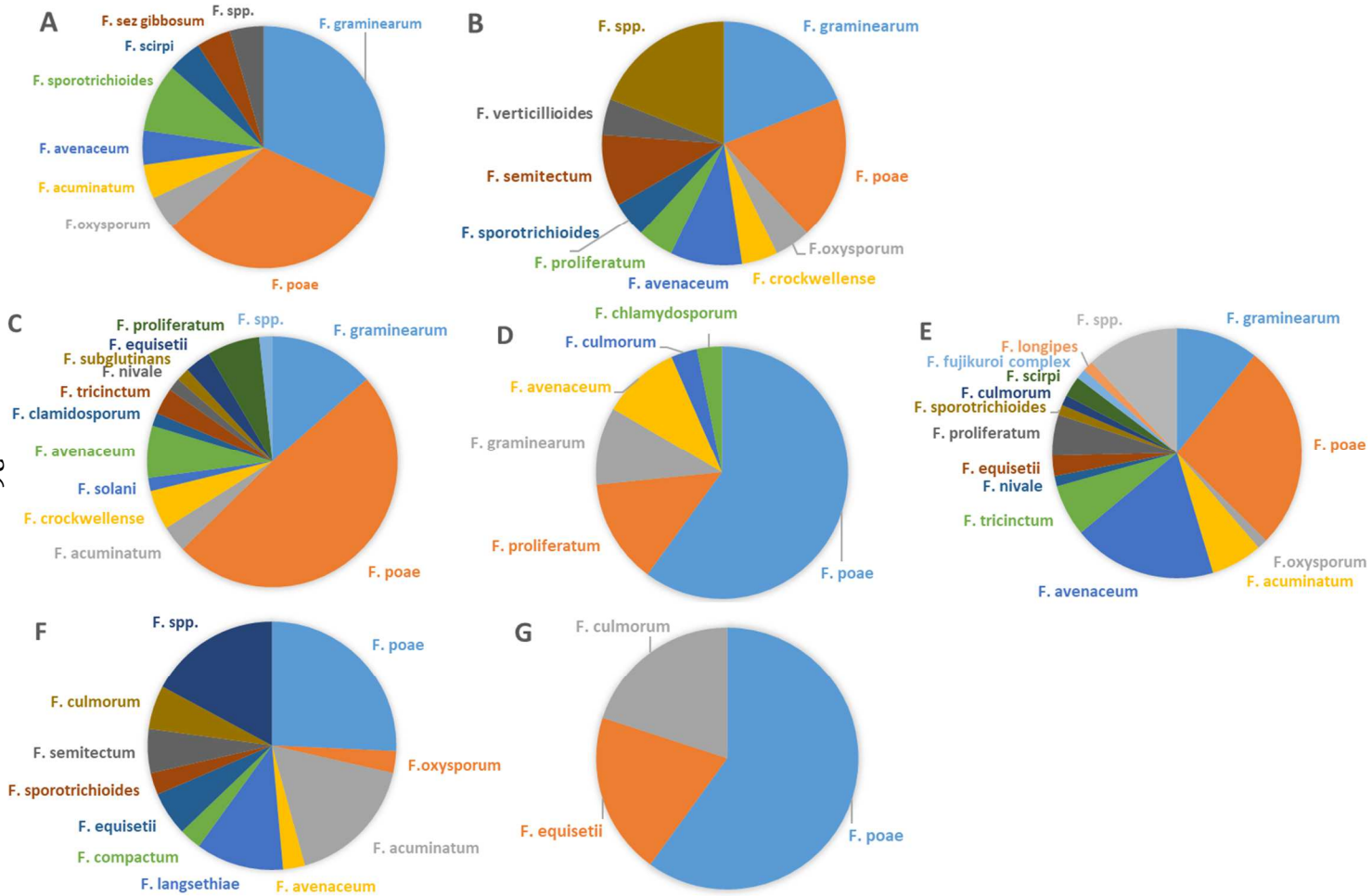


Figure 1.2 Distribution of fungal isolates from wheat in Northern, Central and Southern Italy… *Continue at page 37*

...the graphics shown the relative percentage of infected samples for each *Fusarium* species isolated from kernels, in particular: in Northern Italy 2013-2014 (A); 2 in Northern Italy 2014-2015 (B); in Central Italy 2013-2014 (C); in Central Italy 2014-2015 (D); in Central Italy 2015-2016 (E); in Southern Italy 2013-2014 (F); in Southern Italy 2014-2015 (G).

The presence of toxigenic fungi in a crop is not always synonym of the associated toxins. In a similar way, the absence of toxigenic fungi isolates does not imply the absence of the associated toxin, since the toxins could have been already produced in foodstuffs.

In this survey it was possible to underline the correlation between mycotoxigenic fungi and the produced mycotoxins in wheat samples where contamination levels found were above the permitted limits. In fact, in the three samples contaminated with DON above limits, the kernels were infected by *F. graminearum* species, a DON producer. Conversely, for three samples contaminated with ZEA over limits, it was not possible to find the *Fusarium* species that produced this mycotoxin. Finally, for T2+HT2 toxins, in the eighteen wheat samples with levels above the recommended limits, the associated producer, *F. langsethiae*, *F. acuminatum*, *F. poae* were found in twelve of the infected samples (Supplementary S1, S2 and S3).

Previous studies (Bertuzzi *et al.*, 2014) reported that wheat from Northern Italy (Emilia Romagna) collected in 2010, 2011 and 2012 demonstrated that DON was the most widespread toxin with the highest level of contamination. As in our reported results, significantly different contaminations were found between years, for example they found DON levels above the regulated limits in 2010 and 2011 but not in 2009. Type A trichothecenes, T-2 and HT-2, and ZEA were always found below the limits, as in our case. Conversely, NIV showed higher levels than our measures.

Data regarding the presence of trichothecenes in durum wheat in Central Italy (Umbria) (Covarelli *et al.*, 2015), during the years 2009 and 2010, showed that samples were contaminated by DON in both years, without reaching the fixed levels, and were concordant with our findings despite we detect one sample exceeding the limits. Opposite to the data of this report, they found NIV content ubiquitously at appreciable concentrations during both years and the average contaminations of T-2 and HT-2 toxins were above the level of 100 µg/kg in 2009. The species composition was noticeably different, in fact, we found *F. poae* as the most frequent species while they found mostly *F. graminearum* and with a lesser extent *F. culmorum*, *F. avenaceum* and *F. poae*.

1.5 Conclusions

The present study reports the results of a survey on the presence of mycotoxigenic *Fusarium* species and their mycotoxins in wheat samples harvested in some regions of Northern, Central and Southern Italy during the growing seasons 2013-2014, 2014-2015 and 2015-2016.

This study shows that a real mycotoxin risk related to FHB exists in all areas of Italy. They vary according to the geographical areas and year of sampling. The data shown a different occurrence for several mycotoxins in the regions considered. The higher levels of DON and NIV were found in the samples of wheat coming from Central and Northern Italy, instead those from Southern Italy were principally contaminated by T2, HT2 toxins and ZEA.

The investigated samples were often contaminated by more than one single mycotoxin, although it is not always possible to detect the related *Fusarium* species.

Chapter 2

Bioremediation of aflatoxin B1 by *Pleurotus eryngii*

2.1 Abstract

Aflatoxin B1 (AfB1) is the most harmful mycotoxin produced by filamentous fungi *Aspergillus flavus* and *A. parasiticus*, which can occur as natural contaminant of many agricultural commodities, particularly maize. Several approaches have been experimented for the removal of aflatoxin from contaminated food and feed, but none of them have found a practical application, yet.

We investigated the capability of the white-rot and edible fungus *Pleurotus eryngii* (king oyster mushroom) to degrade AfB1 both in culture media, solid or liquid, and in a laboratory-scale mushroom cultivation, using a substrate like the one routinely used in mushroom farms. Moreover, we explored the AfB1-degradative activity of a crude extract (CE) from spent mushroom substrate (SMS) that may contain a source of bioactive molecules, such as ligninolytic enzymes, potentially useful for aflatoxin degradation.

In malt extract broth (MEB), degradation of AfB1 (500 ng/mL) by nine isolates of *P. eryngii* ranged from 81 to 99% after 10 days of growth, to reach 100% after 30 days, for all the isolates. The growth of *P. eryngii* on solid medium (malt extract agar, MEA) was significantly reduced when AfB1 concentrations was 500 ng/mL or higher. However, the addition of 5% wheat straw and 2.5% (w/v) maize flour to the culture medium (MEASM), increased the tolerance of *P. eryngii* to AfB1 and no inhibition was observed at 500 ng/mL. Degradation of AfB1 in MEASM was 71– 94% after 30 days of growth. Further, the AfB1 degradation by *P. eryngii* strain ITEM 13681 was tested in a laboratory-scale mushroom cultivation. The mushroom growth medium contained 25% (w/w) of maize spiked with AfB1 to the final content of 128 µg/kg. *Pleurotus eryngii* degraded up to 86% of the AfB1 in 28 days, with no significant reduction of either biological efficiency or mushroom yield. Neither the biomass produced on the mushroom substrate nor the mature basidiocarps contained detectable levels of AfB1 or its metabolite aflatoxicol, thus, ruling out the translocation of these toxins through the fungal thallus. Spectrophotometrically has been determined that the crude extract from SMS, shown as a high level of laccase activity (5 U/g DW) and a low level of Mn-peroxidase activity (0.6 U/g DW), thus, permitting to degrade 90% of AfB1 after only 7 days of incubation, in liquid medium.

These findings make a notable contribution towards the development of a novel technology for the remediation of AfB1-contaminated corn for aflatoxin-free feed production, through the exploitation of the degradative capability of *P. eryngii* and from the CE obtained from SMS, that proved to be a suitable source of aflatoxin-degrading enzymes.

Note: Some of the contents of this chapter were previously published in the following article:
Branà M.T., Cimmarusti M. T., Haidukowski M., Logrieco A. F. and Altomare C. (2017). Bioremediation of aflatoxin B_1-contaminated maize by king oyster mushroom (*Pleurotus eryngii*). PlosOne, 8, 2-14.

2.2 Introduction

Aflatoxin B1 (AfB1) is a mycotoxin, produced mainly by isolates of the species *Aspergillus flavus* and *A. parasiticus*, which has potent hepatotoxic, carcinogenic and mutagenic effects on humans and animals (Williams *et al*., 2004). Beside AfB1, other aflatoxins that are structurally correlated to AfB1, occur as natural contaminants of foods and feeds, or are generated from the metabolic transformation of AfB1, but have considerably lower incidence and toxicity than AfB1 itself (Cullen and Newbern, 1993). AfB1 has been listed as a group I agent (carcinogenic to humans) by the International Agency for Research on Cancer and epidemiological studies have correlated the incidence of hepatocellular carcinoma in humans to the consumption of AfB1-contaminated food in some world regions (IARC, 1993). Human exposure to aflatoxins can result directly from ingestion of contaminated food or indirectly from the consumption of products from animals that have been fed with contaminated feed. Because of ingestion of such feeds, aflatoxins are transformed into metabolites that contaminate meat, eggs and dairy products, such as milk and cheese (Bennet and Klich, 2003). Aflatoxin occurrence is a major problem in several crops, including cereals, groundnuts, legumes and cotton seeds, which can be contaminated at any stage of production, processing, transportation, and storage (Ruston, 1997). Amongst the cereal grains, aflatoxin contamination concerns primarily maize and maize by-products (Miller, 1995).

Several approaches have been attempted for the removal of aflatoxins from contaminated commodities, including the degradation of the toxins (Vanhoutte *et al*., 2016). Degradation of aflatoxins requires the alteration of one or both the molecule sites that are important for their toxic properties, namely the double bond of the difuran ring and the lactone ring of the coumarin moiety (Basappa and Shantha, 1996). Chemical and physical methods have been found to be effective in detoxification of AfB1 from various substrates (Méndez-Albores *et al*., 2005; Herzallah *et al*., 2008) but their practical use is limited, due to safety issues, possible loss of nutritional value of the treated commodities and cost implications (Kabak *et al*., 2006). Microbial degradation of aflatoxins has been attempted with some success, although in most cases only in axenic cultures (Mishra and Das, 2003; Hormisch *et al*., 2004; Tejada-Castaneda *et al*., 2008; Petchkongkaew, *et al*., 2008; Topcu *et al*., 2010; Gao *et al*., 2011). The microbial degradation of aflatoxin is achieved by the activity of enzymes, capable to break

down the recalcitrant polyheterocyclic molecule of aflatoxin. Among fungi, the so called "white-rot" fungi are known to possess very efficient enzymatic systems for degradation of polycyclic aromatic hydrocarbons (Pickard *et al.*, 1999). Indeed, the enzymes produced by the white-rot fungi have the crucial role of breaking down the complex molecules of lignin and other plant raw materials into low molecular weight compounds that can be assimilated by the organism. This process involves multiple ligninolytic enzyme systems consisting in extracellular oxido-reductases (Hatakka *et al.*, 1994). Encouraging results in aflatoxin degradation have been obtained with specific enzymes purified from *Pleurotus spp.*, a genus that includes several edible and cultivable mushroom species. Motomura and colleagues (Motomura *et al.*, 2003) reported the degradation of AfB1 by culture supernatants of *P. ostreatus* and isolated a novel enzyme with aflatoxin-degradation activity. More recently, has been shown (Yehia, 2014) that a Mn-peroxidase (MnP) purified from *P. ostreatus* was able to detoxify up to 90% of AfB1, depending on enzyme concentration and exposure time. Other enzymes produced by *Pleurotus* spp. that have received attention because of their aflatoxin-degradative capability are laccases, a group of enzymes with low specificity, that catalyze the oxidation of phenolic substrates *via* the reduction of molecular oxygen to water. Alberts and colleagues (Alberts, *et al* 2009) reported a significant correlation between laccase activity and AfB1-degradative capability of *P. ostreatus* isolates. Loi and colleagues (Loi *et al.*, 2016) purified a laccase isoform (Lac2) from *P. pulmonarius* and discover that AfB1 degradation by Lac2 *via* direct oxidation was 23%, which raised up to 90% in the presence of natural phenols acting as redox mediators. *Pleurotus eryngii* is a cultivable edible white-rot fungus, commonly known as king oyster mushroom (KOM). The KOM is cultivated worldwide and is highly appreciated for its firm texture, taste, flavor and nutritional value (Mau *et al.*, 1998). The goals of the work herein presented were: a) investigate the capability of *P. eryngii* strains that are exploited for commercial production of edible mushrooms to degrade AfB1; b) carry out a study on bioconversion of aflatoxin-contaminated maize into valuable feeds by *P. eryngii* in a laboratory-scale cultivation that mimicked the mushroom farm cultivation process; c) assess the aflatoxin content in the residual mushroom's growth medium and investigate the translocation of AfB1 or its toxic metabolite aflatoxicol (AFOL) through the thallus to the basidiocarps (fruit bodies) of *P. eryngii*; d) explore the AfB1 – degradative activity of a crude extract

(CE) from a spent mushroom substrate (SMS) and undertook a study for the enzymatic characterization of CE.
The results obtained represents the first contribution towards the development of a novel technology for remediation of AfB1-contaminated corn and its bioconversion into safe materials intended for feeds.

2.3 Materials and Methods

2.3.1 Fungal strains

The nine isolates of *P. eryngii* used in the study are commercially exploited for edible mushrooms' production. The isolates were obtained from the culture collection of the Institute of Sciences of Food Production (ITEM Collection, http://www.ispa.cnr.it/Collection/), Bari, Italy and were characterized as belonging to the variety *eryngii* (strains ITEM 13662, ITEM 13676, ITEM 13681, ITEM 13682, ITEM 13696, ITEM 13697, ITEM 13688, ITEM 13730, ITEM 17015) or variety *ferulae* (ITEM 13688) by sequencing of partial regions of the genes *ef1*-α and *rpb2* (Villani *et al.*, 2015). Fungal cultures were maintained in purity on malt extract agar (MEA, Oxoid, Basingstoke, UK).

2.3.2 Chemical and Materials

AfB1, aflatoxicol (AFOL) chemical standards (purity > 99%), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and sodium phosphate powder were supplied by Sigma-Aldrich (Milan, Italy). All solvents (HPLC grade) were purchased from VWR International Srl (Milan, Italy). All aqueous solutions were prepared using water obtained from a Water Millipore Milli-Q system (Millipore, Bedford, MA, USA). Mycosep® 224 AflaZon column was obtained from Romer (Romer Labs®, Getzersdorf, Austria). Paper filters Whatman no. 4 were given from Whatman (Maidstone, UK), regenerated cellulose membranes filters (RC 0.2 µm) were obtained from Alltech Italia-Grace Division (Milano, Italy) and 0.45-µm- pore-size cellulose filters from Labochem Science, (Sant'Agata Li Battiati, Italy). Malt extract agar (MEA), malt extract broth (MEB) and potato dextrose agar (PDA) media were supplied by Oxoid (United Kingdom). Magenta™ vessels were acquired from Sigma (77 × 77 ×77 mm).

2.3.3 Determination of aflatoxin B_1 and aflatoxicol

The standard solution of AfB1 was prepared, into amber silanized vials, by dissolving the commercial powder of the toxin in toluene-acetonitrile (9:1, v/v) to obtain a 1 mg/mL solution. The exact concentration of aflatoxin solution was determined spectrometrically according to AOAC Official Method 971.22 (AOAC, 2000). Aliquots of the stock solution were transferred to 4 mL amber silanized glass vials and evaporated to dryness under nitrogen stream at 50˚C. The residues were dissolved with water-methanol (60:40, v/v) to obtain calibrant standard solutions of AfB1 0.2, 0.4, 1.2, 2.0, 4.0, 5.0, 10.0 ng/mL. The standard solution of AFOL in acetonitrile, was transferred into amber silanized glass vials and dried under a stream of nitrogen at 50˚C, then reconstituted with water-methanol (50:50, v/v), to obtain calibrant standard solution of AFOL 2.0, 5.0, 10.0, 25.0, 50.0, 100 ng/mL. Standard solutions were stored at -20˚C and warmed to room temperature before use.

AfB1 was determined by using UPLC apparatus with Acquity UPLC system (Waters, Milford, MA, USA). Data acquisition and instrument control were performed by Empower 2 software (Waters). The column used was a 100 mm × 2.1 mm i.d., 1.7 µm, Acquity UPLC BEH RP-18, with an Acquity UPLC column in-line filter (0.2 µm), detected by fluorometric detector without post column derivatization. The fluorometric detector was set at wavelengths of 365 nm (excitation) and 435 nm (emission). The mobile phase was a mixture of water-acetonitrile-methanol (64:18:18, v/v/v) at a flow rate of 0.4 mL/min. The temperature of the column was maintained at 40˚C. AfB1 was quantified by measuring the peak areas at the retention time of aflatoxin standard and comparing these areas with the calibration curve of AfB1 in the range 0.2 to 10.0 ng/mL. With this mobile phase, the retention time of AfB1 was about 3.7 min. The limit of quantification (LOQ) of the method was 0.2 ng/mL for AfB1, based on a signal to noise ratio of 10:1.

Analyses of AFOL were performed with a HPLC Agilent 1260 Series (Agilent Technology, SantaClara, CA, USA) with post column photochemical derivatization (UVE™, LC Tech GmbH, Dorfen, Germany). The analytical column was a Luna PFP (150 × 4.6 mm, 3 µm) (Phenomenex, Torrance, CA, USA) preceded by a Security Guard™ (PFP, 4×3.0 mm, Phenomenex).

One hundred microliters samples were injected into the HPLC apparatus with a full loop injection system. The mobile phase consisted of a mixture of waters-acetonitrile (70:30, v/v) and the flow rate was 0.8 mL/min. The retention time

was about 16 min. Calibrant standard solutions were prepared in the range 2.0-100 ng/mL. The limit of quantification (LOQ) values were 20 µg/kg (signal to noise 10:1).

2.3.4 Stability of AfB1 in malt extract broth

A preparatory study was carried out to assess the decay of AfB1 during the incubating conditions utilized for the subsequent experiments. AfB1 was dissolved in 20 mL of MEB to the final concentration of 1000 ng/mL and the solution was placed at 30 ± 1˚C for 30 days. Triplicate of 1 mL samples were collected from the batch solution of AfB1 after 0, 1, 5, 10, 15 and 30 days of storage at 30 ± 1˚C and the AfB1 content of the samples was determined by UPLC/FLD.

2.3.5 Inhibitory effect of AfB1 on growth of P. eryngii

The inhibitory effect of AfB1 on growth of *P. eryngii* was studied in agar media supplemented with a range of concentrations of AfB1, *i. e.* 60, 120, 250, 500 and 1000 ng/mL. The media used were MEA, MEA supplemented with 5% (w/v) wheat straw (MEAS), and MEA supplemented with 5% wheat straw and 2.5% (w/v) maize flour (MEASM).

Aliquots of 1 mg/mL solution of AfB1 in toluene: acetonitrile (9:1, v/v) were added to melted MEA (50˚C) to obtain the desired test dilutions (60 to 1000 ng/mL). The dilutions were thoroughly mixed and poured into 9 cm-diameter Petri dishes (12 ml per dish). For preparation of MEAS and MEASM, wheat straw obtained from a local dealer was fragmented in pieces ≤ 5 mm long and maize kernels were finely ground in a laboratory mill (mulino Cyclone, International PBI, Milano, Italy) to particles of ≤ 0.2 mm; 0.5 g of straw and/or 0.25 g of ground maize were transferred into test tubes (2.5 cm diameter and 15 cm length) and autoclaved at 121˚C for 30 min. After cooling, the glass tubes were filled with 12 mL of melted sterile MEA supplemented with the test dilutions of AfB1, thoroughly mixed and poured into 9 cm-diameter Petri dishes. Then, each plate was inoculated with an 8 mm-diameter mycelial plug from a 20-day-old culture of *P. eryngii* on MEA and incubated at 30 ± 1˚C in the dark for 30 days. Five replicates per each tested isolate were prepared. Control plates were prepared with *P. eryngii* on MEA, MEAS and MEASM not supplemented with AfB1. The growth of *P. eryngii* was assessed by the colony diameter of three

representative isolates (ITEM 13681, ITEM 13697, ITEM 13688), measured with a ruler, with the help of a stereo microscope, every 48 hours.

2.3.6 Degradation of AfB1 by P. eryngii

The capability of different isolates of *P. eryngii* to degrade AfB1 was tested in both liquid and solid cultures.

Liquid cultures of *P. eryngii* were prepared in MEB supplemented with the toxin. The assays were carried out in 12-well plates. Each well was filled with 2 mL of MEB containing 500 ng/mL of AfB1 and inoculated with an 8 mm diameter mycelial plug from a 20-day-old culture of *P. eryngii* on MEA. Wells, in triplicate, were prepared considering each treatment and each sampling time and the cultures were incubated at 30 ± 1˚C for 30 days in the dark. After 10, 20 and 30 days of incubation, 1 mL of culture medium was withdrawn from each well, filtered through 0.45-µm- pore-size cellulose filters and stored at -20˚C until the analysis. Five hundred microliters of each sample were diluted with 500 µL of ultrapure water filtered through 0.2- µm-pore-size RC filters and 10 µL of the filtrate were injected directly into the UPLC apparatus through a full loop injection system. The percent degradation (D) was calculated by the formula:

$$D\ (\%) = \left(\frac{Ci - Cf}{Ci}\right) \cdot 100$$

where Ci was the concentration of AfB1 in the non-inoculated control and Cf was the concentration of AfB1 in culture filtrates 10, 20 and 30 days post inoculation of *P. eryngii*.

Solid cultures of *P. eryngii* were grown on MEASM supplemented with 500 ng/mL of AfB1.

The medium was prepared and inoculated as described above (section 2.3.5). Non-inoculated plates containing the medium supplemented with AfB1 were used as controls. After 15 and 30 days of growth, six 10 mm-diameter plugs of culture were excised with a cork-borer along the radius of the colony, at regular distances from the initial inoculation point to the edge of the colony and transferred to a test tube. The samples (approx. 1 g) were precisely weighted and extracted with 5 mL of a methanol-water (80:20, v/v) solution in a KS 4000i orbital shaker (IKA Werke GmbH & Co. KG., Staufen, Germany) at 250 rpm for 60 min. at room temperature. Five hundred microliters of each extract were then

diluted, processed and analyzed for AfB1 by UPLC/FLD as described for liquid cultures. The percent degradation (D) was calculated like for liquid cultures.

2.3.7 Cultivation of P. eryngii in a AfB1-contaminated mushroom medium

Pleurotus eryngii was cultivated on a substrate similar to the one used for the production of commercial mushrooms, which contained ground maize spiked with aflatoxin. To produce AfB1, the aflatoxigenic *A. flavus* strain ITEM 7764 was cultured on PDA for 7 days at 25˚C in the dark; a conidial suspension in sterile distilled water was prepared from the PDA cultures and used to inoculate 200 g of autoclaved (121˚C for 30 min) maize kernels in 1000 mL Erlenmeyer flask, to reach a final concentration of $1 \cdot 10^4$ conidia/g. Flasks were incubated for 21 days at 25˚C in the dark and analyzed for aflatoxin content. To extract aflatoxins, 300 mL methanol-water (80:20, v/v) were added into each flask, shaken on a rotary shaker for 60 min at 250 rpm and filtered through Whatman No. 4 filter paper. Five hundred microliters of extract were diluted with 500 µL of water, filtered through RC filters and 10 µL of the extract was injected into the UPLC apparatus for aflatoxin quantification. The extract was used to spike not contaminated ground maize till the final AfB1 concentration of 500 µg/kg of maize.

The substrate used for cultivation of *P. eryngii* contained 50% wheat straw, 25% spiked maize, 12.5% sugar beet and 12.5% field beans (*Vicia faba minor*). All the raw materials were obtained from a local supplier (Gruppo I.F.E srl, Matera, Italy). Wheat straw was fragmented in pieces about 2–3 mm long, sugar beet and field beans were ground in a blender Sorvall Omnimixer (Dupont Intruments, Newton, CT, USA) for 2 min. Magenta vessels were filled with 18 g of growth substrate with 1% (w/w) of calcium carbonate. Dry ingredients were carefully mixed, and 32 mL of tap water were added to reach approx. 65% (w/w) of moisture content; this mix was finally autoclaved at 121˚C for 60 min. Once cooled, the substrate was inoculated with 3 g of spawn of *P. eryngii* ITEM 13681, prepared as described by Estrada *et al.*, (2009), with some minor modifications. Briefly, 100 g of durum wheat kernels were mixed with 70 mL of warm tap water in Magenta vessels and then incubated for 28 days at 30 ± 1˚C in the dark. The AfB1 content in the growth substrate was assessed by UPLC/FLD 0, 7, 14, 21, and 28 days post inoculation (d.p.i.), in triplicate vessels. Non-inoculated vessels containing aflatoxin-contaminated substrate were used as controls.

To assess the possible carry-over of AfB1 or its metabolite AFOL in the basidiocarps, 28 d.p.i. vessels were opened, covered with a thin layer (1–2 cm) of soil and placed in greenhouse at 26˚C/ 21˚C day/night with a 12 h photoperiod to promote the fruiting of carpophores. The cultures were maintained constantly moist by nebulization of the adequate amounts of water. Mushrooms were harvested after 15 additional days of growth, when the basidiomata was ripe and the mushroom cap was flat. Fresh mushrooms from each vessel were individually weighed to determine the yield and the biological efficiency (BE) by the ratio: fresh mushroom weight/dry weight of the substrate, expressed as percentage. The spent substrate and the basidiocarps were analyzed for AfB1 and AFOL. Cultures prepared with non-contaminated maize were used as controls. The experiment was performed in triplicate and repeated once.

2.3.8 Determination of AfB1 and AFOL in spent mushrooms substrate and basidiocarps of P. eryngii

The aflatoxin-contaminated growth substrate of *P. eryngii* from each vessel was dried at 50˚C until constant weight. A portion of 20 g of substrate from each replicate was extracted with 100 mL of acetonitrile-water (84:16, v/v) by blending at high speed for 3 min with a Sorvall Omni-mixer. The extract was filtered through Whatman No. 4 paper filters and 50 μL of acetic acid were added. Aliquots of 8 mL were purified with Mycosep 224 AflaZon column. The extract was diluted 1:1 with pure water and 10 μL were injected into the UPLC apparatus (Zhaohui *et al*., 2008). The percentage of degradation (D) of AfB1 in *P. eryngii* growth substrate was calculated with the same formula of liquid cultures.

To investigate the carry-over of AfB1 and AFOL in the basidiocarps, 2 g of dry fruit bodies from each replicated vessel were extract in a Sorvall Omnimixer for 3 min with 50 mL of acetonitrile-water (84:16, v/v). The extract was then filtered, processed and analyzed as described for the growth substrate above.

2.3.9 Preparation of a crude extract of spent mushroom substrate

The CE was extracted with PBS 0.1 M, pH 7.3 from commercial SMS. Laccase and Mn-peroxidase activity from CE were spectrophotometrically determined using ABTS and $MnSO_4$ as substrates, respectively (Li *et al*., 2008; Boer *et al*., 2006). Enzyme activities were expressed in Units per gram of SMS dry weight (U/g DW). (Jönsson, *et al.,* 1997, Loi *et al*., 2016). To evaluate the optimum of

CE laccase activity at different pH values and temperatures, reactions were set as described in Boer *et al*., 2006.

2.3.10 AfB1 degradation assay with CE

One milliliter of extract was incubated with 1 µg/mL of AfB1 at 25 °C, under continuous shaking at 120 rpm for 3, 6, 12, 24, 48, 72 and 168 hours, were performed in triplicate. For each sampling time, control reactions were done in triplicate too. The aflatoxin content was determined by UPLC/FLD, as described above.

2.3.11 Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and Tukey–Kramer multiple comparison test. The statistical analyses were performed using the GraphPad Instat 3.0 software (GraphPad Software, San Diego, CA).

2.4 Results and discussion

The experiments to test the stability of AfB1 in MEB showed no significant degradation compared to the control after 5, 10, 15 and 30 days at 30 ± 1˚C in the dark. The results were highly reproducible (coefficient of variation = 97.2 ± 3.5%).

Although the three isolates showed a different sensitivity to AfB1, all exhibited a statistically significant growth inhibition on MEA when exposed to 500 ng/mL or higher concentration of AfB1 (Figure 2.1). Concentrations of AfB1 of 250 ng/mL or lower did not significantly affect the mycelial growth; *P. eryngii* ITEM 13688 was the most sensitive isolate, showing 60 ± 2% and 30 ± 2% of growth inhibition when exposed for 15 days to 1000 and 500 ng/mL of AfB1, respectively; *P. eryngii* ITEM 13681 was the most tolerant isolate, showing a growth inhibition of 37± 1% and 20 ± 3% at 1000 and 500 µg/mL of AfB1 respectively (Figure 2.1).

For all the isolates tested, the inhibition of growth on MEAS and MEASM was lower than on MEA in the presence of 1000 ng/mL of AfB1 and not statistically significant at the concentrations of 500 ng/mL or lower (Figure 2.2).

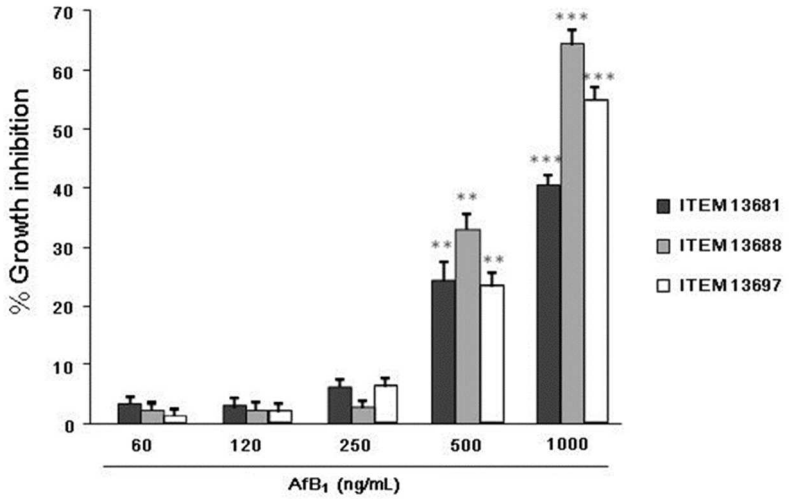


Figure 2.1. Inhibitory effect of AfB1 on growth of *P. eryngii*. The isolates ITEM 13681, ITEM 13688 and ITEM 13697 were grown for 15 days at 30 ± 1˚C in the dark on malt extract agar (MEA) containing different concentrations (60, 120, 250, 500, 1000 ng/mL) of AfB1. Data are the means ± SD (n = 5) of the percent reduction in colony diameters with respect to the control. Statistically significant differences with the control are indicated by asterisks (*** = $P < 0.001$, ** = $P<0.01$; One-way Anova).

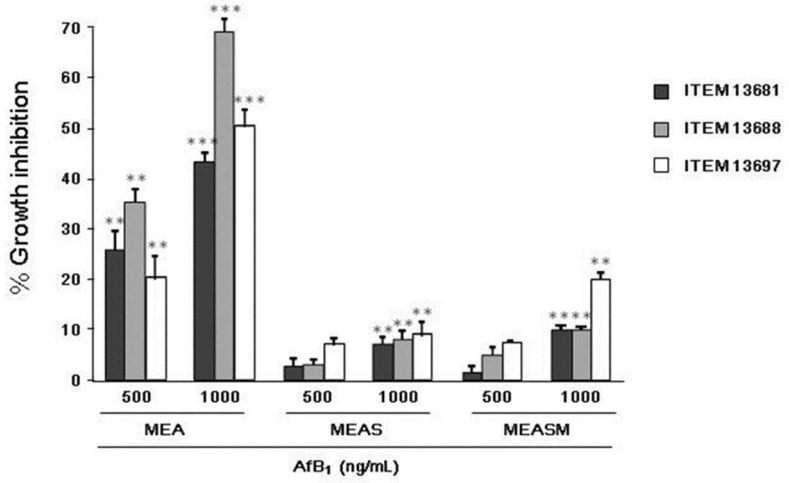


Figure 2.2 Reduction of the inhibitory effect of AfB1 on *P. eryngii* in the presence of wheat straw. The isolates ITEM 13681, ITEM 13688 and ITEM 13697 were grown on malt extract agar (MEA), MEA supplemented with 5% (w/v) wheat straw (MEAS) and MEA supplemented with 5% wheat straw and 2.5% (w/v) maize flour (MEASM) containing 500 and 1000 ng/mL of AfB1. Data are the means ± SD (n = 5) of the percent reduction in colony diameters with respect to the controls after 15 days of growth at 30 ± 1˚C in the dark. Statistically significant differences with the control are indicated by asterisks (*** = $P < 0.001$, ** = $P<0.01$; One-way Anova).

Degradation of AfB1 in MEB are shown in Table 2.1. After 10 days of growth the isolates ITEM 13730, ITEM 17015 and ITEM 13662 degraded 99% of AfB1. The isolates ITEM 13682 and ITEM 13696 degraded AfB1 by 95% and 94%, respectively, and the isolates ITEM 13681, ITEM 13697 and ITEM 13676 showed approximately 90% degradation. The isolate that showed the lowest degradative capability (80%) was ITEM 13688. However, after 20 days of incubation the differences among the strains were not statistically significant and after 30 days all the isolates totally degraded AfB1.

Isolate	10 Days			20 Days			30 Days		
	AfB1[(a)] (ng/mL)		D[(b)] (%)	AfB1[(a)] (ng/mL)		D[(b)] (%)	AfB1[(a)] (ng/mL)		D[(b)] (%)
Control	518 ± 18	A		501 ± 22	A		519 ± 22	A	
ITEM 13662	6 ± 1.0	F	99	1 ± 0.1	B	100	1 ± 0.1	B	100
ITEM 13676	47 ± 4.9	CD	91	4 ± 0.3	B	99	1 ± 0.2	B	100
ITEM 13681	47 ± 1.6	CD	91	17 ± 2.3	B	97	3 ± 0.4	B	100
ITEM 13682	28 ± 6.6	DF	95	2 ± 0.5	B	97	0 ± 0.2	B	100
ITEM 13688	98 ± 7.2	B	81	22 ± 6.3	B	96	3 ± 0.7	B	100
TEM 13696	30 ± 4.7	DE	94	9 ± 0.3	B	98	1 ± 0.2	B	100
ITEM 13697	59 ± 10.9	C	89	5 ± 1.0	B	99	1 ± 0.1	B	100
ITEM 13730	11 ± 2.3	EF	98	3 ± 0.2	B	99	1 ± 0.1	B	100
ITEM 17015	6 ± 0.5	F	99	1 ± 0.1	B	100	1 ± 0.2	B	100

Table 2.1 Degradation of AfB1 by strains of *P. eryngii* grown in MEB supplemented with 500 ng/mL of AfB1, after 10, 20 and 30 days of incubation at 30 ± 1˚C in the dark.

[a] Concentration of AfB1 detected in the culture filtrate. Data represent the mean values of AFB1 in 3 replicated cultures ± SD. Values in column followed by different letters are significantly different for $P < 0.001$ (Tukey–Kramer Multiple Comparison Test).

[b] Percent degradation of AfB1

Degradation of AfB1 by the *P. eryngii* isolates grown on the agar medium (MEASM) was analyzed in 15 and 30 days cultures (Table 2.2). After 15 days of incubation, all the strains significantly degraded the AfB1 supplemented to the medium, in percentages that ranged from 43 to 59%. After 30 days the degradation of AfB1 by the strain ITEM 17015 increased to 84%, and that of the strains ITEM 13696 and ITEM 13662 were 83 and 82% respectively. The strains ITEM 13688 and ITEM 13676, the least effective strains in degradation of AfB1, degraded AfB1 in the medium by 71 and 65% respectively.

Isolate	15 Days			30 Days		
	AfB1[(a)] (ng/mL)		D[(b)] (%)	AfB1[(a)] (ng/mL)		D[(b)] (%)
Control	419 ± 4.9	A		436 ± 5.8	A	
ITEM 13662	183 ± 5.0	BC	56	74 ± 2.1	D	82
ITEM 13676	192 ± 5.3	BC	54	147 ± 7.0	B	65
ITEM 13681	215 ± 19.7	BC	49	88 ± 5.6	C	79
ITEM 13682	204 ± 8.0	BC	51	86 ± 1.0	C	79
ITEM 13688	237 ± 4.6	B	43	120 ± 13.4	B	71
ITEM 13696	185 ± 13.5	BC	55	69 ± 11.8	D	83
ITEM 13697	192 ± 26.1	BC	54	119 ± 6.2	BC	72
ITEM 13730	188 ± 13.5	BC	59	79 ± 11.4	CD	81
ITEM 17015	173 ± 9.5	C	59	66 ± 6.8	D	84

Table 2.2 Degradation of AfB1 by *P. eryngii* grown on malt extract-agar plus wheat straw and maize flour (MEASM) supplemented with 500 ng/mL of AfB1, after 15 and 30 days of incubation at 30 ± 1˚C in dark.

[a] Concentration of AfB1 detected in the culture medium. Data represent the mean values of AfB1 in 3 replicated cultures ± SD. Values in column followed by different letters are significantly different for $P < 0.001$ (Tukey–Kramer Multiple Comparison Test).

[b] Percent degradation of AfB1.

The artificially contaminated cultivation substrate contained 128 ± 6 µg/kg of AfB1 at the time of inoculation with *P. eryngii* ITEM 13681 (day 0). Seven d.p.i. degradation was 13 ± 6% and 28 d.p.i. the level of degradation reached the maximum value of 86 ± 0.2% that did not change significantly until the end of the experiment (42 d.p.i). AFOL was not detected in the fungal biomass grown in the contaminated mushroom substrate. The analysis of basidiocarps of *P. eryngii* obtained on the contaminated mushrooms substrate did not reveal the presence of either AfB1 or AFOL.

To evaluate a possible effect of AfB1 on production of basidiocarps, yield and BE were assessed when the basidiocarps were fully ripe and the mushroom cap was flat (Figure 2.4).

Yield and BE of *P. eryngii* ITEM 13681 grown on the AfB1 contaminated substrate were 9 ± 1 grams and 72 ± 11% respectively, not significantly different from values of the control cultures (9 ± 0,8 g; 70 ± 5%).

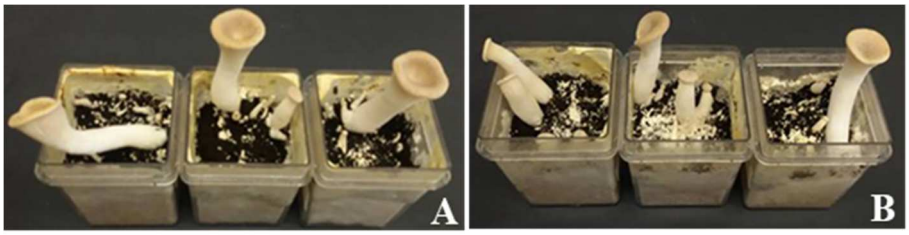


Fig 2.4 *Pleurotus eryngii* cultivated in a AfB1-contaminated mushroom medium. Production of basidiocarps (fruit bodies) by *P. eryngi*i ITEM 13681 in a mushroom cultivation medium; A) medium contaminated with 128 µg/kg of AfB1; B) non-contaminated control. In both the conditions *P. eryngii* ITEM 13681 produced well-developed basidiocarps, as well as growing immature fruit-bodies and fruit primordials in 42 days

The crude extract contained a high level of laccase activity, quantified in 5 Units per gram of spent substrate mushroom dry weight (U/g DW) and low level of Mn-peroxidase (0.6 U/g DW), as determined by spectrophotometric assays.
The laccase activity has been monitored varying temperature and pH to determine the optimal activity of this enzymatic extract. In the first case, the temperature was varied from 5 to 75°C. The laccase activity was higher at 5°C reaching 3.8 U/g·DW and decrease until to 0.5 U/g·DW at 45 °C. From 45 to 75°C, the laccase activity reached 0 U/DW because of enzymatic denaturation process (Figure 2.5A). In the latter case, the pH was changed from 3 to 7. The laccase activity obtained the value of 1.8 U/g·DW at pH 3 and increased until 6 U/g·DW at pH 4 (Figure 2.5B).

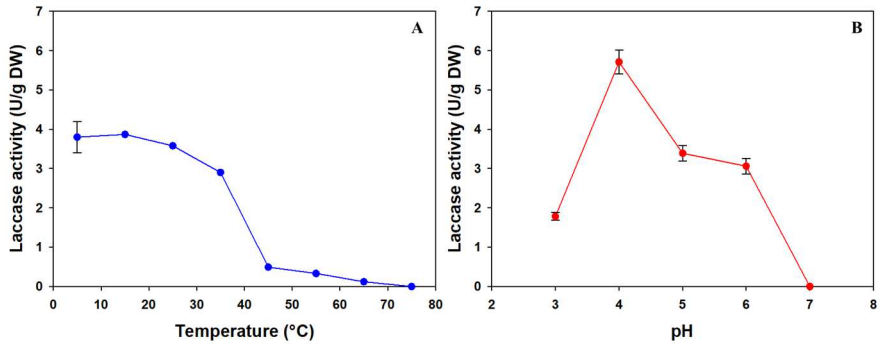


Figure 2.5 Effect of temperatures and pH varation on CE laccase activity . In A) is shown the laccase activity in base of temperature, while in B) is shown the laccase activity in base of pH.

The crude extract was incubated with AfB1 at concentration of 1 µg/mL for 3, 6 12, 24, 48, 72, 168 hours, and the degradation of aflatoxin (%) was determined by UPLC/FLD.
After 1 day of incubation, the crude extract was able to degrade about 50%±2 of AfB1 and after 3 and 7 days of incubation the percentage of degradation reached the values of 69%±8 and 92%±1, respectively (Figure 2.6).

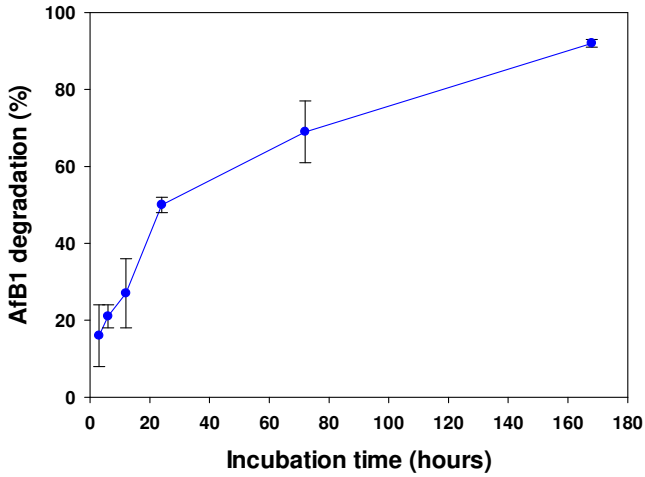


Figure 2.6: Time course of AfB1 degradation (%) after 3, 6 12, 24, 72 and 168 hours of incubation of crude extract with mycotoxin.

Previous research has proven the capability of some white-rot fungi to detoxify aflatoxins. In particular, the genus *Pleurotus*, which comprises edible cultivable mushrooms such as *P. ostreatus* and *P. eryngii*, produce the enzymes laccase and peroxidase that have been proved to degrade aflatoxins (Motomura *et al.*, 2003; Yehia, 2014; Alberts *et al.*, 2009; Muñoz *et al.*, 1997a; Martinez *et al.*, 1996).
The aim of this work was to investigate the AfB1-degrading capability of the species *P. eryngii* and to explore the potential of this mushroom for development of practical technologies aimed to recover and valorize aflatoxin-contaminated cereal wastes. Based on these preliminary results, the crude extract of spent mushrooms substrate proved to be a suitable source of aflatoxin-degrading enzymes and its use appears as a conceivable technology for aflatoxin-free feed production. Further research is needed to improve the stability of spent mushrooms substrate extracts and to implement their use in the pipeline of feed processing.

Aflatoxin B1 has a dose dependent inhibitory effect on the growth of *P. eryngii*. The tolerated level of AfB1 varies among *P. eryngii* isolates and depends on the substrate composition. It is not clear if the individual variation in the tolerance to AfB1 is due to: differences in mycelium sensitivity, or different AfB1-degrading capability of the strains. The latter hypothesis seems more likely to be the right one, since the most affected isolate (ITEM 13688, Figure 2.1) was also the least effective in AfB1 degradation (Tables 2.1 and 2.2). The presence of 5% wheat straw in the growing medium doubled (from 250 to 500 ng/mL) the concentration of AfB1 that was tolerated without resulting in any significant growth inhibition (Figures 2.1 and 2.2). The ligninolytic enzymes involved in AfB1 detoxification are inducible enzymes (Muñoz *et al.*, 1997; Elisashvili and Kachlishvili, 2009; Piscitelli *et al.*, 2011; Sànchez, 2010) therefore the higher tolerance to AfB1 shown by *P. eryngii* when grown of wheat straw may be conceivably due to the increase in the synthesis of such enzymes that is induced by the lignocellulosic materials in the culture medium.

Differences in the AfB1-degrading capability among the nine tested *P. eryngii* strains were found both in liquid culture on a semi-synthetic medium (MEB), and on agar medium supplemented with wheat straw and maize (MEASM) (Tables 2.1 and 2.2), even if the differences among the isolates were not dramatic. In liquid medium, where the degrading enzymes can diffuse freely, the levels of degradation were quite high (81 ± 1.4 to 99 ± 0.2%) already after 10 days of growth, to reach 100% in all the tested strains after 30 of growth. On the contrary, in the agar medium, where the enzymes presumably diffused more slowly through the agar layer, AfB1 was degraded by 71–94% after 30 days of growth.

Based on these results, the capability to degrade AfB1 exhibited by *P. eryngii* appears to be widely distributed in the members of this species. Individual differences in the efficiency of degradation, however, exist and might be due to differences in the kinetics of biosynthesis of single enzymes, in the composition of the enzyme pool implicated in AfB1 degradation (laccase and Mn-peroxidases), or in the degradative efficiency of different enzyme isoforms (Muñoz *et al.*, 1997b).

The results obtained with CE from SMS, showed that this extract contained more laccase activity (5 U/g·DW) compared to Mn-peroxidase activity (0.6 U/g·DW). The optimum of laccase activity of this crude extract has been further investigated varying pH and temperature. The maximum activity obtained was 3.8 U/g·DW

in the range of temperature from 5 to 25 °C and 6 U/g·DW at pH 4. This CE was able to degrade AfB1 until 92% in 7 days of incubation (Figure 2.5).
One more goal of our work was the exploitation of the AfB1-degrading capability of *P. eryngii* for the development of a novel technology aimed to recover and valorize contaminated cereals. So far, cultivation of mushrooms is the only biotechnological process that allows for bioconversion of lignocellulosic organic waste into protein-rich and high nutritional value nutriment, thus contributing to waste reduction (Sànchez, 2010). We have demonstrated that this technology may also be applied to the recycling of aflatoxin-contaminated cereals, which should be otherwise destroyed or directed to alternative uses, such as production of bioethanol. In a laboratory-scale cultivation on a growth medium that is routinely used in mushroom farms, the isolate *P. eryngii* ITEM 13681 was able to bioconvert up to 86% of the AfB1 in the medium (128 μg/kg) in 28 days. Although the mushroom growth medium contained 25% (w/w) of maize contaminated with 500 μg/kg of AfB1, *P. eryngii* did not show any significant reduction of either biological efficiency or mushroom yield.
AFOL is a derivative of AfB1 that originates from the reduction of the cyclopentanone carbonyl of the coumarine moiety (Detroy and Wand Hesseltine, 1970). The biological conversion of AfB1 to AFOL by intracellular enzymes has been demonstrated for several fungi. Although AFOL is 18 times less toxic than AfB1, it maintains a significant toxicity and can be reconverted to AfB1 (Nakazato *et al*., 1990), thus still representing a safety risk. In this study AFOL was not found, as a by-product of enzymatic or pH-dependent reduction of AfB1 by *P. eryngii*, in the fungal biomass. Also, the mature basidiocarps did not contain detectable levels of either AfB1 or AFOL, thus ruling out translocation or "carry-over" of these toxins through the fungal thallus.

2.6 Conclusions

Our results show that *P. eryngii* is able to degrade aflatoxin B1 in both liquid and solid media. The biodegradation of AfB1-contaminated cereals by *P. eryngii* that we have demonstrated to occur in a laboratory-scale mushroom cultivation needs appropriate validation prior to become a practical technology. Nevertheless, our results highlight that it may be regarded as a candidate process for the bioconversion of contaminated staple cereals into valuable products intended for animal feeding. These findings make a notable contribution towards the

development of preventative strategies to reduce AfB1 contamination of feeds and animal-derived foods. Further research will be aimed at the identification of the degradation products of aflatoxins and to the assessment of the technical and economic sustainability of remediation of aflatoxin-contaminated commodities through mushroom bioconversion into non-toxic feeds. Further research is needed to improve the stability of SMS extracts and to implement their use in the pipeline of feed processing.

Chapter 3

Evaluation of the mushroom *Pleurotus eryngii* mycelium as biosorbent for aflatoxin B1

3.1 Abstract

The present study explores the adsorption potential, of the ground not-viable mycelium, of the edible fungi *Pleurotus eryngii* (ITEM 13681) to absorb AfB1.
For the preparation of biosorbent material, the mycelium was autoclaved, lyophilized and finely ground. High performance liquid chromatography (HPLC) with florescence detector was used to define the efficiency of adsorption of this biomaterial.
Preliminary studies were based on the evaluation of the influence of different parameters: pH (5, 7), AfB1 concentrations (50 and 1000 ng/mL), time (30 and 120 min), temperature (25, 37°C), fungal mass (50 and 1000 mg), on the absorption capability of *P. eryngii* mycelium. This biomaterial seems able to absorb AfB1 at 37°C, irrespective of the pH, to values that depend on mycotoxin concentration and biosorbent mass. AfB1 mass balance demonstrated that mycelium extraction with methanol and ethyl acetate was respected, in fact mycotoxin recovery has been 108±6% and 91±6%, respectively.
Optimal absorption was obtained at the lowest AfB1 concentration and the highest fungal mass. Stability of AfB1-biosorbent binding and desorption studies, were carried out varying, respectively, the pH at 7 and 3, for 24 hours of incubation at room temperature, in the dark. Results showed that the percentage of the released mycotoxin were 6±2% at pH 7 and 10±4% at pH 3. These assays revealed that AfB1 was stably and spontaneously absorbed by *P. eryngii* mycelium, over time.
Finally, Langmuir model, better described the absorption mechanism at isothermal conditions, and the thermodynamic perameters showed thet the adsorption was a spontaneous and physical process.
Our results suggest that biosorbent obtained from the mycelium of *P. eryngii* could represent a cheap and effective feed additive for AfB1 detoxification.

3.2 Introduction

Mycotoxins are secondary metabolites produced by toxigenic fungi on a wide variety of agricultural products (Bryden, 2007). Nearly 25% of agricultural products are contaminated with mycotoxins making them unfit for human consumption, with most of the contaminated cereal grains being diverted to animal feed production (Pittet, 1998). The most toxic mycotoxin is aflatoxins B1, a potent carcinogen to humans and classified as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC, 1993). Biological, physical and chemical methods (Huwig *et al*., 2001) have been proposed to decontaminate mycotoxins and keep their presence within allowable limits.

Physical methods have gained much importance because of their low cost and applicability to larger quantities of samples. The addition of adsorbents to mycotoxin-contaminated diets with the aim of reducing the toxin bioavailability in the gastrointestinal tract of animals is a widely used method for detoxifying animal feed (Rustom, 1997). Adsorbents previously reported for the sequestration of aflatoxins are activated charcoal, aluminosilicates and some specific polymers (Decker and Corby 1980; Ramos *et al.* 1996; Grant and Phillips 1998; Jard *et al.* 2011). All of these adsorbents, suffer from a major disadvantage of either, high cost or non-specificity. Furthermore, their use is limited because they are non-degradable and cause environmental deposition problem when excreted in manure (Huwig *et al.* 2001).

Biomasses, derived from bacteria, fungi, algae and other biological materials, could be used to remove unwanted molecules through absorption mechanism (Maurya *et al*., 2006). Most of the studies concentrated on biodegradation and biosorption properties of living fungi. There are a few studies on the using of dead fungal biomass. Although, both living and dead fungi have been shown to be capable to attract several compounds, due to the presence of various functional groups on the biomass (Crini *et al*., 2006; Fu *et al*., 2001). However, dead cells offer several advantages over living cells. Firstly, the use of dead fungal cells obviates the need for nutrients requirements to their growth. In addition, dead fungal biomass can be stored easily and kept for prolonged periods (Low *et al*., 2008).

One of the most recent approaches to the problem is the use of biosorbents in the diet of animals that can sequester the mycotoxins and reduce their bioavailability for gastrointestinal absorption, thereby avoiding toxicosis (Castellari *et al*., 2001;

Bejaoui *et al*., 2005; Ringot *et al*., 2005; Yiannikouris *et al.* 2006; Quintela *et al.* 2012; Avantaggiato *et al*., 2014). The utilisation of biosorbents provides dual advantages: firstly, it creates a use for an available and renewable biomass that would otherwise be discarded and, secondly, it provides required dietary fibre content essential for animal health (Hetland *et al*. 2004; Achak *et al*. 2009).
Fungal mycelium has notable adsorbing properties, mainly due to the ability of the polysaccharides constituting the cell wall to form hydrogen, ionic or hydrophobic interactions with organic and inorganic molecules (Huwing *et al*., 2001). These properties are the subject of research with practical applications in different contexts, including bioremediation of soils and wastewater from heavy metals and organic pollutants (Kahraman *et al*., 2012), and it is conceivable their use also for the detoxification of agricultural products, in particular cereals and grains, contaminated with mycotoxins.
Some studies have demonstrated the ability of some strains of lactic acid and bifidus bacteria to efficiently bind AfB1 (El Nezami *et al*., 1998; Peltonen *et al*., 2001), through a chemical and physical phenomenon associated to the features of structural elements of the bacterial cell wall, such as peptidoglycans and polysaccharides (Kabak, 2006).
In this study, the adsorbent capacity of fungal mycelium for *in vitro* removal of aflatoxin B1, has been investigated. Various parameters including pH, adsorbent dosage, contact time and temperature were evaluated in order to achieve maximum mycotoxin removal. Results of this study will provide a basis for further research on the use of fungal biosorbents as possible adsorbents for the removal of mycotoxins. Proven in its efficacy *in vivo*, could be a low-cost decontamination method for incorporation in animal feed.

3.3 Materials and methods

3.3.1 Reagents

All solvents (grade HPLC) were purchased by V.W.R. International S.r.l (Milan, Italy), water was of Milli-Q quality (Millipore, Bedford, MA, USA). Regenerated cellulose membranes filters (RC 0.2 μm) were obtained from Phenomenex (Bologna, Italy). The filter paper used was Whatman no. 4 (Whatman, Maidstone, UK). Sodium chloride, sodium dihydrogen phosphate, potassium hydrogen phosphate, tri-sodium citrate 2-hydrate, potassium chloride, sodium malonate hydrate, 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonate (ABTS)

and AfB1 mycotoxin were purchased by Sigma Aldrich (Milan, Italy). Malt extract agar (MEA), and malt extract broth (MEB) were bought by Oxoid (Basingstoke, UK).

3.3.2 Preparation of the standard solution

The standard solution of AfB_1 at 1 mg/mL was prepared by dissolving the solid commercial mycotoxin in toluene/acetonitrile (9:1, v/v). The stock solution was diluted, at a concentration of 10 μg/mL and quantified according to AOAC Official Method 971.22 (AOAC, 2000). The stock solution was evaporated at 50 °C with air stream and dissolved in appropriate buffers (pH 5 or 7) at a concentration of 500 ng/mL. The calibration solutions were obtained by diluting at 0.6, 1.2, 2.4, 5.7, 11.0, 23.0, 57.0 ng/mL. The solutions were stored at -20 °C and warmed to room temperature before use. The 0.1 mol/L phosphate buffer (PBS) was prepared by dissolving sodium chloride, sodium dihydrogen phosphate, potassium hydrogen phosphate and potassium chloride in water and adjusted to pH 7 or to pH 8. The 0.01 mol/L acetate buffer (pH 5) was prepared by dissolving tri-hydrate sodium acetate in water adjusted to pH 5 with acetic acid. The 1 mmol/L citrate buffer (pH 3) was prepared by dissolving tri-sodium citrate 2-hydrate in water and adjusted to pH 3 with citric acid.

3.3.3 Preparation of the Pleurotus eryngii mycelium

The isolate of *P. eryngii* ITEM 13681 used in this study, was obtained from the collection of Institute of Sciences of Food Production (ITEM Collection, http://www.ispa.cnr.it/Collection/, Bari, Italy). The culture was grown in purity on malt extract agar (MEA) slants, for 30 days at 28 °C, and used as sources of inoculum for subsequent cultures in malt extract broth (MEB). Five mycelial plugs (8 mm-diameter), were transferred onto Roux flasks filled with 200 mL of MEB and incubated under static conditions for 20 days at 28 °C. After incubation, the mycelium was separated from the culture broth by filtration through filter paper by applying vacuum and then washed four times with 25 mL of sterile distilled water. The biomass collected was then inactivated by autoclaving at 121 °C for 20 min, lyophilized and finally ground with mortar and pestle, then sieved to collect a fine powder (particle size ≤ 500 μm).

3.3.4 Dosage of laccase activity

A 100 mmol/L sodium malonate buffer (pH 4.5) was prepared by dissolving sodium malonate hydrate in distilled water. The solution was adjusted to pH 4.5 with 100 mmol/L malonic acid. An amount of 0.1 grams of ground autoclaved mycelium was extracted with 5 mL of 0.1 M phosphate buffer (PBS) pH 7.3 and incubated for 60 min, at 25 °C in a rotary shaker at 150 rpm. The extract obtained was filtered and used for the enzymatic assay. The laccase activity was determined spectrophotometrically by oxidation of 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonate (ABTS) at 37 °C as described by (Li *et al.*, 2008). The reaction was performed in 100 mM of sodium malonate buffer (pH 4.5), 2 mM of ABTS and an appropriate amount of enzyme extract in a final volume of 1.5 mL. The oxidation of the ABTS was evaluated spectrophotometrically (Varian Cary 50) by the increase of absorbance at 420 nm. One laccase unit was defined as the quantity of enzyme able to oxidize 1 µmol of ABTS in a min, given a molar extinction coefficient ε_{420} = 36000 M^{-1} cm^{-1}.

3.3.5 Analysis of Aflatoxin B1

The chromatographic analysis of the AfB1 was performed by HPLC (Agilent Technology Series 1260) coupled to a fluorescence detector (FLD). The mycotoxin was detected by photochemical post-column derivatization reaction (UVE™ LCTech GmbH, Obertaufkirchen, Germany). A Synergi 4U MAX-RP 80A reverse phase column (150x4.6 mm, 4.0 µm) (Phenomenex, Torrance, California, USA) was used, preceded by a pre-column (MAX-RP, 4 x 3.0 mm, Phenomenex) thermostatically controlled at 40 °C. The mobile phase consisted of water:acetonitrile 60:40 (v/v), with a flow rate of 1 mL/min. The fluorometric detector was set at the wavelengths of 365 nm (excitation) and 435 nm (emission). Under these analytical conditions, the retention time of the AfB1 was about 6 min. AfB1 was quantified by measuring the peak area and comparing it with the calibration curve obtained with standard solutions. The quantification limit of the method (LOQ) was 0.6 ng/mL, based on a 10:1 signal to noise ratio.

3.3.6 Preliminary test

Preliminary test has been done evaluating different amounts of powdered biomass (25, 50, 75, 100, 150, 200 mg), while other parameters remained constant, such as temperature and pH.

This study was tested in triplicate. The biosorbent was weighted in a 15 mL test tubes and suspended 8 mL of AfB1 500 ng/mL in acetate and PBS buffer. The suspension was shaken at 250 rpm, for 90 minutes, at 25 and 37 °C. At the end of the incubation period, the samples were centrifuged at 10000 rpm for 10 minutes. Supernatant was removed, and the pellet was washed three times, with the related buffer solution, and analyzed by HPLC/FLD. The efficiency of adsorption (% A) were calculated using following equation:

$$Ads\% = \frac{C_0 - C_e}{C_0} \cdot 100 \qquad (1)$$

Where C_0 and C_e are the initial and the final concentration (supernatant plus washing solution) of AfB1 after adsorption process. A blank control was prepared using the mycotoxin working solution in buffer without mycelium.

3.3.7 Variables affecting the adsorption and optimization of the process

A full factorial design 2^4 was adopted. All the experiments were done in duplicate, arranged in random blocks to avoid systematic errors and were performed in two different working days. The variables studied were pH of solution (5 and 7), time of interaction (30 and 120 min), mass of adsorbent (50 and 500 mg) and concentration of AfB1 (50 and 500 ng/mL). The *p*-values calculated from the analysis of variance (ANOVA) were used to check the significance ($p < 0.05$) of the effect of different parameters and the interactions between variables (Kavak, 2009).

Optimization of the process was carried out, considering the two most significant parameters obtained from the previous analysis: mass of adsorbent and concentration of AfB1. A completely randomized factorial experimental design 3^2 was used for optimization, in which the mass of adsorbent (400, 700, 1000 mg) and AfB1 concentration (50, 525, 1000 ng/mL), were investigated at 3 levels and 5 centerpoints.

Samples preparation were performed as described in paragraph 3.3.6.

3.3.8 Desorption

To assess the desorption of AfB1, aliquots of *P. eryngii* powdered mycelium were weighed and subjected the adsorption treatment at pH 5. After recovery of the supernatant and the washing solutions, the remaining pellet was treated with 8 mL of either citrate buffer (pH 3) or PBS buffer (pH 7). The tubes were kept at room temperature in the dark for 48 h and subsequently centrifuged for 10 min

at 10000 rpm at 25 °C. The supernatant was recovered and analyzed by HPLC/FLD. The experiments were carried out in triplicate.

The percentage of desorption was determined by comparing the quantity of mycotoxin relased (q_{des}) and that adsorbed ($q_{ads,}$) on mycelium, according to the following equation (2):

$$\%D = \frac{q_{des}}{q_{ads}} \cdot 100 \qquad (2)$$

The mycotoxin relased (q_{des}) per gram of biomass was calculated from the concentration of mycotoxin after desorption (C_{des}), equation:

$$q_{des} = C_{des}\frac{V}{m} \qquad (3)$$

Where V was the volume of the solution and m was the weight of the biosorbent.

3.3.9 Mass Balance

To test the biosorbent for reusability, a new absorption experiment was carried out as described in paragraph 3.3.6. At the end of the process, AfB1 recovered supernatant, washing solutions, were analyzed by HPLC/FLD. The residual AfB1 adsorbed to the pellet, was extracted in tubes with methanol and with ethyl acetate, kept at 40 °C in the dark for 1 hour and subsequently centrifuged for 10 min at 900 rpm at 25 °C. The supernatant was recovered and analyzed by HPLC/FLD.

3.3.10 Adsorption Isotherms

The adsorption isotherms were determined to study the effect of the amount of adsorbent (isotherm I) and of the AfB1 concentration (isotherm II) on the mycotoxin binding by mycelium. Experiments were set up using 30 min as contact time at pH 7. For the isotherm I the fixed concentration of AfB1 was 200 ng/mL and the amount of mycelium varied from 600 to 1200 mg. For isotherm II the fixed amount of mycelium was 250 mg and the concentration of AfB1 varied from 200 to 2000 ng/mL.

The amount of adsorbed mycotoxin was calculated as, the difference between the amount of mycotoxin in the supernatant of the blank tubes without mycelium and, the amount of mycotoxin found in the supernatant of the experimental tubes with adsorbent. This amount was related then to the quantity present in the supernatant of the blank tubes and expressed in percent. The amount of bound

mycotoxin per unit mass of fungal mycelium was calculated using the following equation:

$$Qeq = \left(\frac{C_0 - C_{eq}}{m}\right) \cdot V \qquad (4)$$

where Q_{eq} is the quantity of mycotoxin adsorbed per nanogram of fungal mycelium (ng/mg); C_0 is the concentration of mycotoxin in the supernatants of the blank tubes with no mycelium (ng/mL); C_{eq} is the residual mycotoxin concentration in the supernatant of the experimental tubes with adsorbent at equilibrium (μg/mL); V was the volume of solution (mL); and m was the mass of fungal mycelium (mg).

Langmuir and Freundlich isotherm (equations 5 and 6) were used to describe the adsorption equilibrium (Freundlich 1906; Langmuir 1916):

$$Qeq = Qmax \frac{K_L \cdot Ceq}{1 + K_L \cdot Ceq} \qquad (5)$$

Where Q_{max} is the constant related to maximum mycotoxin uptake and K_L is constant related to the energy of adsorption and affinity of the adsorbent,

$$Qeq = K_F \cdot Ceq^{1/n} \qquad (6)$$

Where K_F is the constant related to capacity of the adsorbent for the mycotoxin and n is adsorption intensity.

Adsorption isotherms were obtained by plotting the amount of mycotoxin adsorbed per unit of mass of adsorbent (Qeq) against the concentration of the toxin in the external phase (Ceq), under equilibrium conditions (Qeq = f(Ceq)). These data were transferred to OriginPro and fitted by the Langmuir and Freundlich isotherm models.

The adsorption isotherms were obtained by plotting the values of the amount of mycotoxin (mg/g) adsorbed at the equilibrium (Q_{eq}) as a function of the amount of residual mycotoxin in solution (ng/mL) at the equilibrium (C_{eq}), and reporting the percentage of adsorption as a function of the dosage of the adsorbent (mg/mL). The data were fitted by the Langmuir and Freundlich isotherm models. A dimensionless constant, known as the separation factor (K_R), derived from the Langmuir equation (K_L is the Langmuir constant) was used to assess the favorability of adsorption:

$$K_R = \frac{1}{1 + K_L C_0} \qquad (7)$$

The Gibbs free energy change (ΔG^0, kJ/mol), the standard enthalpy (ΔH^0, kJ/mol) and the standard entropy (ΔS^0, kJ/mol·K) were calculated according to Kavak (2009).

3.3.11 SEM characterization

For scanning electron microscope (SEM) investigations, the samples were previously fixed on an aluminum stub with a carbon-based, electrically conductive, double sided adhesive disc and then sputtered with a 30 nm thick carbon film using an Edwards Auto 306 thermal evaporator.
Images of the samples were taken with a secondary electrons (SE) detector mounted on a SEM of LEO, model EVO50XVP. Operating conditions of the SEM were: 7.5 kV accelerating potential, 500 pA probe current and 9 mm working distance.

3.3.12 Statistics

The results obtained were subjected to one-way ANOVA with a significance level of $p < 0.05$. The data processing software used was OriginPro 2017 (OriginLab Corporation, Northampton, Massachusetts, USA). The statistical software used was STATGRAPHICS® centurion XVII (Statpoint Technologies, Inc. The Plains, Virginia, USA).

3.4 Results and discussion

In order to rule out the occurrence of enzymatic degradation, in the reduction of AfB1 concentration in the solutions exposed to the adsorbent, mycelium of *P. eryngii* was autoclaved to obtain denaturation of the proteins, grounded and subsequently extracted with PBS (pH 7.3). The extract was then analyzed for laccase activity. No laccase activity was found in the *P. eryngii* mycelium subjected to the heat treatment. This allowed to clarify that the removal of AfB1 in the working solutions treated with autoclaved *P. eryngii* mycelium was not due to enzymatic degradation.
Preliminary studies were based on the evaluation of the influence of different parameters on the AfB_1 absorption capacity of the mycelium of *P. eryngii*. The maximum percentage of AfB1 adsorption at 37°C has been 64±6% at pH 5 and 68±3% at pH 7 (Figure 3.1) Instead at 25°C was 22±6% at pH 5 and 26±3% at pH 7 with 200 mg of adsorbent (data not shown).

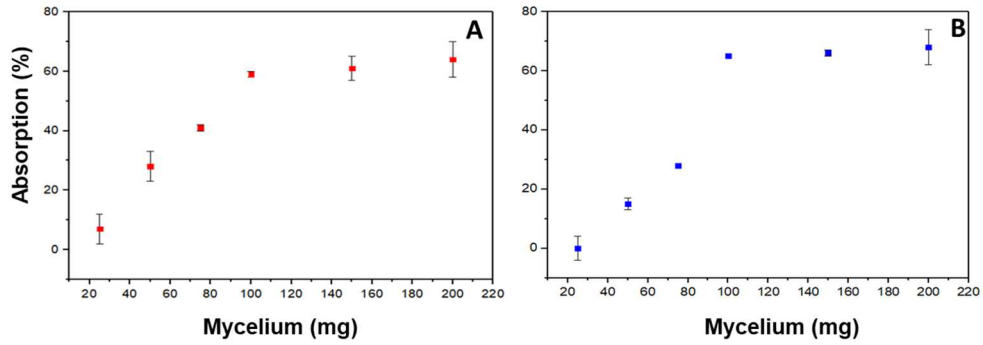


Figure 3.1 Absorption of AfB1 at 37°C, at pH 5 (A) and pH 7 (B).

A factorial design was employed to reduce the total number of experiments needed to achieve the optimization of the system. The adopted design, determined which factors have significant effects on the system response and, how the effect of one factor was related to the levels of the other factors (interactions). The analysis of variance (ANOVA) was employed to analyze the role of different variables (pH, time, mass of the adsorbent and concentration of AfB1) on the adsorption process. The pH is not envolved in the absorption process, because AfB1 is a non-ionizable molecule. While, the main factors and interaction effects are shown in Table 3.1. Only two factors, the mass of adsorbent and the concentration of AfB1, were significantly different, with a confidence level of 95.0% ($p < 0.05$). Time, pH and interaction between factors were not statistically significant. The Pareto chart of standardized effects at $p < 0.05$ is presented in Figure 3.2. The same factors, the mass of adsorbent and the concentration of AfB1, presented an absolute value higher than 2.3 ($p < 0.05$), which were located at right of the dash line, were statistically significant.

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A: pH	69.0313	1	69.0313	0.20	0.6613
B: m	3260.28	1	3260.28	9.34	0.0062
C: AfB1	16607.5	1	16607.5	47.57	0.0000
D:t	247.531	1	247.531	0.71	0.4097
pH x m	57.7813	1	57.7813	0.17	0.6885
pH x AfB1	11.2813	1	11.2813	0.03	0.8591
pH x t	13.7813	1	13.7813	0.04	0.8445
m x AfB1	205.031	1	205.031	0.59	0.4524
m x t	344.531	1	344.531	0.99	0.3324
AfB1 x t	0.03125	1	0.03125	0.00	0.9925
blocks	38.2813	1	38.2813	0.11	0.7440
Total error	6982.13	20	349.106		
Total (corr.)	27837.2	31			

Table 3.1 Effects of pH, mass of mycelium (m), AfB1 concentration (AfB1) and time (t), and interactions on AfB1 adsorbtion by *P. eryngii* mycelium. The effects are statistically significant if the P-Value is < 0.05 (95% confidence level).

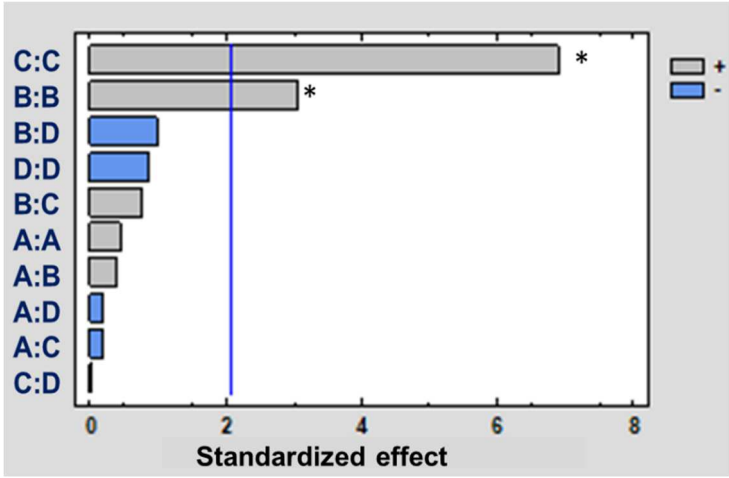


Figure 3.2 Standardized Pareto Chart for Absorption. A is pH, B is adsorbent mass, C is mycotoxin concentraton and D is time. * P-Value < 0.05

Mass of adsorbent and concentration of AfB1 were identified as effective factors of adsorption and their effects were optimized by a factorial experiment, in which, the two variables were investigated at 3 levels, as reported in paragraph 3.3.7.

The 3^2 factorial design matrix and the results of the experiments are shown in Table 3.2.

Run	Mycelium (mg)	AfB1 (ng/mL)	Absorption (%)
1	1000	525	62
2	700	525	58
3	700	1000	59
4	700	525	59
5	1000	50	96
6	700	525	61
7	700	525	61
8	400	1000	46
9	700	50	72
10	400	50	78
11	700	525	62
12	400	525	53
13	1000	1000	60
14	700	525	60

Table 3.2 Full Factorial Design results

The model espressed by equation (8), where the variables are expressed in their original units, represents the removal efficiency of AfB1 (*Ads %*) as a function of m and AfB1.

$$Ads\ \% = 70.1995 + 0.0154816m - 0.0640237AfB_1 + 0.00000784314m^2 - 0.00000701754mAfB_1 + 0.0000385856{AfB_1}^2 \quad (8)$$

The model equation is useful for indicating the direction in which the variables should be changed in order to optimize the AfB1-removal efficiency of the adsorbent. The ANOVA is presented in Table 3.3. The statistical significance of each coefficient was determined by *P*-values: the smaller the *P*-values, the more significant is the coefficient. This implies that the first-order main effects of mass of adsorbent and mycotoxin concentration are more significant than their quadratic main effects. However, the quadratic main effect of AfB_1 concentration is more significant than other second main effects.

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
m	280.167	1	280.167	10.65	0.0115
AfB1	1093.5	1	1093.5	41.57	0.0002
m^2	1.41176	1	1.41176	0.05	0.8226
mAfB1	4.0	1	4.0	0.15	0.7067
$AfB1^2$	214.745	1	214.745	8.16	0.0212
Total error	210.422	10	26.3027		
Total (corr.)	1867.21	13			

Table 3.3 The analysis of variance of the significant of each coefficient

The fit of the model was checked by determination of the coefficient (R^2). In this case, the value of the determination coefficient (R^2 = 0.8873) indicated that the 11.27% of the total variable was not explained by the model.

The effects of the initial concentration of AfB1 and the quantity of the mycelium, on mycotoxin removal efficiency, were shown in Figure 3.3. The working conditions at the optimum point for removal efficiency of AfB1 were determined as follows: m = 1000 mg, AfB_1 = 50 ng/mL.

As a result, the optimum removal efficiency of AfB1 was calculated as 90.07%. The experimental removal efficiency was 85±13%.

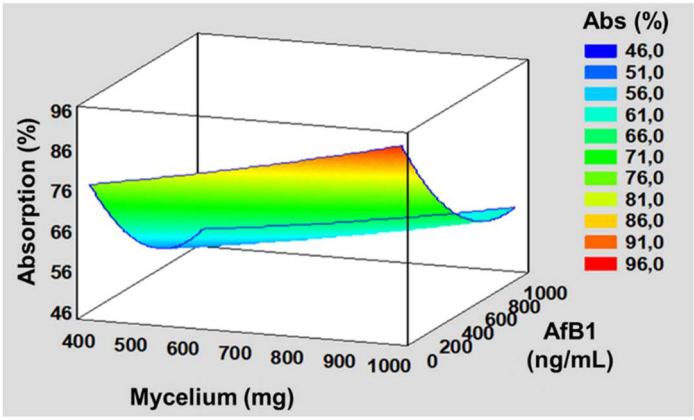


Figure 3.3 Estimate response surface plot for the effect of mass of adsorbent and mycotoxin concentration on the AfB1 removal.

A SEM micrograph of the *P. eryngii* mycelium was shown in Fig. 3.4 The surface appears rough and sponge-like. The approximate pore size of 5-15 μm, was measured from SEM micrographs.

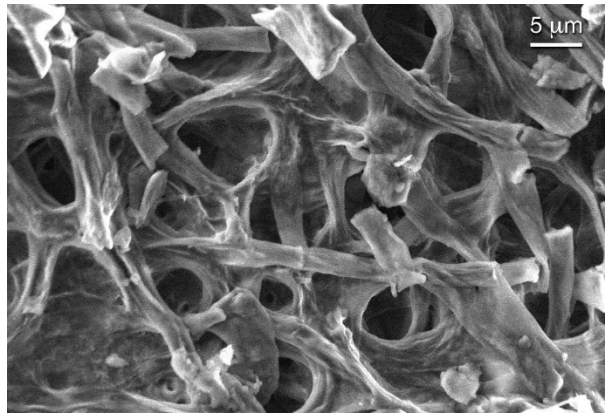


Figure 1.4 SEM micrograph of *P. eryngii* mycelium.

Several adsorption isotherms models were frequently used to describe experimental adsorption data. Among them, the Langmuir and Freundlich models are the most employed. In this work, both models were used to study the effect of mycotoxin concentration, keeping constant temperature (37 °C) and pH (7), by testing a fixed amount of mycelium (250 mg) and increasing mycotoxin concentration (from 200 to 2000 ng/mL) (Figure 3.5 A). Also, the effect of adsorbent dosage was evaluated, at constant temperature (37 °C) and pH (7), by testing a fixed amount of mycotoxin (200 ng/mL) and increasing adsorbent dosage (from 600 to 1200 mg) (Figure 3.5 B).

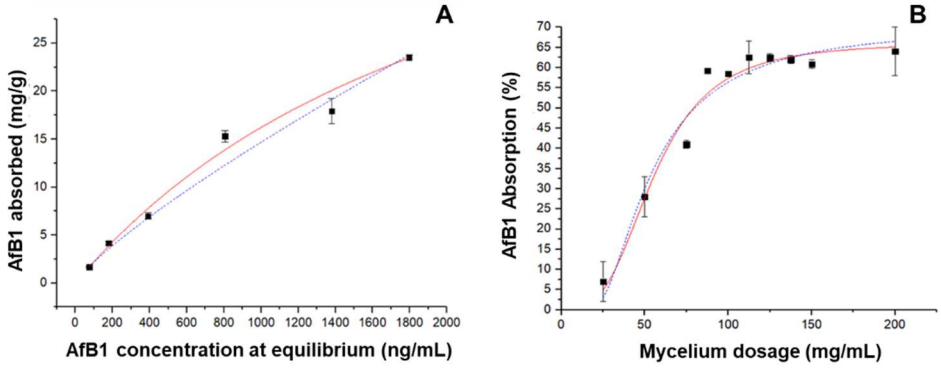


Figure 3.5 A) Effect of AfB1 concentration on adsorption by mycelium. B) Effect of mycelium dosage on AfB1 adsorption. In red the curve of Langmuir, in blue of Freundlich.

The linear regression analysis was applied to assess the goodness of fits and, to calculate the parameters involved in the absorption mechanism (Table 3.4). The

results obtained by comparing R^2 and SS_{res} showed that, for both the effect of adsorbent quantity and the effect of AfB1 concentration, the isotherm that fits the experimental data is the Langmuir isotherm. This suggests that the AfB1 adsorption mechanism is monolayer and that occurs at a finite (fixed) number of definite equivalent sites (Langmuir, 1918). The model describes a homogeneous adsorption in which each molecule has enthalpy and activation energy of the constant process and is graphically characterized by a plateau, such as a saturation point where each molecule occupies a site and there can be no further adsorption.

The Langmuir model can be used to predict whether the adsorption system is favorable or unfavorable by calculating the dimensionless constant K_R (Weber *et al.*, 1974). For favorable adsorption the K_R value should fall in the range 0 to 1. The adsorption is considered unfavorable when $K_R > 1$, the isotherm is linear when $K_R = 1$ and the adsorption is irreversible when $K_R = 0$. In this study, the values of K_R for AfB1 adsorption on *P. eryngii* mycelium are comprised between 0 and 1, which suggests a favorable process for the system.

model	parameter	Effect of AfB1 concentration	Effect of adsorbent dosage
Langmuir	K_L (±SE)	$(4.3 \pm 0.6)\cdot 10^{-4}$	$(3 \pm 7)\cdot 10^{-6}$
	q_m (±SE)	53 ± 19	66 ± 3
	R^2	0.9976	0.9698
	SS_{res}	16.47	100.70
Freundlich	K_F (±SE)	$(50 \pm 8)\cdot 10^{-3}$	70 ± 6
	1/n (±SE)	0.82	2.2 ± 0.5
	R^2	0.9942	0.9580
	SS_{res}	39.03	140.20

Figure 3.4 Effect of adsorbent dosage on AfB1 adsorption by mycelium. Equilibrium adsorption isotherms were obtained at constant temperature (37 °C) and pH (7) by testing a fixed amount of mycotoxin with increasing adsorbent dosage.

The effect of temperature on the adsorption of AfB1 by *P. eryngii* mycelium was investigated. The uptake of AfB1 was found to increase when temperature increase: 66% ±3 at 22 °C, 67 %±0 at 37 °C and 85% ±3 at 50 °C. This indicated the endothermic nature of the adsorption process, as confirmed also by a positive ΔH^0.

Molar free energy change of the adsorption process (ΔG^0), standard enthalpy change (ΔH^0) and standard entropy change (ΔS^0) are showed in Table 3.5.
The negative ΔG^0 values are indicative of a spontaneous adsorption process. The ΔG^0 values decreased as the temperature raised, which is an indication of a physical nature of the adsorption process. Generally, the free energy variation for the physical adsorption is between -20 and 0 kJ / mol, while in the chemisorption the range is -80 -400 kJ / mol (Kavak, 2009). Beside of the physical nature of the process, the experimental data show that the adsorption process needs to be activated by a moderately high temperature. This implies that the process is reversible and that the material can be regenerated by an appropriate treatment.

Temperature (°C)	$\ln K_0$	ΔG^0 (kJ/mol)	ΔH^0 (kJ/mol)	ΔS^0 (kJ/mol K)
22	0.65	-1.59		
37	0.71	-1,83	30.62	0.11
50	1.77	-4.76		

Table 3.2 Thermodynamic parameters for the AfB_1 adsorption

To verify the stability of the system over time, the percentage of desorption of the mycotoxin adsorbed on *P. eryngii* mycelium was assessed at room temperature and at the pH values of 3 and 7.
Desorption studies, showed a very low desorption after 48 h at 25 °C, at all the pH values tested. The percentage of desorption was 10 ± 4 % at pH 3 and 6 ± 2 % at pH 7. These results indicate a good stability of the system.
AfB1 mass balance demonstrated that mycelium extraction with methanol and ethyl acetate was respected, in fact, mycotoxin recovery has been 108±6% and 91±6%, respectively. These results supported a possible re-utilization of the adsorbent after use, by regeneration of the adsorbing properties with an appropriate chemical treatment.

3.5 Conclusions

The results presented in this study show that *P. eryngii* mycelium is an efficient biosorbent of AfB1.
The study was conducted to identify the major factors involved in the absorption process. The concentration of mycotoxin in the solution and the quantity of adsorbent material were identified as determinants of the process. The pH of the

solution was irrelevant in a range from 5 to 7. In addition, the system worked with no significant variation in the time lapse from 30 to 120 min of exposure. This allowed to obtain a system that reached the equilibrium in a short time (30 min) and that, as assessed by desorption measurements, remained stable, with reduced desorption for 48 h at room temperature.

The thermodynamic study of the process showed that this is a spontaneous process, with ΔG^0 = -2.73 kJ/mol (average of ΔG^0 at three temperatures 22, 37 and 50 °C), endothermic (ΔH^0 = 30.62 kJ/mol and ΔS^0 = 0.11 kJ/mol·K) and that it is a physical adsorption, regulated by weak and reversible interactions, whereby the material can be regenerated with an appropriate treatment with organic solvent.

Optimization of biosorption resulted in 85 ± 13% of AfB1 removal efficiency by *P. eryngii* mycelium. This biological and edible material is therefore completely different from the inorganic materials currently used in the feed industry. These results prompt us to pursue the experimentation of the use of *P. eryngii* biomass as an adsorbent material to be used as feed additive for bioremediation of AfB1-contaminated feeds.

Chapter 4

Mycotoxins degradation by Ery4 laccase from *Pleurotus eryngii* and Lac2 laccase from *Pleutotus pulmonarius* with redox mediators

4.1 Abstract

Laccases are multicopper oxidases that find application as versatile biocatalysts for the, so called, "green" bioremediation of environmental pollutants and xenobiotics. In this study we elucidated the degrading activity of Lac2 pure enzyme form *Pleurotus pulmonarius* towards aflatoxin B1 (AfB1) and M1 (AfM1); and Ery4 from *Pleurotus eryngii* to degrade AfB1 and other mycotoxins, such as, fumonisin B1 (FB1), ochratoxin A (OTA), deoxynivalenol (DON), zearalenone (ZEA) and T-2 toxin. Adding redox mediators to the reactions, were found to drastically increase the degradation efficiencies of those enzymes. In fact, mediator addition enhances Lac2 activity, till 90% of AfB1 degradation, while AfM1 was completely degraded. Ery4 laccase-mediator system (LMS), were able to degrade AfB1, FB1, OTA, ZEN and T-2 toxin at 73%, 74%, 27%, 100% and 40%, respectively. No degradation was observed for DON. LMSs were evaluated combining AfB1 and ZEA, or FB1 and T-2 toxin, that were simultaneously degraded by 86% and 100%, or 25% and 100%, respectively.
The novelty of this study relies on the identification of a pure enzyme as Lac2 capable to degrade AfB1 and, for the first time, AfM1, this evidenced that, the mechanism for the effective degradation occurs via mediators' mediation. LMS proved, once more, to be a promising approach to enhance degradation properties of laccase enzymes useful for the potential development of a multi-mycotoxin reducing method. These results gave new perspective for Lac2 and Ery4 application in the food and feed supply chains as a biotransforming agent of the main mycotoxins.

Note: Some of the contents of this chapter were previously published in the following articles:

Loi M., Fanelli F., Cimmarusti M. T., Mirabelli V., Haidukowski M., Logrieco F., Caliandro R., Mulè G. (2018) *In vitro* single and combined mycotoxins degradation by Ery4 laccase from *Pleurotus eryngii* and redox mediators. Food Control, 90, 401.

Loi, M., Fanelli, F., Zucca, P., Liuzzi, V. C., Quintieri, L., Cimmarusti, M. T., Monaci, L., Haidukowski, M., Logrieco, A. F., Sanjust, E., Mulè G. (2016). Aflatoxin B_1 and M_1 Degradation by Lac2 from *Pleurotus pulmonarius* and Redox Mediators. Toxins, 8, 245.

4.2 Introduction

Food and feed contamination by mycotoxins is a concerning issue worldwide, due to their adverse health effects and their important economic impacts (Rocha *et al*, 2014). Biological degradation is a strategy which can be used to mitigate mycotoxins contamination through a mild, sustainable and environmental friendly approach. Mycotoxins bioremediation by enzymes has been recently reviewed (Loi *et al*., 2017), with a focus on laccases. Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are multicopper oxidases widely distributed in plants, bacteria, insects, and fungi (Claus, 2004). Among fungi, white rot basidiomycetes, such as *Pleurotus spp*. are the most efficient producers of laccases (Osma *et al*., 2010). Laccases catalyze the oxidation of phenols, aromatic amines, and other non-phenolic compounds through one-electron oxidation of the reducing substrate, and enzyme reoxidation by means of molecular oxygen, which is in turn reduced to water. Laccase activity can be further extended to non-phenolic substrates by the use of synthetic or natural redox mediators (Zucca *et al*., 2015). The mediators, after being oxidized by laccase, diffuse out of the active site and oxidize recalcitrant compounds which possess high redox potential or high molecular weight. Being structurally diverse, different mediators may act on chemically-unrelated compounds, widening laccase substrate range (Baiocco *et al*., 2003).

Several molecules have been used as redox mediators in the laccase mediator system (LMS). Synthetic mediators such as 2,2-azino-bis-[3-ethylbenzo-thiazolin-sulfonate] (ABTS), 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO), and 1-hydroxybenzotriazole (HBT), have been widely used in many biocatalytic processes (Camarero *et al*., 2005; Moldes, *et al*., 2008) However, their use is limited due to their elevated cost, toxicity, and the high mediator-substrate molar ratio needed. In LMS, oxidative capability is enhanced by the generation of a new, often radical, species; oxidation mechanisms are diversified according to the nature of the mediator used; the use of a free effector, the oxidized mediator, circumvent steric hindrance. Indeed, the degradation of recalcitrant and chemically heterogeneous compounds, including aflatoxin B1 (AfB1), aflatoxin M1 (AfM1) and Zearalenone (ZEN), by LMS has been previously reported (Banu *et al*., 2013; Loi *et al*., 2016, 2017). Additionally, laccases have been already used in food processing to improve the techno-functional properties of bakery, dairy and meat products (Osma *et al*., 2010). To this purpose, a scientific risk

assessment on one laccase from *Trametes hirsuta* by EFSA is currently in progress (EC, 2016).

In the current study we tested the degrading activity of purified Lac2 from *Pleurotus polmonarius* towards AfB1 and AfM1, elucidating the effect of direct and mediated oxidation using a model synthetic mediator, ABTS, and two naturally-occurring phenols, acetosyringone (AS) and syringaldehyde (SA).

In addition, we describe the application of Ery4 laccase from *Pleurotus eryngii* coupled with various redox mediators for the in vitro degradation of AfB1, fumonisin B1 (FB1), ochratoxin A (OTA), deoxynivalenol (DON), ZEA and T-2 toxin. At the end, the simultaneous degradation of a combination of AfB1 and ZEA, and FB1 and T-2 toxin was assessed in order to investigate the feasibility of a potential laccase and LMS application in food and feed.

4.3 Materials and methods

4.3.1. Chemicals

2-azino-di-[3-ethylbenzo-thiazolin-sulphonate] (ABTS), acetosyringone (AS), syringaldehyde (SA), p-coumaric acid (p-CA), 1-hydroxybenzotriazole (HBT) 2,2,6,6-tetramethylpyperidyloxil (TEMPO), phenol red (PhR), chlorogenic acid (CGA), ferulic acid (FA), mycotoxin standards (purity > 99%) of OTA, AfB1, AfM1, DON, T-2 toxins, and ZEA were purchased from Sigma Aldrich (Milan, Italy). FB1, α-zearalenol (α-ZON) and β-zearalenol (β-ZON) were purchased from Biopure (Romer Labs Diagnostic GmbH). Regenerated cellulose membranes filters (RC, 0.2 µm) were obtained from Alltech Italia-Grace Division (Milano, Italy). All solvents (HPLC grade) were purchased from J. T. Baker Inc. (Deventer, The Netherlands). Ultrapure water was produced by Milli-Q system (Millipore, Bedford, MA, USA).

4.3.2 Laccase production and purification

Lac2 laccase purification was performed according to Zucca *et al*., (2011) with slight modifications (Loi, *et al* 2016).

Ery4 laccase was produced from *Saccharomyces cerevisiae* ITEM 17289 of the Agri-Food Microbial Fungi Culture Collection of the Institute of Sciences of Food Production (www.ispa.cnr.it/Collection), subsequently engineered with pYES2 vector (Invitrogen, USA) bearing ery4 gene sequence from *Pleurotus eryngii*, as described in Loi *et al*, 2018.

4.3.3 Laccase spectrophotometric activity assay

Laccase activity was photometrically measured (Ultraspec 3100pro, Amersham Pharmacia Biotech Italia, Cologno Monzese, Italy). The reaction was performed in 100 mM sodium acetate pH 4.5, 5 mM ABTS and the appropriate amount of enzyme solution in a final volume of 1 mL. The oxidation of ABTS was determined after 10 min by photometric assay at 420 nm (ε_{420} = 36,000 M^{-1} cm^{-1}). One unit was defined as the amount of enzyme which oxidized 1 µmol of substrate per min (Bleve *et al*., 2008).

4.3.4 In vitro degradation assays

Lac2 and Ery4 degradation capability assays were performed in 500 µL of sodium acetate buffer 1 mM, pH 5. One µg/mL of AfB1 and 2.5 U/mL of Lac2 were added to each reaction. As redox mediators, ABTS, AS, and SA were independently tested at 1 and 10 mM. With respect to AfM1, degradation assays were performed by incubating 0.05 µg/mL of AfM1, 2.5 units of Lac2 and ABTS, AS, or SA as redox mediators at 10 mM.

To evaluate the degrading capability of Ery4, 1 µg/mL of AfB1, FB1, ZEA, DON and T-2 toxin and 0.5 µg/mL of OTA, in combination with 3 or 9 U/mL of laccase enzyme, were added to each reaction and gently mixed. Subsequently, eight different redox mediators at two different concentrations (1 and 10 mM) were independently tested, in combination with Ery4 (5 U/mL) to assess their ability to degrade AfB1, FB1 and OTA. In particular, four natural mediators (AS, SA, p-CA and FA) and four artificial ones (ABTS, TEMPO, HBT, PhR) were tested. ZEA, DON and T-2 toxin degradation were assayed in the presence of three redox mediators, SA, TEMPO and ABTS, respectively. Coupled degradation, like AfB1/ZEA was assessed using either SA, TEMPO or both; while FB1/T-2 only with TEMPO. For each toxin, the starting concentration was 0.5 µg/mL, while the final mediator concentration was 10 mM.

In control samples, the enzymatic solution was replaced by an equal volume of buffer. Reactions were incubated at 25°C for three days in the dark. Each experiment was performed in triplicate.

4.3.5 Chemical analyses

Different chemical methods were performed to quantify the residual mycotoxin concentration in the samples.

AfB1 analyses were performed by high performance liquid chromatography with fluorescence detection (HPLC-FLD) as previously described by Loi *et al.* (2016). The limit of quantification (LOQ) was 0.4 ng/mL, based on a signal to noise ratio of 10:1.

AfM1 analyses were performed by high performance liquid chromatography with fluorescence detection (HPLC-FLD) as previously described by Loi *et al.* (2016). The limit of quantification (LOQ) was 1 ng/mL, based on a signal to noise ratio of 10:1.

Samples containing FB1 were filtered using RC (0.20 µm) filters and quantified by HPLC-FLD after derivatization with o-phtaldialdehyde (OPA) (Haidukowski *et al.*, 2017). LOQ was 20 ng/mL based on a signal to noise ratio of 3:1. Standards of partially hydrolyzed fumonisins (PHFB1) and hydrolyzedfumonisin (HFB1) were prepared from pure FB1 according to the procedure described by De Girolamo (2014). Multimycotoxin calibration solutions were opportunely prepared by diluting with acetonitrile-water (1:1, v/v) to obtain calibrant solutions with concentration in a range of 10-1000 ng/mL for FB1, 100-1000 ng/mL for PHFB1 and 1000-5000 ng/mL for HFB1. Determination of PHFB1, HFB1 and FB1 was performed by ultraperformance liquid chromatography with an AcquityQDa mass detector (UPLC-QDa). The chromatographic separation was performed on an Acquity UPLC BEH C18 column (2.1 x 100 mm, 1.7 µm) preceded by an Acquity UPLC® in-line filter (0.2 µm). Column temperature was set at 50 °C. The flow rate of the mobile phase was set at 0.4 mL/min. Eluent A was water, and eluent B was methanol, both containing 0.1% acetic acid. An elution gradient was applied by changing the mobile phase composition from 10% to 50% of eluent B in 10 min, kept constant for 4 min, then linearly increased up to 90% in 3 more min, and finally kept constant for 4 min. For column re-equilibration, Eluent B was decreased to 10% in 1 min and kept constant for 3 min. For LC/MS analyses, the ESI interface was used in positive ion mode, with the following settings: desolvation temperature 600 °C; capillary voltage 0.8 kV, sampling rate 5 Hz.

The mass spectrometer operated in full scan (100-800 m/z) and in single ion recording (SIR) mode, by monitoring the individual masses of each compound (FB1 722.40 m/z, PHFB1 564.00 m/z, HFB1 406.30 m/z). Retention time for HFB1, PHFB1 and FB1 were about 14, 15, 16 min, respectively. Toxins were quantified by measuring peak areas and comparing these values with a calibration curve obtained from standard solutions. Empower™ 2 Software (Waters) was

used for data acquisition and processing. LOQ values were 10 ng/mL for FB1, 100 ng/mL for HFB1 and 1000 ng/mL for PHFB1, based on a signal to noise ratio of 10:1.

OTA quantification by HPLC-FLD was performed as described by Ferrara *et al.* (2014) and De Bellis *et al* (2015). LOQ for OTA and OTα were 0.5 ng/mL, based on a signal to noise ratio of 10:1.

DON levels were determined by ultra performance liquid chromatography linked with photodiode array detector (UPLC-PDA) (Pascale *et al.*, 2014). The LOQ was 100 ng/mL, based on a signal to noise ratio of 10:1.

The quantification of T-2 and HT-2 toxins by UPLC-PDA was performed as described by Pascale *et al.* (2012). LOQ for T-2 and HT-2 toxins were 50 ng/mL.

A novel chromatographic method was performed to quantify ZEA. ZEA stock solution (1 mg/mL in methanol) was diluted in methanol to a concentration of 10 µg/mL. The exact concentration of ZEA stock solution was spectrophotometrically determined (ε= 12623 L/mol cm at λ= 274 nm in methanol). The stock solution was evaporated to dryness under a stream of nitrogen at 50 °C. The residue was dissolved in water:acetonitrile (50:50, v/v) and diluted, in order to obtain a standard calibration curve (10-300 ng/mL). Alpha and β-ZON stock solutions (10 mg/mL in acetonitrile) were diluted with water to obtain a concentration of 5 mg/mL in acetonitrile:water (1:1, v/v). The standard calibration curve ranged from 10 to 300 ng/mL. A volume of 100 µL was injected in the HPLC system (Agilent 1260 Series, Agilent Technology, Santa Clara, CA, USA) with a full loop injection system. The analytical column was a Luna C18 (150 x 4.6 mm, 5 µm) (Phenomenex, Torrance, CA) preceded by a guard column inlet filter (0.5 µm x 3 mm diameter, Rheodyne Inc. CA, USA). The column was thermostated at 30 °C. The mobile phase consisted of a water/acetonitrile (50:50, v/v) eluted at a flow rate of 1.0 mL/min. The fluorometric detector for ZEA was set at wavelengths, λ_{ex}= 274 nm, λ_{em}= 440 nm, DAD detector at 236 nm for α-ZON and 240 nm for β-ZON. Data acquisition and instrument control were performed by LC Openlab software (Agilent). With this mobile phase, the retention time of α-ZON, β-ZON and ZEA were about 4.4 min, 3.5 min and 7.3 min, respectively. ZEA, α-ZON and β-ZON and were quantified by measuring peak areas, comparing them with the calibration curves obtained with standard solutions for each mycotoxin. The LOQ of the method was 15 ng/mL for α-ZON, β-ZON and ZEA, based on a signal to noise ratio of 10:1.

4.3.6 Statistical analysis

All data are mean ± standard deviation of three independent replicates. Data were expressed as mean percentage ± standard deviation with respect to the control. Results were analyzed through Student's t-test (paired comparison) performed using STATISTICA software for windows, ver. 7 (Statsoft, Tulsa, and Okhla). Differences between samples and relative control were considered significant for a P value < 0.05 or <0.01.

4.4 Results and discussion

Lac2 and Ery4 degrading capability of different mycotoxins were investigated without mediators or with different LMSs. In particular, AS, SA, p-CA and FA are the natural phenols deriving from siringyl and cynnamic acids, while HBT, TEMPO, ABTS and PhR are the artificial compounds, used as mediators in LMS. Among the three different mechanisms described so far, the natural phenols and HBT follow the Hydrogen Atom Transfer (HAT) mechanism, TEMPO follows the ionic route, while ABTS the Electron Transfer (ET) mechanism (Baiocco *et al*., 2003; Fabbrini *et al*., 2001).

Direct oxidation of AfB_1 by means of Lac2 alone accounted for 23%. The addition of a redox mediator resulted in a very effective degradation of the toxin. The lowest concentrations of ABTS and AS (1 mM) were able to double the degradation percentage compared to Lac2 alone (45% and 42%, respectively), while the presence of SA mediator at 1 mM, lowered the degradation percentage (13%). AfB1 degradation, enhanced furtherly, when the concentration of each mediator was 10 mM, reaching 90% for AS, 81% for ABTS and 72% for SA, respectively. With regards to AfM1, Lac2 proved to completely degrade the toxin with all the mediators added at 10 mM, since, AfM1 was not detected by HPLC with fluorescence detector (LOD 0.12 ng/mL) (Figure 4.1).

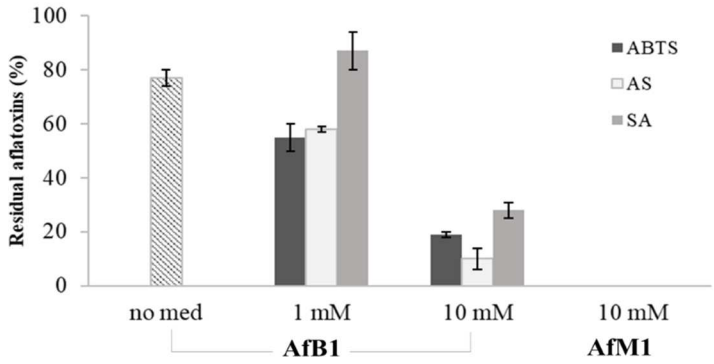


Figure 4.1. Residual AfB1 and AfM1 (%) after 3 days of incubation at 25°C, performed by Lac2 and the respective redox mediator in sodium acetate buffer 1 mM, pH 5. Values are the mean of three replicates and the error bars represent the standard error measured between independent replicates.

Conversely, Ery4 laccase was not able to directly oxidize AfB1, suggesting that this toxin is not a substrate of Ery4 (data not shown). AfB1 degradation was achieved by using different mediators. Syringyl-type phenols (AS and SA) were the best performing mediators, followed by ABTS and cynnamic acid derivatives (FA, p-CA), while HBT, PhR and TEMPO were ineffective. Higher degradation percentages were reported for AS, SA, p-CA and PhR, when 10 mM mediator was used compared to 1 mM (73% vs 51% for AS, 68% vs 48% for SA, 22% vs 0% for p-CA and 11% vs 0% for PhR). By contrast, 10mM was detrimental in the case of ABTS (39% with 1 mM vs 25% with 10 mM) and FA (24% with 1mM vs 17% with 10 mM) (Figure 4.2).
The results confirmed the efficacy of these LMSs for AfB1 degradation.
Lac2 from *P. pulmonarius* efficiently degradaded AfB1 by 90%, 72% and 81% using 10 mM of AS, SA and ABTS redox mediators, respectively (Figure 4.1).
Addictionally, Ery4 obtained the best results with AS and SA, degrading AfB1 by 73% and 68%, respectively (Figure 4.2).

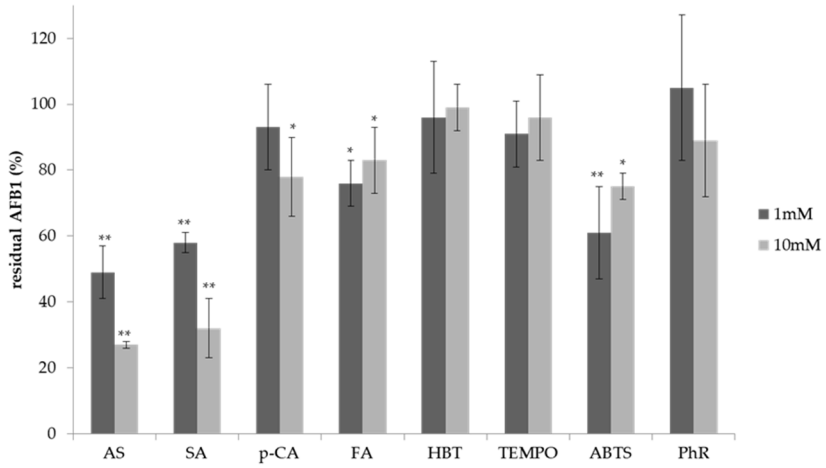


Figure 4.2 Residual AFB_1 in samples treated with different mediators. A *P* value <0.05 was considered statistically significant (*) and a P value <0.01 is considered highly statistically significant (**).

The binding architecture of AfB1 within the catalytic site of laccase is responsible for the success of AfB1 oxidation and varies among laccase enzymes and within their isoforms, too. Indeed, Lac2 from *Pleurotus pulmonarius* was able to directly oxidize AfB1, though with low efficacy (Figure 4.1). An in-silico study on laccase isoforms from *Trametes versicolor (*Dellafiora *et al*,. 2017) proposes that AfB1 degradation is isoform-dependent. Also, laccase limited oxidative capacity might hinder direct AfB1 oxidation. In fact, oxidoreductive enzymes with greater oxidative capacity than laccase, such as peroxidases from *Pleurotus spp.* and *Armoracia rusticana*, were reported to efficiently degrade AfB1 (Chitrangada *et al*., 2000; Yehia *et al*., 2014).

AfB1 degradation was putatively achieved through the HAT mechanism. After an initial hydrogen atom removal, further electronic rearrangements lead to the loss of the coumarin and/or lactone moieties, responsible for AfB1 characteristic fluorescence.

FB1 was not degraded by direct Ery4 oxidation (data not shown). However, a statistically significant degradation ($P < 0.01$) was achieved using TEMPO 10 mM (74%), PhR 10 mM (30%) and SA 1 mM (25%) (Figure 4.3). The other concentrations applied, or the other mediators tested were ineffective or not statistically significant. The known FB1 biotransformation method relies, for example, on esterases activity, which hydrolyse the two ester bonds of FB1 (Duvick *et al*., 2001; EFSA, 2014). Since no other enzymatic method was

reported for FB1 degradation, and no known hydrolysed products were detected in the samples, the complete mechanism underlying FB1 oxidation with laccase and TEMPO has not been clarified, yet.

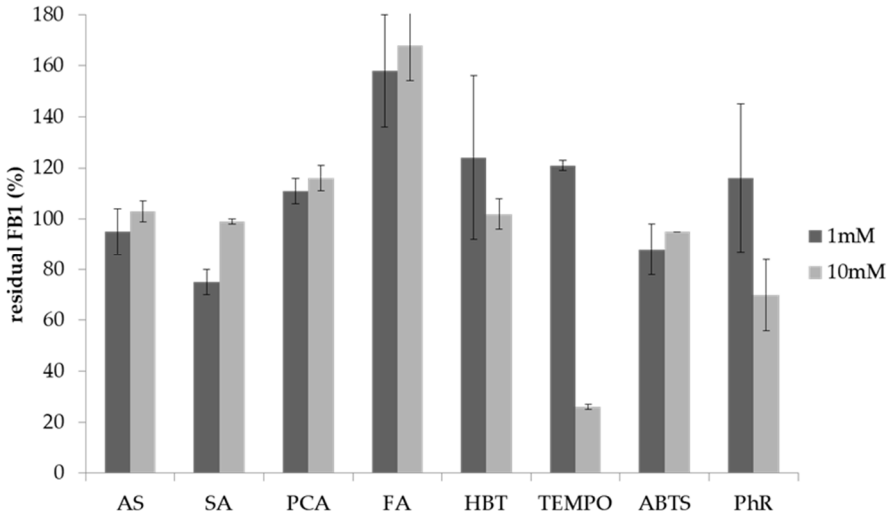


Figure 4.3 Residual FB_1 in samples treated with different laccase mediator systems. A P value <0.01 is considered highly statistically significant (**).

TEMPO acts through an ionic route. Specifically, once oxidized into the oxoammonium ion by laccase, the nitrogen atom of TEMPO becomes susceptible to the nucleophilic attack by an oxygen atom, such as a primary hydroxyl group, as reported for the oxidation of the model compound 4-methoxybenzyl alcohol to the corresponding aldehyde (Fabbrini *et al.*, 2001). Nonetheless, one or more hydroxyl groups of the aminopolyol backbone of FB1 ought to be involved in the first steps of the degradation process.

Ery4 was not able to directly degrade OTA (data not shown), nevertheless a slight reduction was observed in the presence of redox mediators (Figure 4.4). Natural phenols were the best performing mediators, with degradation percentages of 27% for FA (10 mM), 25% for SA and p-CA (1 mM) and 24% for AS (1 mM), respectively. Regarding the artificial compounds, degradation percentage was 22% for HBT (1 mM), 20% for TEMPO (1 mM) and 18% for PhR (1 mM), respectively. Only FA proved to be more efficient when added at higher concentration (10 mM).

LMS oxidation of OTA was not as effective as other enzymatic methods, by which 80 to 100% of OTA degradation could be obtained in few hours of assay

(Abrunhosa *et al.*, 2010). Currently, the peferred enzymatic methods for OTA degradation, rely on specific peptidases, able to break the amide bond releasing OTα and phenylalanine. In particular, OTα is considered a non-toxic compound with a half life, 10 times shorter than OTA, in humans (Klimke *et al.*, 2015). No other enzymatic method was reported for OTA degradation.

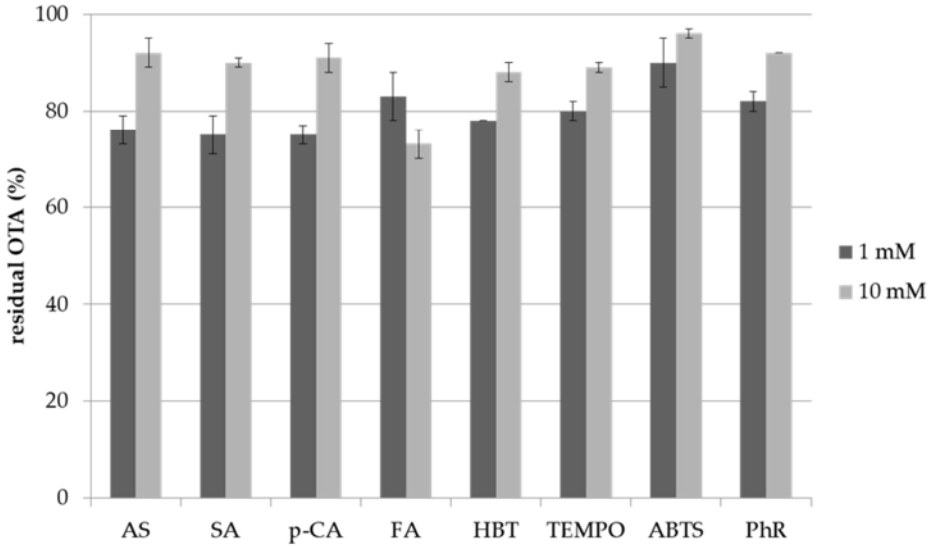


Figure 4.4 Residual OTA in samples treated with different laccase mediator systems. AS: acetosyringone, SA: syringaldehyde, p-CA: p-coumaric acid, FA: ferulic acid, HBT: 1hydroxybenzotriazole, TEMPO: 2,2,6,6-tetramethylpiperidin-1-yl)oxyl, ABTS: 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), PhR: phenol red. All data are statistically significant ($P< 0.01$).

Ery4 laccase was not able to directly oxidize ZEA, DON and T-2 toxin (data not shown).

According to the preliminary data regarding AfB1, FB1 and OTA, the mediator's screening for ZEA, DON and T-2 toxin was reduced to SA, ABTS and TEMPO, at 10 mM, the representatives of the three different LMS mechanisms.

ZEA was completely removed with all the tested mediators (data not shown). This result is in agreement with Banu *et al.* (2013), despite enzyme, mediator and toxin concentrations were significantly lower than those used in our study, they reported that ZEA was degraded up to 81.7% using 0.16 mM ABTS as redox mediator. As expected, considering laccase oxidative nature, in LMS treated samples, nor α or β-zearalenol were detected (data not shown), indeed, they originated from ZEA reduction.

DON is considered the most recalcitrant toxin to degrade. Under the tested conditions no LMS was effective towards DON (data not shown). Peroxidases from *Aspergillus oryzae* and *Rhizopusoryzae* were positively correlated with DON degradation in submerged fermentation (Buffon *et al.*, 2011), meaning that a higher redox capacity than that of laccase or LMS tested may be necessary to degrade DON. T-2 toxin was slightly degraded by LMS (data not shown). A statistically significant degradation ($P < 0.01$) was obtained using TEMPO (40%), and ABTS (13%), while SA was ineffective. Since LMS was not able to degrade DON, a comparative structural investigation of the two trichothecenes could suggest a possible starting point for the degradation of T-2 toxin. Laccase-TEMPO system ought to firstly act on the acetoxy group in C15 or the ester in C8 positions. In accordance to this hypothesis, de-acetylation to HT-2 toxin was excluded, as it was not found in degraded samples. No biotransformation of T-2 toxin with enzymes, including laccases, has been reported so far.

Results of combined mycotoxin degradation for AfB1/ZEA are shown in Figure 4.5. AfB1 and ZEN were simultaneously degraded when SA, or SA and TEMPO were used as redox mediators. In accordance with the results reported before, AfB1 was significantly degraded only in presence of SA, while ZEA was almost completely removed either with SA, TEMPO or both. Since degradation percentages are comparable to those reported for the single toxin experiments, no relevant synergistic, or additive effects could be hypothesized to occur in the presence of both mediators.

Regarding the simultaneous degradation of FB1/T-2 toxin by Ery4 and TEMPO, T-2 toxin degradation was greatly enhanced with respect to the single degradation assay (100% vs 40%, $P < 0.001$), pointing to a strong additive effect. By contrast, an antagonistic effect was reported for FB1, whose degradation was drastically reduced from 74% to 25%, with respect to the single degradation assay.

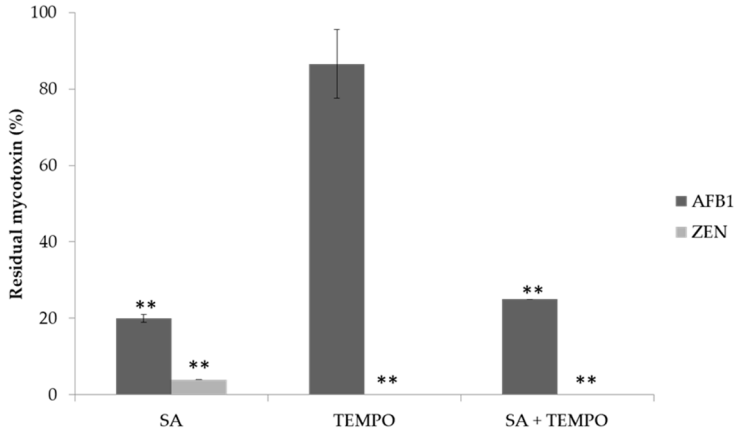


Figure 4.5 Residual AfB_1 and ZEA in samples treated with different laccase mediator systems. TEMPO: 2,2,6,6-tetramethylpiperidin-1-yl)oxyl, SA: syringaldehyde. A *P* value < 0.01 is considered statistically significant (**).

4.5 Conclusions

In this study, for the first time, an extensive *in vitro* screening towards multiple toxins was performed with two pure laccases enzymes and different LMSs. Mediators were found to drastically increase catalytic efficiency of Lac2 and Ery4. The degradation activity of Lac2 towards two aflatoxins: AfB1 and AfM1 using three different mediators, were respectively, more than 70% for AfB1, while AfM1 was completely degraded.

The degradative action of Ery4 towards AfB1, FB1, OTA, ZEA and T-2 toxin were 73%, 74%, 27%, 100% and 40%, respectively. By contrast, no degradation occurred for DON with any of the LMSs tested.

Another novelty presented in this work is the use of LMS for the simultaneous degradation of multiple toxins, possibly with the use of a single mediator. AfB1 and ZEA were simultaneously degraded by 86% and 100%, while FB1 and T-2 by 25% and 100%, respectively. A strong additive effect was found for the T-2 degradation in the presence of FB1, enforcing the advantages of using LMS to selectively degrade toxins.

This study represents a starting point for the development of methods to counteract the natural co-occurrence of multiple mycotoxins in raw materials, or in food and feed by means of an efficient, environmental friendly and versatile laccase enzyme.

Chapter 5

Screening of Laccase-Mediator Systems to reduce mycotoxin levels by liquid chromatography-mass spectrometry (LC-MS/MS)

5.1 Abstract

The natural co-occurrence of mycotoxins in food and feed is a well-known problem, and can be explained by at least three reasons: (i) most fungi are able to produce several mycotoxins concurrently; (ii) food commodities can be contaminated by several fungi simultaneously or in quick succession and (iii) animal diets are usually made up. Most of the published data has concerned the major mycotoxins aflatoxins (AFs), ochratoxin A (OTA), zearalenone (ZEA), fumonisins (FUM) and trichothecenes (TCTs), especially deoxynivalenol (DON). Concerning cereals and derived cereal product samples, among the 127 mycotoxin combinations described in the literature, AFs+FUM, DON+ZEA, AFs+OTA, and FUM+ZEA are the most observed. Strategies for the reduction of mycotoxins contaminations can adopt methodologies, one of the most promising consist in the use of enzymes, particularly laccases.

The action of laccase with different type of mediators as acetosyringone (AS), syringaldehyde (SA), and 2,2,6,6-tetramethyl-piperidinyl-oxyl (TEMPO) towards seven mycotoxins, simultaneously, showed the reduction of multiple toxic molecules at various levels. A liquid chromatography/tandem mass spectrometry method was developed for the simultaneous determination of the seven mycotoxins: aflatoxins B1 (AfB1), ochratoxin A (OTA), fumonisins B1 (FB1), deoxynivalenol (DON), fusaric acid (FA), zearalenone (ZEA) and T-2 toxins. For each laccase-mediator system considered, different results were obtained in base of mediator studied. The laccase-AS system reacted with DON, FB1, AfB1, OTA and ZEA with following percentage of degradation: 57%, 24%, 87%, 100% and 58%, respectively. In the laccase-SA system, DON, FB1, AfB1and ZEA, were degraded by 70%, 22%, 51% and 94%, respectively. At the end, the reduction observed for laccase-TEMPO were more than 90% for DON, 70% for FA, 60% for FB_1 and AfB1, respectively.

This study proves for the first time the possible applications of laccase-mediator systems to efficiently detoxify more mycotoxins, at the same time.

5.2 Introduction

Mycotoxins are toxic secondary compounds synthesized under specific conditions by certain fungal species capable of growing in a wide variety of food and feed commodities. The main mycotoxins are: aflatoxin B1 (AfB1), ochratoxin A (OTA), fumonisin B1 (FB1), zearalenone (ZEA). trichothecenes such as: deoxynivalenol (DON) and T2 toxin (T2). These metabolites are produced mainly by fungi of the genera *Aspergillus*, *Fusarium* and *Penicillium* (Anfossi, *et al* 2016). A mycotoxin is generally produced by closely related species; for example, aflatoxins are produced by a few species in the genus *Aspergillus*. Instead, OTA is produced by *Aspergillus spp.* or *Penicillium spp*. depending on the commodity and environmental conditions. In some cases, a single *Fusarium spp*. can form more than one mycotoxin, for example, DON and ZEA.

Other secondary fungal metabolites can exert their toxicity toward plants and animals, such as fusaric acid (FA) produced from several *Fusarium* species. This picolinic acid derivative is known for its high phytotoxicity, it causes diseases in a great variety of plants (tomato and banana for example) and it is directly related to the severity of damping off, vascular wilt and root rot diseases of numerous vegetable crops. In addition, it exhibits toxicity towards animals, including notochord malformation in zebrafish and neurochemical effect in mammals. Many plant species can metabolize FA into less phytotoxic products (Karlovsky, 1999). Some foods and feeds are often contaminated by numerous mycotoxins, but most studies have focused on the occurrence and toxicology of a single mycotoxin. Regulations throughout the world do not consider the combined effects of mycotoxins. However, several surveys have reported the natural co-occurrence of mycotoxins from all over the world.

Concerning cereals and cereal derived product samples, among the 127 mycotoxin combinations described in the literature, AFs+FB, DON+ZEA, AFs+OTA, and FB+ZEA are the most observed (Smith *et al*., 2016).

Co-occurrence of multiple mycotoxins in cereal grains and their derived products is an important aspect that need to be futher investigated. The development in analytical techniques, such as, the increased availability of liquid chromatography coupled with mass spectrometry instrumentation, enabled the detection of multiple mycotoxins, simultaneously.

Next to the availability of powerful analytical methods that let to simultaneously detect multiple mycotoxins, new strategies capables to conteract the contamination of food and feed by more mycotoxins, are necessary. Among them, the possible approaches are represented by chemical, physical and biological methods. The latter, appear to be the more promising, because it could selectively remove toxic compounds without altering the palatability of food, matching both safety and quality characteristic, thus, improving consumer satisfaction. Among the emerging biological strategies, the use of laccase enzymes seems to have a wide action spectrum towards different molecules, that comprise fungal secondary metabolites, such as mycotoxins. Laccasses, widely produced by plants, fungi, bacteria, and some insects, catalyze the oxidation of a broad number of phenolic compounds and aromatic amines by using molecular oxygen as the electron acceptor, which is reduced to water (Reinhammar and Malstrom, 1981). To extend the activity of this enzyme to non-phenolic substrates, such as mycotoxins, it is possible adding the appropriate redox mediator to the reaction. Natural molecules as syringaldehyde (SA) and acetosyringone (AS) or synthetic compounds like 2,2,6,6-tetramethyl-piperidinyl-oxyl (TEMPO), are the most common mediators capable to interact with laccase enzyme in the first step of the reaction, and once oxidized, react with mycotoxins.

In this survey, the simultaneous action of laccase mediator systems (LMSs) toward FA and the most common mycotoxins, was investigated for the first time. For this purpose, several degradation assays were performed, screening the effect of different redox mediators on the activity of laccase from *Trametes versicolor* (EC 1.10.3.2) towards FA and mycotoxins, such as: DON, T2, FB_1, AfB1, OTA and ZEA. As redox mediator, AS, SA and TEMPO were used. Degradation action by laccase mediator systems were evaluated setting up an analytical method with separation by chromathography reverse phase and detection with triple quadrupole (QQQ) operating in multiple reaction monitoring (MRM) mode.

5.3 Materials and Methods

5.3.1 Chemical and Materials

Methanol (LC-MS grade), ethyl acetate, formic acid, sodium acetate trihydrate, FA were supplied by Carl Roth GmbH (Karlsruhe, Germany). 2,20-azino-bis (3-

ethylbenzothiazoline-6-sulfonic acid) (ABTS), AS, SA, TEMPO, laccase from *Trametes versicolor*, AfB1, T2 toxin were obtained from Merck KGaA (Darmstadt, Germany). DON, OTA were given from AppliChem GmbH (Darmstadt, Germany). FB_1 come by Enzo Life Science GmbH (Lörrach, Germany). ZEA from Romer Labs Diagnostic GmbH (Tulln, Austria). Water Millipore Milli-Q system (Sartorius, Gottingen, Germany).

5.3.2 Laccase activity

Laccase enzymatic activity was spectrophotometrically determined monitoring the absorbance at 420 nm, that describe the oxidation rate of ABTS substrate to ABTS radical cation ($ABTS^{\bullet+}$). The reaction was performed in a final volume of 1 mL, with 100 mM sodium acetate buffer (pH 5), 5 mM ABTS and an appropriate amount of enzyme solution. Therefore, enzyme unit (U) is defined as the amount of enzyme that catalize the oxidation of one µmol of substrate per minute, at 25°C, under the tested conditions. Laccase activity (U/mL) is calculated on the base of Lambert-Beer law with some modification, through the following equation:

$$\frac{U}{mL} = \frac{\Delta Abs \times Vr \times df}{d \times \varepsilon \times t \times Ve}$$

where ΔAbs represent the absorbance of the sample compared to blank control; Vr is the reaction volume expressed in mL; df is the dilution factor; d is the light path (1 cm); ε is the extinction coefficient (36 $M^{-1}cm^{-1}$); t is the reaction time expressed in minute and Ve is the enzyme volume added to the reaction.

5.3.3 LMS-multiple mycotoxins assay

Degradation assay was performed in a final reaction volume of 200 µL. The samples were prepared as follows: 1 UI of enzyme, 10 mM of AS, SA and TEMPO mediator and 1 µg/mL of each mycotoxins (DON, T2, FB1, AfB1, FA, OTA and ZEA) dissolved in 1 mM sodium acetate buffer (pH 5). Reactions were incubated at 25°C in the dark, with mild shaking and sampled at 0 hour, 1, 2, 3 and 6 days, replicated two times. The percentage of degradation for each mycotoxin, was evaluated on the basis of the positive control (toxins and mediator) and was calculated by the following formula:

$$D(\%) = \frac{Ci - Cf}{Ci} \times 100$$

where Ci was the concentration of mycotoxin in the positive control and Cf was the concentration of mycotoxin in the sample.

To stop the reaction and remove protein interferences, three volumes of acetonitrile were added to each sample after the incubation time. Solutions were vortexed for 2 min, left to stand for 20 min at 4°C and centrifuged for 20 min at 15000 rpm, 4°C. The supernatant was recovered and dried using a SpeedVac vacuum concentrator system. After the evaporation, the pellet was resuspended in water: methanol (75:25, v/v).

5.3.4 Multiple mycotoxins analysis

Standard solution containing the 7 mycotoxins were prepared at the following concentration: 6.25, 62.5, 125, 250, 500, 1000 ng/mL in water: methanol (75:25, v/v).The analytical column utilized was a Kinetex (5 x 2 mm, 2.5 µm, Phenomenex, Torrance, CA). Five µL of samples were injected using an Agilent 1290 Infinity Binary Pump LC coupled to an Agilent 6460 triple quadrupole MS/MS with Jet Streaming technology and electrospray ionization (ESI) using Agilent MassHunter software. MS source parameters were as follows: gas temperature, 350 °C; gas flow 13 L/min; nebulizer 60 psi; capillary voltage 4000 V. Data acquisition was performed in Dynamic MRM mode with positive ESI using one principal MRM transition for quantitation and one additional transition to serve as a qualifier for each analyte (Table 5.1). The mobile phase consisted of water with 0.1% of formic acid as solvent A and methanol as solvent B, and the flow rate was 0.2 mL/min. A gradient elution was applied by changing the mobile phase composition from 15% to 98% of eluent B in 9 min, and kept constant for 1 min, and after 30 sec to 15%, to re-equilibrate the column. The temperature of the column was maintained at 40 °C. With this mobile phase, the retention time were for DON about 2 min; FA 3.7 min, AfB1 5.3 min, FB_1 6.2 min; T2 6.7 min, ZEA 7.2 min, OTA 7.3 min (Figure 5.1). Limit of detection (LOD) were the following mycotoxins: DON 2.5 ng/mL, FA 1 ng/mL, AfB1 0.1 ng/mL, FB1 10 ng/mL, T2 2 ng/mL, OTA 0.01 ng/mL and ZEA 0.1 ng/mL. Data acquisition and instrument control were performed by Quantitative Analysis (version B.08.00) from Agilent Technologies.

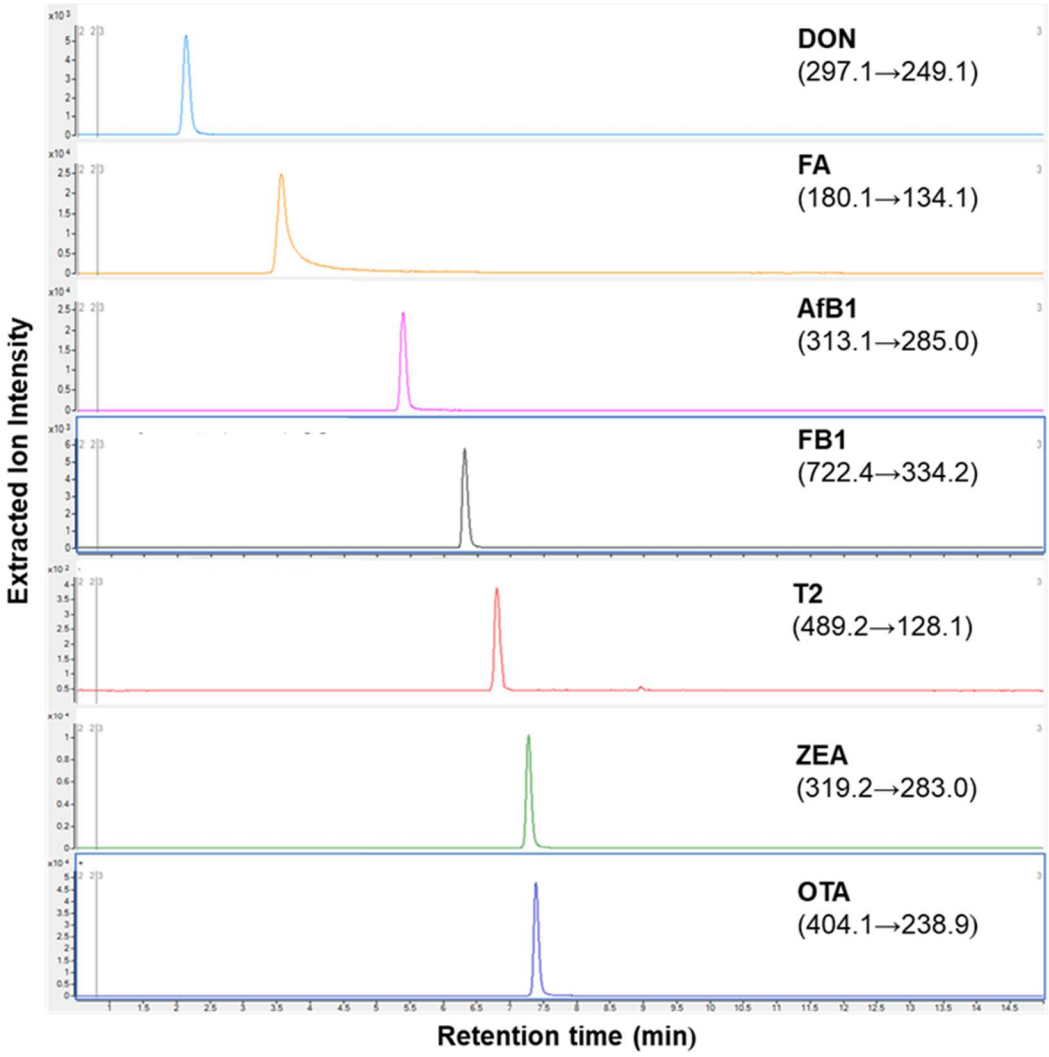


Figure 5.1 Extracted ion chromatogram (quantification transitions). For each mycotoxin is shown the multiple reaction monitoring (MRM) used for quantification.

Analyte	Precursor ion	Q1 (m/z)	Q3 (m/z)	CE (V)	Fragmentor (V)
DON	$[DON+H]^+$	297.1	91.2	64	100
			249.1*	4	100
FA	$[FA+H]^+$	180.1	162.1	15	90
			134.1*	15	90
AfB1	$[AfB1+H]^+$	313.1	269.0	27	135
			285.0	27	135
FB_1	$[FB_1+H]^+$	722.4	352.2	45	160
			334.2*	45	160
T2	$[T2+Na]^+$	489.2	115.0	142	170
			128.1*	98	170
ZEA	$[ZEA+H]^+$	319.2	301.1	12	90
			283.0*	12	90
OTA	$[OTA+H]^+$	404.1	358.0	17	110
			238.9*	17	110

Table 5.1 LC-MS/MS parameters for mycotoxins detection. (Q1 first quadrupole, Q3 third quadrupole, * transitions used for quantification, CE collision energy).

5.4 Results and discussion

The laccase-AS system and laccase-TEMPO system reacted with most of the mycotoxins evaluated, compared to laccase-SA system. After the proteins removal step, the recoveries were calculated for each mycotoxin (1 µg/mL), considering each LMSs.

For laccase-AS system, the recoveries were the following: 63% (± 2%) for AfB1, 68% (± 3%) for FA, 117% (± 9%) for FB_1, 86% (± 3%) for DON, 98% (± 4%) for ZEA, 103 % (± 4%) for OTA, and 98% (± 3%) for T2, respectively.

For laccase-SA system, the recoveries were the following: 57% (± 3%) for AfB1, 67% (± 3%) for FA, 108% (± 9%) for FB_1, 65% (± 4%) for DON, 87% (± 5%) for ZEA, 91 % (± 4%) for OTA, and 86% (± 3%) for T2, respectively.

For laccase-TEMPO system, the recoveries were the following: 61% (± 5%) for AfB1, 89% (± 4%) for FA, 114% (± 7%) for FB_1, 81% (± 3%) for DON, 91% (± 5%) for ZEA, 97 % (± 5%) for OTA, and 91% (± 5%) for T2, respectively.

The laccase-AS system reacted with DON, FB1, AfB1, OTA and ZEA with following percentage of degradation: 57%, 24%, 87%, 100% and 58%. Beside those values, only for OTA and AfB1 was possible to observe a time dependent trend of degradation (Figure 5.2). Otherwise, for the other mycotoxins, no changes in reduction percentage were found, from the first to the last time point, even if the reactions did not reach the completition (data not shown).
No degradation was observed for FA and T2 toxin, in the tested conditions.

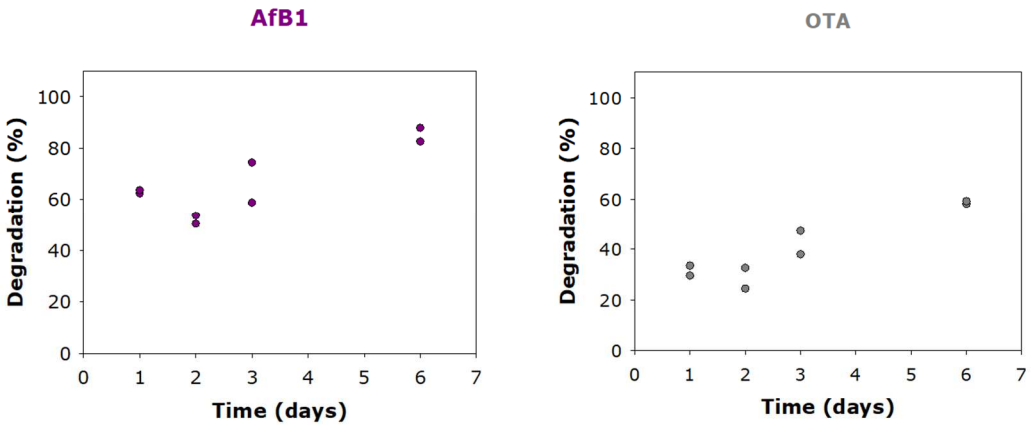


Figure 5.2 Percentage of degradation of AfB1 and OTA at 1, 2, 3 and 6 days, with laccase-AS system. Two replicates for each time point were analyzed.

In the laccase-SA system, DON, FB1, AfB1and ZEA, were degraded by 70%, 22%, 51% and 94%, respectively. As discussed before, degradation was not time dependent, since no variation was observed throughout each time points (data not shown). Moreover, no degradation for T2 toxin, FA and OTA was measured.
In the laccase-TEMPO system, the percentage of degradation was time dependent and varied, depending on the considered mycotoxin. ZEA was degraded up to 100% after only 2 days of incubation. After 6 days of incubation, the reduction observed for the other compounds were more than 90% for DON, 70% for FA, 60% for FB_1 and AfB1, respectively (Figure 5.3). No degradation was observed for OTA and T2 toxin by laccase-TEMPO system in the tested conditions.

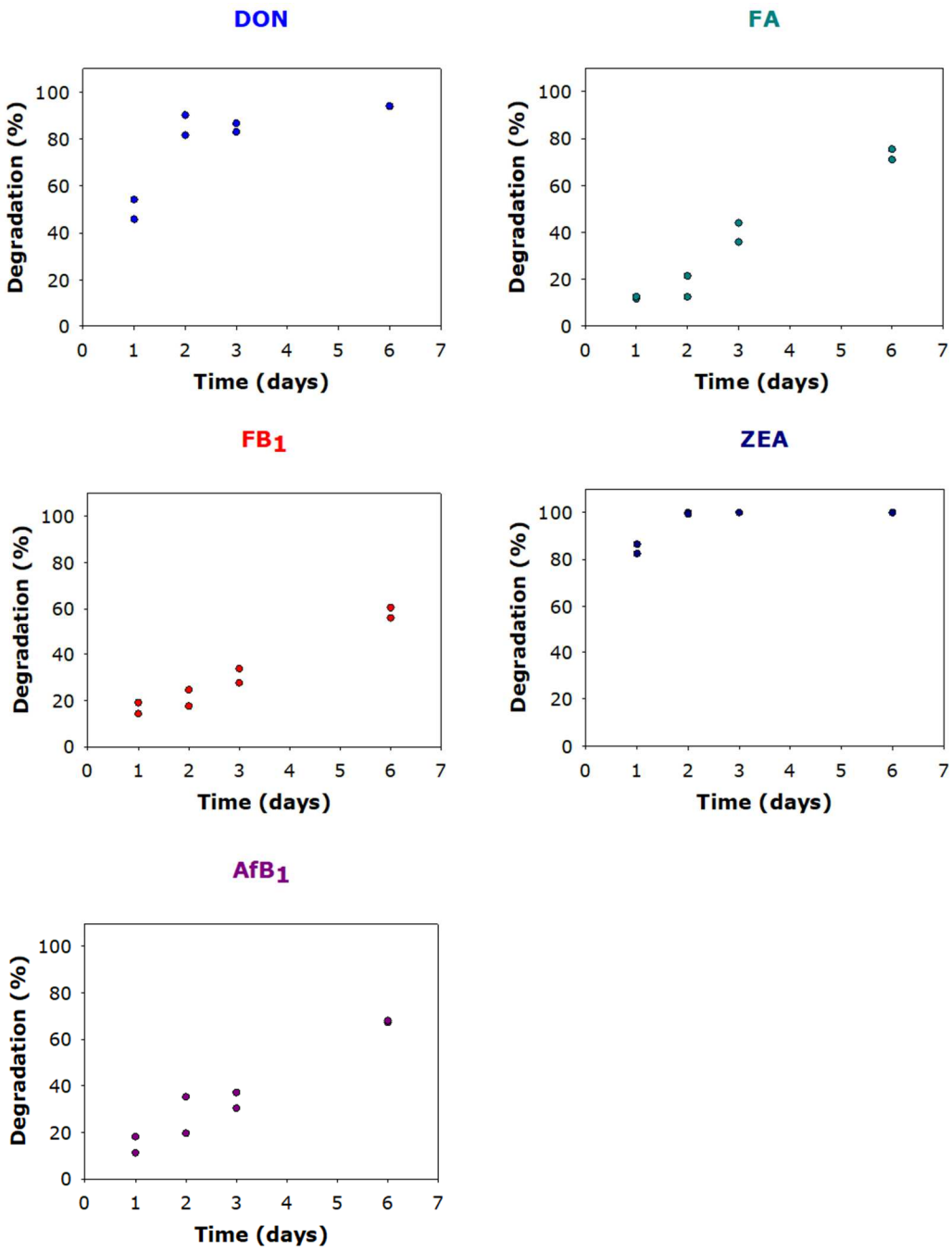


Figure 5.3 Percentage of degradation of DON, FA, AfB1, FB_1, and ZEA at 1, 2, 3 and 6 days, with laccase-TEMPO system. Two replicates for each time point were analyzed.

DON, AfB1, FB1 and ZEA were degraded, at different levels, by each LMS evaluated, while, T2 was the only mycotoxin never degraded, FA was degraded only by laccase-TEMPO system, and OTA only by laccase-AS system. Thus, underlining the different capabilities of each mediator to extent the laccase activities toward various molecules, simultaneusly. Furthermore, only ZEA proved to be, almost, completely detoxified as fast as in two days by each LMS studied, compared to the other toxins.
As reported in Chapter 4, the action of other laccase enzyme coupled with different mediators toward single mycotoxins, showed different results. For example, DON has never been degraded by Ery4 laccase, with AS, SA and TEMPO mediator. Conversely, T2 toxin was degraded by Ery4 laccase with TEMPO mediator.

5.5 Conclusions

Our preliminary data showed encouraging results regarding the degradation activity of laccase-mediator systems toward the most common mycotoxins and FA. Further studies will be necessary to evaluate the action of those systems in food and feed co-contaminated by mycotoxins.
Many enzymes have been reported to remove or reduce mycotoxin contamination both in vitro and in real matrices. Nonetheless, their application in feed is very limited, due to the lack of information about their influence on nutritional quality of feed. These data are mandatory to be authorized as possible biotransforming agent in Europe (Boudergue *et al.*, 2009).

Chapter 6

Degradation products of zearalenone by liquid chromatography-high resolution mass spectrometry (LC-HRMS)

6.1 Abstract

To identify putative metabolites obtained by laccase-mediator system (LMS) activity toward zearalenone (ZEA) mycotoxin, an untarget approach has been applied.
Liquid chromatography coupled with mass spectrometry is considered the best strategies that facilitates identification and quantitation of metabolites in complex biological samples. Raw data, obtained analizing ZEA degradation products, by quadrupole time of flight mass spectrometer (Q-TOF), were processed by MassHunter Workstation, Mass Profile Professional and MassHunter Molecolar Structure Correlator and, among the 148 entities retrieved, six neutral mass were identified as the most probable metabolites linked to ZEA degradation. Chemical structures of these putative metabolites seem to be not directly correlated as ZEA degradation products. Further analyses were needed to elucidate the products and the mechanism of the laccase-mediator system detoxifying action toward ZEA. Our study gave a first methodological approach toward the comprension of the LMS biological degradation of this mycotoxin.

6.2 Introduction

Different physical, chemical and biological methods have been recommended for detoxification of mycotoxin-contaminated food and feed. Among them, biological degradation gives the possibility to remove mycotoxins under mild conditions, without using harmful chemicals, without significant losses in nutritive value and without reducing the palatability of detoxified food and feed. The use of microorganisms or enzymes, which are able to metabolize, destroy or deactivate toxins into stable, less toxic, up to harmless compounds (Commission Regulation 2015/786), can permit their potential application in food and feed. A mandatory requisite is that, products of mycotoxin degradation have to be stable and non-toxic. The absence of knowledge related to degradation products and their toxicity, represent a barrier that limited the application of these biotransformation strategies into the feed chain.

Identification of unknown metabolites, originated by an enzymatic reaction, require powerful and advanced analytical methodology.

The coupling of liquid chromatography (LC) to mass spectrometry (MS), is one of the most adopted strategies, that facilitates identification and quantitation of metabolites in complex biological samples. Liquid chromatography is the most versatile separation method of compounds with a wide range of polarity, and it is often in line with mass spectrometry by electrospray-ionization (ESI). This has become the method of choice to detect molecules and it is often preferred for profiling ‘‘unknown’’ metabolites, since this ‘‘soft’’ ionization approach forms intact molecular ions and aids the initial identification (Zhou *et al*., 2011).

Mass spectrometry improves sensitivity and signal reproducibility by reducing sample complexity, thereby alleviating matrix interferences in the ionization process.

Meanwhile, versatile mass analyzers, as quadrupole time of flight mass spectrometer (Q-TOF), working in tandem or in hybrid configuration, can further aid metabolite identification by acquiring highly resolved and accurate MS/MS spectra. Some of the biggest advantages of Q-TOF tandem analyzers are their higher mass accuracy, higher resolution and increased scan speed (Chernushevich *et al*., 2001).

Identification of new metabolites, starts with an untargeted analysis to screen potential and putative metabolites of interest. To convert the raw LC-MS data into a peak list which can be easily interpreted and compared across runs,

multiple pre-processing steps need to be performed using specific software, like MassHunter Workstation, Mass Profile Professional and MassHunter Molecolar Structure Correlator. This softwares allow to identify possible brute formulas, to screen two or more sample sets by differential analysis to determine relationships among multiple sample groups and to retrieve putative chemical structure querying ChemSpider. Once a pool of candidates has been hypotesized, these metabolites should be subjected to a targeted analysis for metabolite identity verification, quantitation, functional interpretation, and pathway analysis (Zhou *et al.*, 2011).

An untarget approach was used to identify putative metabolites obtained by laccase-mediator system activity toward zearalenone (ZEA) mycotoxin. ZEA is a macrocyclic β-resorcylic acid lactone produced by numerous species of *Fusarium*. It frequently contaminates corn and cereal products and the biological activity of ZEA is characterized by its pronounced oestrogenicity. Biotransformation of ZEA has been shown to occur in fungi, plants and mammals, and involves both the aliphatic macrocycle and the aromatic ring (Metzler *et al.*, 2010). In mammals, the keto group at C-8 is reduced to two stereoisomeric metabolites of ZEA (α- and β-isomers). The ZEA metabolites, such as α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL) and zearalanone (ZAN), are also produced by fungi, as reported by Bottalico *et al.* (1985) in corn stems infected with *Fusarium*. All members of this group are nonsteroidal estrogenic mycotoxins frequently implicated in reproductive disorders of farm animals and occasionally in hyperestrogenic syndromes in humans.

One of the enzymes ubiquitously present in fungi, plants, insects and bacteria, that has recently gain much attention for its biodegrading properties, is laccase. Laccase catalizes the oxidation of phenolic and aniline groups contained in different compound, using oxygen to oxidase the substrate. Since laccase does not directly catalyse the degradation of non-phenolic compound, like ZEA, the action of a mediator, as the synthetic molecule 2,2,6,6-tetramethyl-piperidinyl-oxyl (TEMPO), is necessary to reduce ZEA levels.

As described in Chapter 5, the action of laccase-TEMPO system is capable to completely reduce 1 µg/mL of ZEA, in only two days of mild shaking incubation at 25°C, in the dark. For this reason, we decided to further investigate the final products of ZEA degradation by laccase-TEMPO system.

6.3 Materials and Methods

6.3.1 Chemical and Materials

Methanol (LC-MS grade), sodium acetate trihydrate, were supplied by Carl Roth GmbH (Karlsruhe, Germany). Laccase from *Trametes versicolor* was obtained from Merck KGaA (Darmstadt, Germany). ZEA, α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), from Romer Labs Diagnostic GmbH (Tulln, Austria). Water Millipore Milli-Q system (Sartorius, Gottingen, Germany).

6.3.2 Laccase activity

Laccase enzymatic activity was spectrophotometrically determined monitoring the absorbance at 420 nm, that describe the oxidation rate of ABTS substrate to ABTS radical cation ($ABTS^{\bullet+}$). The reaction was performed in a final volume of 1 mL, with 100 mM sodium acetate buffer (pH 5), 5 mM ABTS and an appropriate amount of enzyme solution. Therefore, enzyme unit (U) is defined as the amount of enzyme that catalize the oxidation of one µmol of substrate per minute, at 25°C, under the tested conditions. Laccase activity (U/mL) is calculated on the base of Lambert-Beer law with some modification, through the following equation:

$$\frac{U}{mL} = \frac{\Delta Abs \times Vr \times df}{d \times \varepsilon \times t \times Ve}$$

where ΔAbs represent the absorbance of the sample compared to blank control; Vr is the reaction volume expressed in mL; df is the dilution factor; d is the light path (1 cm); ε is the extinction coefficient (36 $M^{-1}cm^{-1}$); t is the reaction time expressed in minute and Ve is the enzyme volume added to the reaction.

6.3.3 Laccase-TEMPO-ZEA assay

The reactions, set up to identify the degradation products of ZEA, contained 20 µg/mL of this mycotoxin dissolved in 1 mM sodium acetate buffer (pH 5), 1 UI of enzyme, 10 mM of TEMPO mediator and were incubated at 25°C in the dark and sampled at 0 hour and 3 days, replicated six times. The negative control reactions were prepared with laccase and TEMPO mediator (without toxin) incubated in the same conditions and sampled at 0 hour and 3 days, in triplicates.

Before the LC-HRMS analysis, all the samples were processed to remove the interfering proteins. Three volumes of acetonitrile were added to each sample at each sampling time. Solutions were vortexed for 2 min, left to stand for 20 min at 4°C and centrifuged for 20 min at 15000 rpm, 4°C. The supernatant was recovered and dried using a SpeedVac vacuum concentrator system. After the evaporation, the pellet was resuspended in water: methanol (90:10, v/v).

6.3.4 ZEA metabolites identification

Standard solution containing ZEA, α-ZOL, β-ZOL, α-ZAL and β-ZAL were prepared at the following concentration: 6.25, 62.5, 125, 250, 500, 1000 ng/mL, in water: acetonitrile (1:1, v/v).

An Agilent 6545 Quadrupole Time of Flight Mass Spectrometer (Q-TOF LC-MS, Santa Clara, CA) was used to identify the possible metabolites of ZEA. Q-TOF MS was operated with an electrospray source in positive ion mode. The main instrument settings for the Q-TOF were as follows: drying gas temperature 320 °C, drying gas flow 8 L/min, capillary voltage 3500 V, fragmentor voltage 175 V. The Q-TOF chromatograms were acquired in a scan range from 100 to 1700 m/z.

For the analysis 5 µL of the extract was injected. Chromatographic separation was achieved on a Polaris 3 C18 Ether column (100 x 2 mm, 3 µm, Agilent, Santa Clara, CA) using a flow rate of 0.2 mL/min at 35 °C during a 38 min gradient (0–1 min to 10 % B, 1–30 min from 10 % to 98% B, 30.00–32.00 min to 98% B, 32.00–32.30 min from 98 % B to 10% B, 32.30-38 to 10% B) using water with 0.1 % formic acid as solvent A and methanol as solvent B. In this analytical condition, the retention time of ZEA is about 24.6 min, while for α-ZOL is about 24.4 min, β-ZOL is about 22.7 min, α-ZAL is about 23.9 and for β-ZAL is about 22.1 min (Figure 6.1).

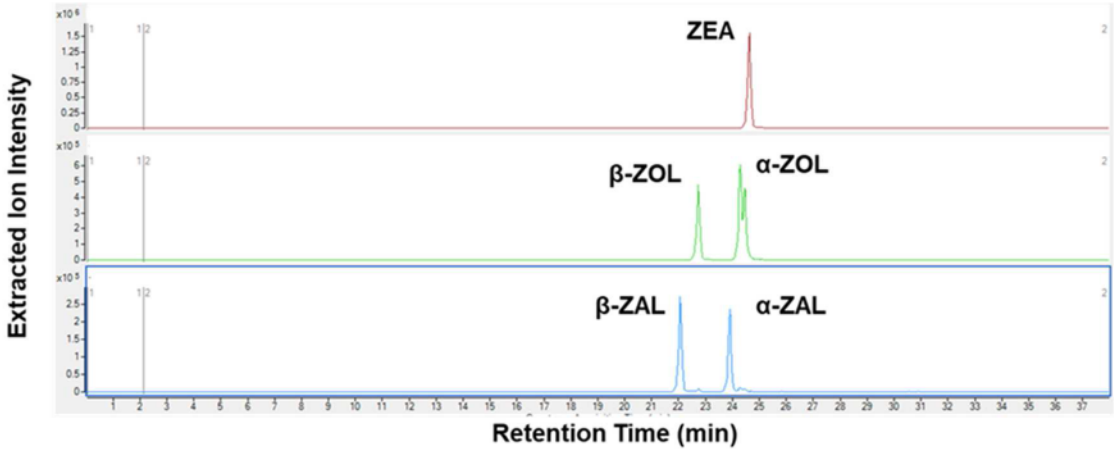


Figure 6.1 Extracted ion chromatograms of standard solution as ZEA, α/β-ZOL and α/β-ZAL (1 µg/mL).

Exact mass value used to detect ZEA, α/β-ZOL, α/β-ZAL were shown in Table 6.1.

Target compound	Elemental formula	Mass m/z
ZEA	$C_{18}H_{22}O_5$	319.154
α/β- ZOL	$C_{18}H_{24}O_5$	321.1697
α/β- ZAL	$C_{18}H_{26}O_5$	323.1853

Table 6.1 Exact mass value calculated on the basis of the elemental formula.

6.3.5 Data processing

Data were processed by MassHunter Workstation Software (Qualitative Analysis Navigator and Qualitative Analysis Workflow, version B.08.00), Mass Profile Professional (version 14.08), MassHunter Molecular Structure Correlator (version B.08.00), from Agilent Technologies.

6.4 Results and discussion

In order to identify products of the reaction with laccase-TEMPO system towards ZEA, the samples have been further investigated by Q-TOF. The results obtained

shown the absence of ZEA and its known metabolites (α-ZOL, β-ZOL, α-ZAL, β-ZAL) (Bottalico *et al.*, 1985). This means that the products of the reaction Laccase-TEMPO-ZEA are, presumably, unknown molecules.

Comparing the total ion chromatogram (TIC) of laccase-TEMPO at 0 hour and 3 days, the different profile caused by the oxidation of TEMPO mediator by laccase enzyme at 3 days was clearly shown (Figure 6.2 A, B). While in the samples: laccase-TEMPO-ZEA at 0 hour was present ZEA (Figure 6.2 C), as shown in extract ion chromatogram (Figure 6.2 D), that disappeared after 3 days of incubation (Figure 6.2 E), but the contribute of TEMPO oxidation by laccase in the TIC profile remains easily recognizable.

The identification of the unknown ZEA metabolites was done by comparing the entities in the blank (mobile phase) with laccase-TEMPO at 0 hour and at 3 days samples (Figure 6.3 a). One hundread and eighty-one entites were filtered in base of the frequency, and 126 entities were obtained. They represent all the entities found in blank, laccase-TEMPO at 0 hour and at 3 days. These entites were, further, compared with the samples at 0 hour and 3 days. One hundread and forty-eight were univocally assigned to the 3 days reaction.

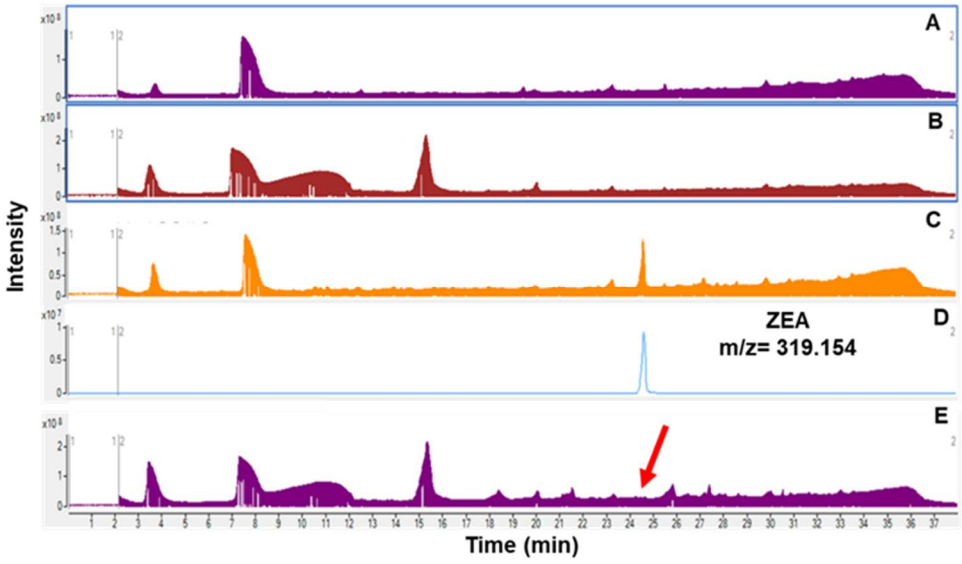


Figure 6.2 Total ion chromatograms (TICs) and extracted ion chromatogram. TIC of laccase-TEMPO at 0 hour (A) and at 3 days (B); laccase-TEMPO-ZEA at 0 hour (C) and 3 days (E). In (D) the extracted ion chromagram of ZEA from laccase-TEMPO-ZEA at 0 hour.

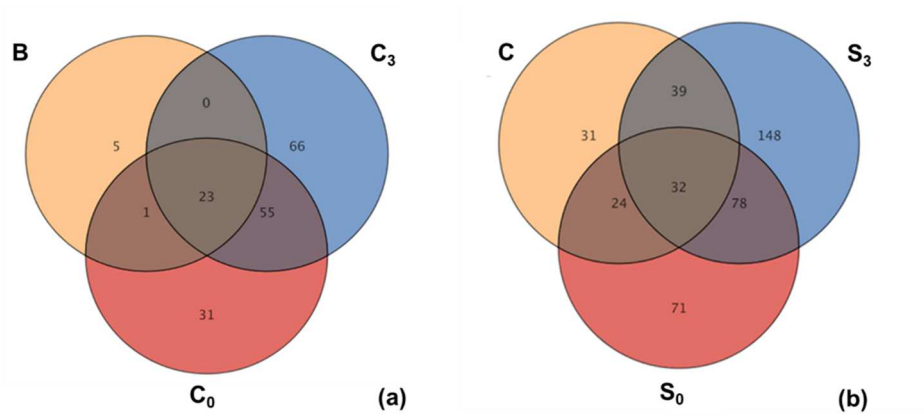


Figure 6.3 Venn diagrams of putative metabolites of ZEA by LC-HRMS.
(a) All the entities coming from the negative controls: B is the mobile phase; while C_0 and C_3 are the laccase-TEMPO at 0 hour and 3 days, respectively. **(b)** Filtered entities coming from the negative controls were summarized in C and compared to, S0 (the laccase-TEMPO-ZEA samples at 0 hour) and S3 (the laccase-TEMPO-ZEA samples at 3 days), respectively.

Among these, 22 entities, filtered on the basis of their frequencies, represents the most probable metabolites. Six different components were found only in the 3 days incubated samples and the neutral masses and retention times were as follows: 341.2569 (14.82 min); 314.257 (20.99 min); 122.1095 (21.30 min); 334.13 (23.39 min); 258.1259 (24.46 min) and 310.2262 (27.79 min). These masses were analyzed with MassHunter Workstation Software to identify possible brute formulas and therefore putative chemical structures were retrieved, querying ChemSpider, by MassHunter Molecular Structure Correlator. All of these chemical structures were manually analyzed to find probable degradation products, but none of them was likely to be a degradation product of ZEA. This means that, the products of the detoxifying reaction of ZEA by laccase-TEMPO system and its mechanism, need to be further investigated. The reaction laccase-mediator towards ZEA is an unspecific reaction; where the TEMPO mediator is oxidized by the enzyme in TEMPO oxoammonium ion. This radical can interact with specific functional groups, for example alcohols (de Nooy *et al.*, 1996), inducing the formation of different products and its conversion to the reduced form, that should be reoxdized by laccase. In its molecular structure, ZEA contains two hydroxyl groups in position 14 and 16, and two ketone groups in C1 and C7, that could be targets of the mediator radical.

6.5 Conclusions

Different putative entities were found to be highly correlated with the degradation reaction of ZEA. Otherwise, to assess the appropiate entities, a more efficient purification of the samples, to remove all the interfering molecules, is required for an accurate identification of the putative metabolites.

Further study with LC-HRMS using ^{13}C-labelled toxin and nuclear magnetic resonance spectroscopy (NMR) are requested to elucidate their structure and, in addition, to evaluate their toxicity.

General Conclusions

Food contamination became a serious health concern for humans and animals with severe economic implications. In recent years, researcher, progressively increased their efforts, to find out the best preventive strategies aimed to avoid fungal contamination and mycotoxin production in crops, and to adopt mitigation actions focused to reduce mycotoxins level in food and feed chain.

In this doctoral thesis, *Fusarium* mycotoxin monitoring was performed and challenging biological and physical strategies to counteract and/or reduce mycotoxins contamination, were explored.

The survey on the presence of mycotoxigenic *Fusarium* species and their mycotoxins in wheat samples harvested in some regions of Italy, vary accordingly to the geographical areas and year of sampling. Higher levels of DON and NIV were found in wheat sampled from Central and Northern Italy, instead those from Southern Italy were principally contaminated by ZEA and T2, HT2 toxins. The investigated samples were often co-contaminated by more than one single mycotoxin. Next to the detection of mycotoxins in foodstuff and/or feedstuff contaminated, is necessary to develop strategies with the aim to guarantee consumer's health. Different approaches were pursued, to reduce the contamination levels of mycotoxins in agricultural commodities, with the goal to emphasize the biological methodologies.

One of the most promising biological player capable to contain and/or reduce mycotoxins level is represented by the edible mushroom *Pleurotus spp.*, thank to the known bioremediation properties exhibited.

We have demonstrated that, *P. eryngii* is able to degrade aflatoxin B1 in both liquid and solid media. Cereals contaminated by AfB1, can be processed in valuable products intended for animal feeding, through the biodegradation achieved by *P. eryngii* laboratory-scale mushroom cultivation. Furthermore, *P. eryngii* extracts, derived from spent mushroom substrate, proved their efficacy to biodegrade AfB1. Thus, it could be economically advantageous to implement the re-use of this waste products, into the pipeline of feed processing.

Other applications of *P. eryngii,* involve the use of not-viable mycelium as adsorbent biomass, to be used as feed additive to bioremediate AfB1-contaminated feeds. This biological and edible material, can replace the inorganic feed additives currently used in the feed industry, providing healthy fiber content for animal diet, too.

Among the *Pleurotus* enzymatic activities, laccases are widely recognized as potential biodegrading enzymes, and their action toward phenolic and aniline compounds, can be easily extended to different molecules, through the use of chemical mediators.

An extensive *in vitro* screening, of laccase mediator systems demonstrated that, the degradative action towards AfB1, AfM1, DON, FB1, OTA, ZEA and T-2 toxin, vary in base of the laccase isoform (Lac2 and Ery4), the redox mediator and the single or combined mycotoxin, considered for the assays.

Laccase from *Trametes versicolor* has been utilized to investigate the simultaneous degradation of multiple mycotoxins: FA, AfB1, DON, FB1, OTA, ZEA and T-2 toxin, evaluating the effect of different mediators.

Our preliminary data showed encouraging results regarding the multiple mycotoxins degradation activity of laccase-mediator systems. Nonetheless, their application in feed is very limited, due to the lack of information about their influence on nutritional quality of feed. These informations, along with data regarding the identification of the degradation products, are mandatory requisites to authorize their usability as biotransforming agent, by European legislation.

A first methodological approach toward the comprension of laccase mediator system biological degradation versus zearalenone mycotoxin, has been done by LC-HRMS.

Different putative entities were found to be highly correlated with the degradation reaction of ZEA, however, further studies are required for an accurate identification of the putative metabolites, and to elucidate their structure.

This study represents a starting point for the development of methods to counteract the natural co-occurrence of multiple mycotoxins in raw materials, food and feed, by means of efficient and environmental friendly biological approaches.

Annex A

Chemical determination of Enniatins (A, A1, B and B1) and Beauvericin production profiles in *Fusarium* species by UPLC-PDA-QDa.

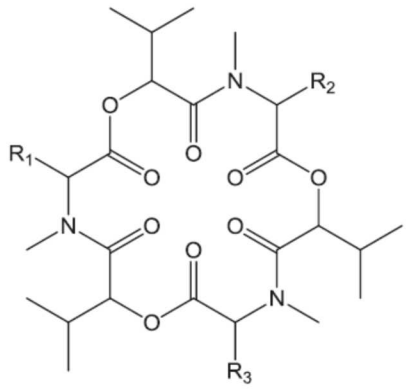
A.1 Abstract

Members of *Fusarium* fungal genus can produce numerous secondary metabolites as beauvericin (BEA) and enniatins (ENNs).
BEA and ENNs have a wide range of biological activities, including insecticidal, antifungal, antibiotic and cytotoxic properties, and acute exposure seems to not indicate concern for human health. Beside those properties, more precise, detection and quantification techniques for their presence were required, to exclude concern due to chronic exposure.
For this reason, a liquid chromatography mass spectrometry method was developed for the simultaneous determination of enniatins (A, A1, B, B1) and beauvericin (BEA), coupled with photodiode array (PDA) and a single quadrupole, Acquity QDa mass detector (QDa). All of the strains studied, were grown in two different media: potato dextrose agar (PDA) and Fusarium defined medium (FDM), in the same conditions. Each strain shown the same chemotype profile in both media. These strains produced quantifiable amounts of ENNs, with higher amounts produced on PDA medium than on FDM medium. Limits of detection is ranged from 0.0015 µg/g of ENN A to 0.7 µg/g of ENN B and B1. Recoveries around 80% were obtained for all tested mycotoxins with, less than 8%, relative standard deviations.

Note: Some of the contents of this chapter were previously published in the following article:
Liuzzi, V. C., Mirabelli, V., Cimmarusti, M. T., Haidukowski, M., Leslie, J. F., Logrieco, A. F., Caliandro, R., Fanelli F. and Mulè, G. (2017). Enniatin and Beauvericin Biosynthesis in *Fusarium* Species: Production Profiles and Structural Determinant Prediction. Toxins, 9, 45.

A.2 Introduction

Enniatins (ENNs) and beauvericin (BEA) are structurally-related mycotoxins produced by several fungal species. These compounds have antibiotic and ionophoric properties and different bioactivities. More than 20 analogues of enniatins have been identified, but only four A, A1, B and B1 are commonly detected in food and feed (Sy-Cordero *et al.*, 2012) (Figure A1). Structurally, BEA and ENNs are non-ribosomal cyclic hexadepsipeptides that consist of alternated D-2-hydroxyisovaleric acid and *N*-methyl L-amino acids. In beauvericin structure, the three amino acid residues, are aromatic *N*-methyl-phenylalanines, whereas in type A and B enniatins, the amino acid residues are aliphatic *N*-methyl-valine, or *N*-methyl-isoleucine, or mixtures of these amino acids (Strongman *et al.*, 1988). The subunits are linked by peptide bonds and intramolecular ester (lactone) bonds, forming a cyclic depsipeptide.



Compound	R_1	R_2	R_3
Beauvericin	$-CH_2C_6H_5$	$-CH_2C_6H_5$	$-CH_2C_6H_5$
Enniatin A	$-CH(CH_3)CH_2CH_3$	$-CH(CH_3)CH_2CH_3$	$-CH(CH_3)CH_2CH_3$
Enniatin A1	$-CH(CH_3)CH_2CH_3$	$-CH(CH_3)CH_2CH_3$	$-CH(CH_3)_2$
Enniatin B	$-CH(CH_3)_2$	$-CH(CH_3)_2$	$-CH(CH_3)_2$
Enniatin B1	$-CH(CH_3)_2$	$-CH(CH_3)_2$	$-CH(CH_3)CH_2CH_3$

Figure A.1 Enniatin and beauvericin chemical structures.

Data on the occurrence of BEA and ENNs in food and feed have recently been reported by the European Food Safety Authority (EFSA, 2014) in the first risk

assessment related to these two mycotoxins. In cereals, beauvericin and enniatins often co-occur. High co-occurrence levels are expected since the two mycotoxin groups are structurally related, may be produced by the same *Fusarium* species and are the products of the same metabolic pathway (Bottalico and Perrone, 2002; Jestoi., 2008). Indeed, some *Fusarium spp*. produce BEA, ENNs or both, with the mechanisms responsible for the end product variation and their regulation remaining unknown. As any other secondary metabolite, their biosynthesis is regulated by genetic, epigenetic and environmental factors. The interaction of these factors determines the type and amount of mycotoxin(s) produced and varies by strain (Shwab and Keller, 2008; Brakhage, 2013). Based on the results of the occurrence of BEA and ENNs, total dietary exposures were estimated and an assessment of the risk of these compounds on human health was performed. As a result, EFSA concluded that the values obtained for acute exposure to BEA and ENNs do not indicate a concern for human health, although there may be concern about chronic exposure.

The emerging importance of BEA and ENNs in cereal contamination and the recent study of their toxic effect(s) on human and animal health, together with the possibility that these compounds may be used as pharmaceutical products (Jan-Garcia *et al*., 2015), require more precise, detection and quantification, techniques.

In this study, we evaluated the production of BEA and ENNs by strains in several *Fusarium* species grown in the same experimental conditions on two agar medium: potato dextrose agar (PDA) and Fusarium defined medium (FDM). Analytical method to detect ENNs and BEA in fungal extract, was developed using ultra-performance liquid chromatography (UPLC) coupled with two detectors: a photodiode array (PDA) setted at 205 nm and a single quadrupole Acquity QDa mass detector (QDa). The mass spectral information combines seamlessly into the same workflow as an LC analysis, providing more complete separation characterization by confirming the identity of components.

A.3 Materials and Methods

A.3.1 Fungal strains

The strains used in this study are listed in Table A.2. They were selected based on the availability of the strain to be used for chemical analysis.

Strains were grown for 5 days at 25 °C on PDA medium. Conidia were harvested, and a conidial suspension was prepared in sterilized distilled water. Spores were counted in a Thoma chamber, and the suspension was diluted to a final concentration of 10^6 conidia/mL. For each experiment, 50 µL of the concentration-adjusted conidial suspension were inoculated at single point agar plates.

A.3.2 Media and growth conditions

The media used in this study were, Potato dextrose agar (PDA) purchased by Oxoid (Rodano (MI), Italy) and an enniatin-inducing medium, Fusarium defined medium (FDM) (Fanelli *et al.*, 2014; Billich and Zoccher, 1987; Madry *et al.*, 1983) containing the following substances per liter of distilled water: 12.5 g of glucose, 4.25 g of $NaNO_3$, 5 g of NaCl, 2.5 g of $MgSO4 \cdot 7H_2O$, 1.36 g of KH_2PO_4, 10 mg of $FeSO_4 \cdot 7H_2O$ and 2.9 mg of $ZnSO_4 \cdot 7H_2O$. A conidial suspension of each strain was inoculated on 9-cm Petri dishes containing 20 mL of either PDA or FDM agar medium. The inoculated plates were incubated at 25 °C for 14 days in the dark. Experiments were performed in triplicate.

A.3.3 Chemicals and preparation of standards solution

All solvents (HPLC grade) were purchased from VWR International Srl (Milan, Italy). Ultrapure water was produced by a Millipore Milli-Q system (Millipore, Bedford, MA, USA). Regenerated cellulose membranes filters (RC, 0.2 µm) were obtained from Alltech Italia-Grace Division (Milano, Italy) and paper filters Whatman no.4 from Whatman (Maidstone, UK). ENN and BEA standards (purity >99%) were supplied by Sigma-Aldrich (Milan, Italy). Standard stock solutions of a mixture of ENNs (A 3% = 0.03 mg/mL, A1 20% = 0.2 mg/mL, B 19% = 0.19 mg/mL and B1 54% = 0.54 mg/mL) and BEA (1 mg/mL) were prepared by dissolving the solid commercial toxin standards in methanol. Adequate amounts of the stock solution were dried under a nitrogen stream at 50 °C and reconstituted with methanol:water (70:30, v/v). Standard solutions for UPLC calibration were prepared by using different concentrations within an appropriate calibration range (Table A.1). Standard solutions were stored at −20 °C and warmed to room temperature prior to use.

Mycotoxin	Elemental Formula $[M + H]^+$	Retention Time (min)	SIR (m/z)	Calibration Range (µg/mL)	LOQ UPLC/PDA (µg/g)
Enniatin A	$C_{36}H_{63}N_3O_9$	11.4	682	0.09-1.20	0.015
Enniatin A1	$C_{36}H_{61}N_3O_9$	10.2	668	0.30-8.00	0.05
Enniatin B	$C_{33}H_{57}N_3O_9$	8.5	640	0.04-7.60	0.07
Enniatin B1	$C_{34}H_{59}N_3O_9$	9.2	654	0.04-21.60	0.07
Beauvericin	$C_{45}H_{57}N_3O_9$	9.8	784	0.02-40.00	0.04

Table A.1 Enniatins and beauvericin experimental parameters. LOQ= limit of quantifications; SIR= single ion recording.

A.3.4 Determination and confirmation of enniatins (A, A1, B and B1) and beauvericin from agar medium

The potential of a subset of 13 *Fusarium spp.* isolates to produce ENNs and BEA *in vitro* was evaluated by ultra- performance liquid chromatography coupled with a photodiode array and Acquity QDa mass detector (UPLC-PDA-QDa).

Five grams of agar culture material for each culture were extracted with 10 mL of methanol on an orbital shaker for 60 min. Six milliliters of the extract were evaporated to dryness under a stream of nitrogen at 50 °C. The residue was dissolved in 500 µL of methanol:water (70:30, v/v) and filtered through a 0.20 µm RC filter. Ten microliters of the extract were injected into the full loop injection system of a UPLC system Waters Acquity (Milford, MA, USA), equipped with an ESI interface, and with a binary solvent manager, a sample manager, a column heater, a photodiode array and QDa detectors. The analytical column was an Acquity UPLC BEH C18 (2.1 × 100 mm, 1.7 µm) preceded by an Acquity UPLC in-line filter (0.2 µm). The temperature of the column was set at 50 °C. The flow rate of the mobile phase was set at 0.35 mL/min. The toxins were determined by both detectors, i.e., the PDA set at 205 nm, and, after the effluent, into the ESI interface, without splitting. The mobile phase consisted of a binary gradient applied as follows: the initial composition (50% (A) water-0.1% formic acid/50% (B) acetonitrile-0.1% formic acid) was kept constant for 2 min, then solvent B was increased linearly up to 75% in 8 min, then linearly increased up to 80% in 2 more min and, finally, kept constant for 4 min. For column re-equilibration, eluent B was decreased to 50% in 1 min and kept constant for 4

min. For LC/MS analyses, the ESI interface was used in positive ion mode, with the following settings: desolvation temperature 600 °C; capillary voltage 0.8 kV, sampling rate 5 Hz. The mass spectrometer was operated in full scan (600–800 m/z) and in single ion recording (SIR) mode, by monitoring the individual masses of each compound (Table A.1). MassLynx® 4.1 mass spectrometry software was used for data acquisition and processing. Retention time for enniatins B, B1, A1, A were about 8.5, 9.2, 10.2 and 11.4 min, respectively, and beauvericin about 9.8 min. Toxins were quantified by measuring peak areas and comparing these values with a calibration curve obtained from standard solutions.

A.4 Results and Discussion

Mean recoveries of fortified agar plugs (n=3) at levels of ENN A (0.3–0.6 µg/g), ENN A1 (2.0–4.0 µg/g), ENN B (1.9–3.8 µg/g), ENN B1 (5.4–10.8 µg/g) and BEA (2.0–4.0 µg/g) were 80.6%, 81.5%, 78.3%, 84.0% and 86.7%, with relative standard deviations of 3.3%, 2.6%, 5.2%, 7.6% and 6.8%, respectively. The chromatograms of beauvericin standard in UPLC/PDA and in single ion recording (SIR) was shown in Figure A.2, while in Figure A.3 shown the chromatogram of combined standard solutions of the four ENNs evaluated in UPLC/PDA and in SIR. As an example, Figure A.4 shows well-resolved chromatograms of ENNs A2 and B2 from the *F. proliferatum* KSU 4854 grown in FDM medium.

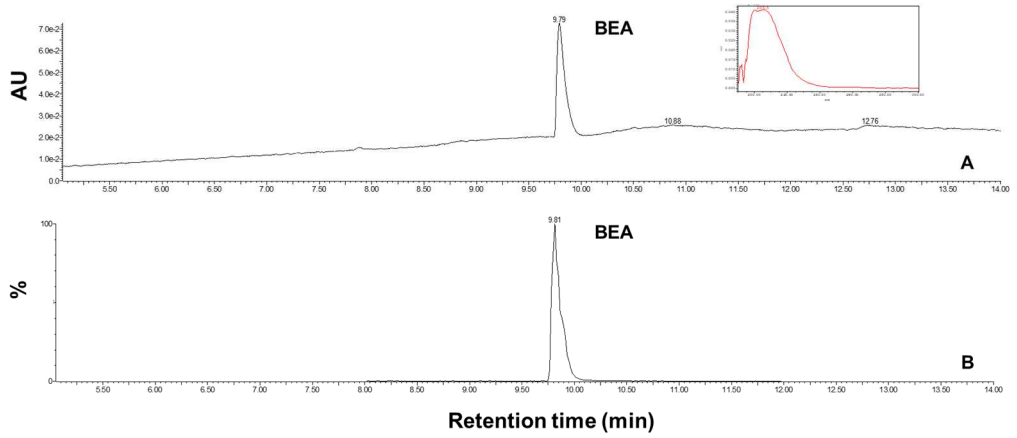


Figure A.2 Chromatograms of beauvericin standard (0.4 µg/mL) in UPLC/PDA (A) and in Single Ion Recording (SIR) (B). In the box, above on the right, the typical UV spectrum of the standard solution are shown.

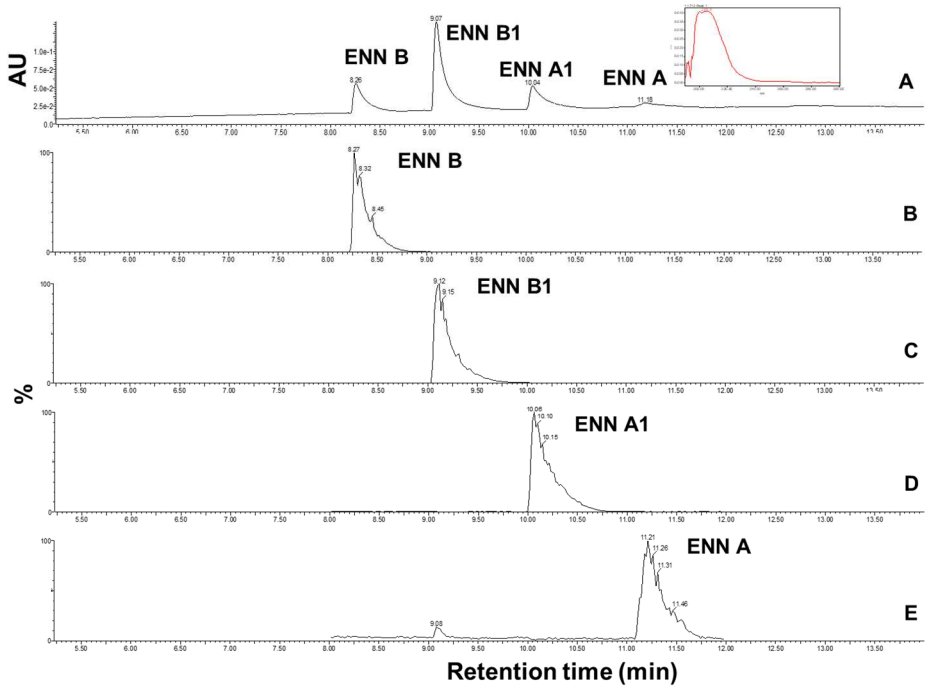


Figure A.3 UPLC/PDA Chromatogram of enniatin standards in UPLC/PDA (A), Single Ion Recording (SIR) of ENN B (7.0 µg/mL) (B), ENN B1 (20 µg/mL) (C), ENN A1 (8.0 µg/mL) (D) and ENN A (1.2 µg/mL) (E). In the box, above on the right, the typical UV spectrum at 205 nm of enniatins is shown.

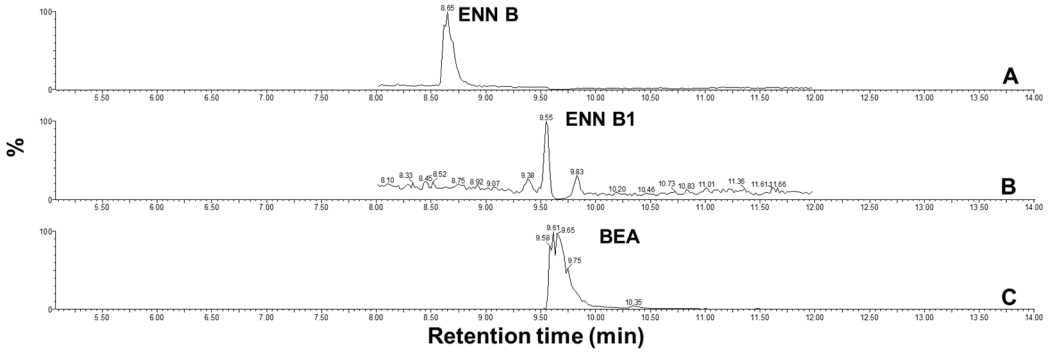


Figure A.4 UPLC/PDA chromatogram of the agar extract from the sample *Fusarium proliferatum* KSU 4854 grown on chemically-defined production medium (FDM). (A) SIR of ENN B (0.08 µg/g), (B) ENN B1 (0.23 µg/g) and (C) BEA (4.87 µg/g).

The limit of quantification (LOQ) values were calculated according to s/n = 10 (Table A.1). The linearity of the standard curves at three determinations of six

concentration levels was reliable between 0.9988 and 0.9997. LOQ was calculated as three-fold LOD.
The chemotype of *Fusarium* isolates known from previous publications and determined by chemical analysis is reported in Table A.2.

Species	Strain	Metabolic Profile
Fusarium fujikuroi	B14	ENN A, ENN B, ENN B1, BEA
	FGSC 8932	ENN A, ENN B, ENN B1, BEA
	KSU 10626	ENN A, ENN B, ENN B1, BEA
	KSU 3368	ENN A, ENN B, ENN B1, BEA
Fusarium verticillioides	FGSC 7600	ENN A, ENN B, ENN B1
	ITEM 10027	ENN A, ENN B, ENN B1
	KSU 488	ENN A, ENN B, ENN B1
	KSU 999	ENN A, ENN B, ENN B1
Fusarium spp.	KSU 3089G	ENN A, ENN B, ENN B1
Fusarium proliferatum	KSU 830	ENN A, ENN B, ENN B1
	KSU 4854	ENN B, ENN B1

Table A.2 Enniatins (ENNs) and beauvericin (BEA) production profile of *Fusarium* strains.

The amount of ENNs and BEA produced in Fusarium defined medium (FDM) and potato dextrose agar (PDA) are indicated in Tables A.2 and A.3. All of the strains used produced quantifiable amounts of ENNs, with higher amounts synthesized on PDA medium than on FDM medium. The chemotype profile of each strain was confirmed in both media. All *F. fujikuroi* strains produced both ENN A, B, B1 and BEA. Among *F. proliferatum*, strain KSU 4854 produced ENN B, B1 and BEA in both medium, in according to Desjardius (2006), while strain KSU 830 produced only ENN A, ENN B in FDM medium and, also, ENN B1 in PDA medium. *Fusarium verticillioides* produced only ENN B, B1 and A.
The ability of *Fusarium avenaceum* to produce both BEA and ENNs has been previously reported (Logrieco *et al*., 2002b; 1998), and for this reason, the strains ITEM 3403 and 3404 were used as positive controls for the chemical analyses. Infact, the data, summarized in Tables A2 and A3, shown that they produce all the ENNs and BEA studied (Tables A2 and A3). The strains ITEM 3403 and 3404 were selected from the Agri-Food Toxigenic Fungi Culture Collection of the Institute of Sciences of Food Production, Italian National Council of Research (CNR), Bari (www.ispa.cnr.it/Collection). They were identified according to the criteria and synoptic keys of Nelson (1983) and by molecular analysis.

Species	**Strain**	**ENN B** Mean±SD (µg/g)	**ENN B_1** Mean±SD (µg/g)	**ENN A** Mean±SD (µg/g)	**ENN A_1** Mean±SD (µg/g)	**BEA** Mean±SD (µg/g)
Fusarium fujikuroi	B14 A	0.10±0.02	0.93±0.31	n.d.	n.d.	49.95±17.31
	FGSC 8932*	0.19±0.06	0.27±0.16	0.12±0.08	n.d.	16.34±20.27
	KSU 10626*	0.09±0.03	0.47±0.23	0.08±0.07	n.d.	16.34±20.27
	KSU 3368*	0.15±0.04	0.65±0.18	0.04±0.02	n.d.	50.26±15.59
Fusarium verticillioides	FGSC 7600	0.09±0.01	0.12±0.11	0.08±0.04	n.d.	n.d.
	ITEM 10027*	0.07±0.01	0.09±0.11	0.04±0.02	n.d.	n.d.
	KSU 488	0.05±0.00	n.d.	n.d.	n.d.	n.d.
	KSU 999	0.07±0.00	n.d.	0.09±0.08	n.d.	n.d.
Fusarium spp.	KSU 3089G*	0.04±0.01	0.10±0.10	n.d.	n.d.	n.d.
Fusarium proliferatum	KSU 830*	0.04±0.00	n.d.	0.02±0.02	n.d.	n.d.
	KSU 4854*	0.08±0.03	0.23±0.11	n.d.	n.d.	4.87±3.96
Fusarium avenaceum	ITEM 3403	0.31±0.06	22.38±11.04	n.d.	0.03±0.00	0.82±0.40
	ITEM 3404	0.23±0.08	17.27±4.68	0.003±0.00	0.05±0.01	0.61±0.42

Table A.2 Enniatin and beauvericin production of *Fusarium* strains on chemically-defined production medium (FDM). n.d. = not detected; * results confirmed in LC/MS.

The developed analytical method, in which, UPLC was coupled with an MS-QDa detector, resulted in a unique chromatographic run that could detect, confirm and quantify BEA and ENN levels in agar samples. We used an extraction procedure, without a sample clean-up step, and the obtained chromatograms could be used to quantify the toxins. This process avoids the risk of unexpected co-elutions and provides analytical confidence for the mass detection.

Species	Strain	**ENN B** Mean±DS (µg/g)	**ENN B_1** Mean±DS (µg/g)	**ENN A** Mean±SD (µg/g)	**ENN A_1** Mean±SD (µg/g)	**BEA** Mean±SD (µg/g)
Fusarium fujikuroi	B14*	0.09±0.06	1.41±0.31	0.08±0.06	n.d.	101.41±14.96
	FGSC 8932*	0.48±0.08	0.67±0.25	0.58±0.48	n.d.	99.85±16.68
	KSU 10626*	0.40±0.22	0.47±0.23	0.24±0.22	n.d.	26.85±7.70
	KSU 3368*	0.24±0.06	0.29±0.11	0.07±0.02	n.d.	33.23±6.78
Fusarium verticillioides	FGSC 7600*	0.24±0.02	0.12±0.11	0.17±0.01	n.d.	n.d.
	ITEM 10027*	0.25±0.05	0.09±0.11	0.27±0.08	n.d.	n.d.
	KSU 488	0.14±0.03	0.10±0.00	0.22±0.04	n.d.	n.d.
	KSU 999	0.17±0.01	0.10±0.00	0.26±0.06	n.d.	n.d.
Fusarium spp.	KSU 3089G	0.32±0.07	0.10±0.00	0.10±0.12	n.d.	n.d.
Fusarium proliferatum	KSU 830	0.12±0.02	0.29±0.04	0.23±0.08	n.d.	n.d.
	KSU 4854	0.25±0.03	0.23±0.11	n.d.	n.d.	7.51±3.34
Fusarium avenaceum	ITEM 3403	0.38±0.14	22.38±11.04	0.02±0.01	0.09±0.03	0.01±0.02
	ITEM 3404	0.12±0.02	17.27±4.68	0.18±0.05	0.03±0.03	13.72±5.91

Table A.3 Enniatin and beauvericin production of Fusarium strains on potato dextrose agar (PDA) medium. n.d. = not detected; * results confirmed in LC/MS.

A.5 Conclusions

The developed analytical method in which UPLC was coupled with two detectors, PDA and MS-QDa, resulted in a unique chromatographic run that could quantify and confirm BEA and ENNs levels in agar samples. Such a methodology is essential for any effort to select strains for higher levels of metabolite production or, for testing the production capabilities of fungal isolates recovered from contaminated substrates. Twelve strains chosen to develop this method, belong to the following species: *F. fujikuroi*, *F. verticillioides*, *F. proliferatum* and *F. avenaceum*; while, one strain, not yet identified, was indicated as spp. Thus, even if further studies are necessary, and our conclusions cannot be generalized to the entire *Fusarium* genus, the method described can be used to easily analize ENNs and BEA production.

Supplementary data

Supplementary table S.1 Incidence and levels of mycotoxins in wheat samples and of species within *Fusarium* genus isolates from wheat in different locations of Italy during the growing season 2013-2014. (n.d.= not detectable).

ID	Region	Origin	DON (µg/kg)	NIV (µg/kg)	ZEA (µg/kg)	T2 (µg/kg)	HT-2 (µg/kg)	T2+HT-2 (µg/kg)	*Fusarium* species (%)
1	Piemonte	Cigliano (VC)	n.d.	n.d.	6	n.d.	n.d.	n.d.	*1% F.poae*
2		Cigliano (VC)	506	n.d.	3	n.d.	n.d.	n.d.	*9% F.graminearum; 2% F.avenaceum; 1% F.oxysporum*
3		Cigliano (VC)	966	n.d.	3	n.d.	n.d.	n.d.	*6%F.graminearum; 1%F.poae*
4	Veneto	Lendinara (RO)	58	n.d.	n.d.	n.d.	n.d.	n.d.	*1% F.poae*
5		Lendinara (RO)	531	n.d.	n.d.	n.d.	n.d.	n.d.	*4% F.graminearum; 2% F.spp;*
6		Lendinara (RO)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
7	Emilia Romagna	Molinella (BO)	382	n.d.	n.d.	n.d.	n.d.	n.d.	*2% F.graminearum; 1% F.sporotrichioides; 1% F.poae*
8		Poggiorenatico (FE)	669	n.d.	12	n.d.	13	13	*1% F.scirpi; 6% F.poae; 1% F.graminearum*
9		Ozzano (BO)	n.d.	n.d.	23	n.d.	n.d.	n.d.	*1% F.poae; 1% F.acuminatum; 7% F.poae; 1% F.graminearum*
10		Argelato (BO)	123	n.d.	n.d.	n.d.	n.d.	n.d.	*1%F.sporotrichioides; 1%sez. Gibbosum; 1%F.graminearum*
11		Ozzano (BO)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*5% F.poae*
12		San Giovanni Persiceto (BO)	n.d.	n.d.	n.d.	n.d.	8	8	

Supplementary table S.1 *continued*

ID	Region	Origin	DON (µg/kg)	NIV (µg/kg)	ZEA (µg/kg)	T2 (µg/kg)	HT-2 (µg/kg)	T2+HT-2 (µg/kg)	*Fusarium* species (%)
13	Marche		n.d.	n.d.	n.d.	n.d.	12	12	*1% F.graminearum; 1% F.poae*
14			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*17% F.poae; 2% F. acuminatum*
15			469	n.d.	n.d.	n.d.	n.d.	n.d.	*1% F.croockellense; 4% F.poae*
16			n.d.	n.d.	3	n.d.	20	20	
17			n.d.	n.d.	n.d.	n.d.	15	15	*6% F.poae*
18			n.d.	n.d.	n.d.	n.d.	21	21	*4% F.graminearum; 15% F.poae; 2% F.spp*
19			n.d.	n.d.	n.d.	n.d.	13	13	
20			n.d.	n.d.	n.d.	n.d.	13	13	*1% F.graminearum; 1% F.acuminatum; 1% F.crookwellense*
21			9129	n.d.	31	n.d.	n.d.	n.d.	*37% F.graminearum; 1% F.crookwellense*
22			n.d.	n.d.	n.d.	23	31	55	*1% F.poae*
23			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1% F.poae*
24			n.d.	n.d.	n.d.	n.d.	13	13	*12% F.poae*
25			n.d.	n.d.	n.d.	n.d.	26	26	*4% F.poae; 2% F.graminearum; 2% F.proliferatum*
26			n.d.	n.d.	n.d.	n.d.	15	15	*5% F.poae*
27			n.d.	n.d.	n.d.	n.d.	63	63	*11% F.poae*

Supplementary table S.1 *continued*

ID	Region	Origin	DON (µg/kg)	NIV (µg/kg)	ZEA (µg/kg)	T2 (µg/kg)	HT-2 (µg/kg)	T2+HT-2 (µg/kg)	*Fusarium* species (%)
28	Marche		n.d.	n.d.	32	n.d.	13	13	*11% F.poae*
29			n.d.	n.d.	54	n.d.	13	13	*2% F.poae; 2% F.tricinctum; 1% F.solani*
30			n.d.	n.d.	31	n.d.	10	10	*6% F.poae; 2% F.nivale; 3% F.proliferatum*
31			1209	71	43	n.d.	n.d.	n.d.	*6% F.poae; 2% F.graminearum*
32			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1% F.gramineraum; 3% F.poae*
33			n.d.	n.d.	3	n.d.	n.d.	n.d.	*3% F.avenaceum;1% F.graminearum*
34			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1% F.avenaceum;2% F. poae*
35			112	40	14	n.d.	n.d.	n.d.	*1% F.poae;1% F.subglutinans;1% F.equiseti*
36			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1% F.avenaceum*
37			n.d.	n.d.	n.d.	15	45	60	*1% F.proliferatum;4% F.poae*
38			n.d.	n.d.	3	19	48	68	*15% F.poae*
39			215	n.d.	n.d.	21	28	50	
40			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
41	Toscana	Cesa (AR)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
42		Cesa (AR)	n.d.	n.d.	148	n.d.	n.d.	n.d.	

Supplementary table S.1 *continued*

ID	Region	Origin	DON (µg/kg)	NIV (µg/kg)	ZEA (µg/kg)	T2 (µg/kg)	HT-2 (µg/kg)	T2+HT-2 (µg/kg)	*Fusarium* species (%)
43	Toscana	Cesa (AR)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1%F.avenaceum; 1%F.equiseti*
44		Cesa (AR)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1%F.poae*
45		Cesa (AR)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
46		Cesa (AR)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
47		Cesa (AR)	n.d.	n.d.	40	n.d.	n.d.	n.d.	*2%F.tricinctum*
48		Cesa (AR)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1%F.poae*
49		Cesa (AR)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
50		Cesa (AR)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1%F.proliferatum*
51		Cesa (AR)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
52		Cesa (AR)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
53		Cesa (AR)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
54		Gambassi Terme (FI)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*2%F.chlamydosporum*
55		Montepaldi (FI)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*2%F.poae*
56		Montepaldi (FI)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
57		Montepaldi (FI)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
58		Montepaldi (FI)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*9%F.poae*

Supplementary table S.1 *continued*

ID	Region	Origin	DON (µg/kg)	NIV (µg/kg)	ZEA (µg/kg)	T2 (µg/kg)	HT-2 (µg/kg)	T2+HT-2 (µg/kg)	*Fusarium* species (%)
59	Toscana	Montepaldi (FI)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*2%F.poae*
60		Montepaldi (FI)	n.d.	47	n.d.	n.d.	20	20	*2%F.poae*
61		Montepaldi (FI)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1%F.poae*
62		Montepaldi (FI)	n.d.	n.d.	23	n.d.	n.d.	n.d.	
63		Montepaldi (FI)	n.d.	50	n.d.	n.d.	n.d.	n.d.	*6%F.poae*
64		Montespertoli (FI)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1%F.poae*
65		Montespertoli (FI)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
66		Montespertoli (FI)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
67		Montespertoli (FI)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
68		Monticchiello (SI)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
69		Monticchiello (SI)	n.d.	65	n.d.	n.d.	n.d.	n.d.	
70		Monticchiello (SI)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
71		San Mauro (FI)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
72	Molise	Castelmauro (CB)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*3% F.poae*
73		Castelmauro (CB)	n.d.	n.d.	8	n.d.	n.d.	n.d.	*2% F.poae*
74	Puglia	Candela (FG)	n.d.	n.d.	n.d.	n.d.	35	35	*2% F.acuminatum*

Supplementary table S.1 *continued*

ID	Region	Origin	DON (µg/kg)	NIV (µg/kg)	ZEA (µg/kg)	T2 (µg/kg)	HT-2 (µg/kg)	T2+HT-2 (µg/kg)	*Fusarium* species (%)
75	Puglia	Foggia	n.d.	n.d.	18	177	104	281	1% *F. langsethiae*; 1% *F. spp*
76		Cerignola (FG)	n.d.	n.d.	5	76	n.d.	76	1% *F. langsethiae*
77		Manfredonia (FG)	n.d.	n.d.	57	120	35	155	1% *F. culmorum*
78		Ascoli Satriano (FG)	n.d.	n.d.	151	139	n.d.	290	1% *F. langsethiae*; 1% *F. oxysporum*; 1% *F. spp*
79		Stornarella (FG)	n.d.	n.d.	44	331	155	486	2% *F. acuminatum*
80	Basilicata	Maschito (PZ)	n.d.	n.d.	325	280	22	302	6% *F. acuminatum*; 1% *F. equiseti*; 1% *F. poae*
81		Melfi (PZ)	n.d.	n.d.	55	384	29	413	1% *F. acuminatum*; 1% *F. poae*
82		Palazzo San Gervasio (PZ)	n.d.	n.d.	24	266	23	289	1% *F. acuminatum*; 1% *F. spp*
83		Rapolla (PZ)	n.d.	n.d.	21	346	n.d.	346	2% *F. poae*; 1% *F. semitectum/sporotrichioides*
84		Venosa (PZ)	n.d.	n.d.	103	252	14	266	
85	Campania	Circello (AV)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*2% F.poae*
86		Bisaccia (AV)	n.d.	n.d.	7	335	n.d.	335	2% *F. langsethiae*
87		Calitri (AV)	n.d.	n.d.	35	327	22	349	1% *F. culmorum*; 1% *F. poae;* 1% *F. semitectum;* 1% *F. avenaceum;* 2% *F. spp*

Supplementary table S.1 *continued*

ID	Region	Origin	DON (µg/kg)	NIV (µg/kg)	ZEA (µg/kg)	T2 (µg/kg)	HT-2 (µg/kg)	T2+HT-2 (µg/kg)	*Fusarium* species (%)
88	Campania	Grottaminarda (AV)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
89		Lacedonia (AV)	n.d.	n.d.	9	236	n.d.	236	1% *F. spp*
90		Savignano Irpino (AV)	n.d.	n.d.	14	310	27	337	3% *F. acuminatum*; 3% *F. compactum/equiseti;* 1% *F. poae;* 1% *F. spp*
91	Sicilia	Roccamena (PA)	n.d.	n.d.	55	385	11	396	
92		Mussomeli (CL)	n.d.	n.d.	4	351	n.d.	351	1% *F. poae*
93		Leonforte (EN)	n.d.	n.d.	12	420	n.d.	420	
94		Raddusa (CT)	n.d.	n.d.	12	375	n.d.	375	
95		Valledolmo (PA)	n.d.	n.d.	11	460	n.d.	460	

Supplementary table S.1

Supplementary table S.2 Incidence and levels of mycotoxins in wheat samples and of species within *Fusarium* genus isolates from wheat in different locations of Italy during the growing season 2014-2015. (n.d.= not detectable).

ID	Region	Origin	DON (µg/kg)	NIV (µg/kg)	ZEA (µg/kg)	T2 (µg/kg)	HT-2 (µg/kg)	T2 + HT-2 (µg/kg)	*Fusarium* species (%)
1	Piemonte	Gerinola (CN)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*18% F.graminearum, 4% F.poae, 2% F.semitectum, 2% F.avenaceum, 41% F.spp*
2		Gerinola (CN)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*3% F.semitectum, 1% F.crookwellense, 1% F.oxysporum, 1% F.proliferatum, 53% F.spp*
3		Gerinola (CN)	2493	76	6	n.d.	n.d.	n.d.	*17% F.graminearum, 8% F.avenaceum, 3% F.verticillioides, 1% F.sporotrichioides, 47%F.spp*
4	Emilia Romagna	Noceto (PR)	n.d.	n.d.	9	n.d.	n.d.	n.d.	*22% F.poae, 16% F.graminearum*
5		Noceto (PR)	n.d.	n.d.	4	n.d.	n.d.	n.d.	*33% F.poae, 6% F.graminearum, 1% F. spp*
6		Noceto (PR)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*25% F. poae*
7	Marche		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1% F. poae; 2% F. avenaceum*
8			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*2% F. poae*
9			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
10			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*6% F. poae*
11			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1% F. poae*
12			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1% F. avenaceum*
13			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*2% F. graminearum; 6 % F. poae*

Supplementary table S.2 *continued*

ID	Region	Origin	DON (µg/kg)	NIV (µg/kg)	ZEA (µg/kg)	T2 (µg/kg)	HT-2 (µg/kg)	T2 + HT-2 (µg/kg)	*Fusarium* species (%)
14			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
15			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1% F. poae; 2% F. avenaceum*
16			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
17			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
18			767	n.d.	n.d.	n.d.	n.d.	n.d.	*7% F. poae*
19			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*3% F. graminearum*
20	Marche		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1% F. proliferaum*
21			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1% F. poae*
22			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1% F. chlamydosporum; 1% F. poae*
23			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*5% F. poae; 1% F. culmorum*
24			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1% F. proliferatum, 1% F. poae*
25			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1% F. poae*
26			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
27	Toscana		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1% F. proliferatum*
28			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*2% F. poae*

Supplementary table S.2 *continued*

ID	Region	Origin	DON (µg/kg)	NIV (µg/kg)	ZEA (µg/kg)	T2 (µg/kg)	HT-2 (µg/kg)	T2 + HT-2 (µg/kg)	*Fusarium* species (%)
29	Toscana		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1% F. poae*
30			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1% F. poae*
31			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
32			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*3% F. poae*
33			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
34			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
35			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
36			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*4% F. poae; 2% F. proliferatum*
37			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*3% F. poae*
38			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*2% F. poae*
39			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
40			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
41			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
42	Basilicata	Venosa (PZ)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
43	Puglia	Deliceto (FG)	n.d.	n.d.	n.d.	16	44	60	

Supplementary table S.2 *continued*

ID	Region	Origin	DON (µg/kg)	NIV (µg/kg)	ZEA (µg/kg)	T2 (µg/kg)	HT-2 (µg/kg)	T2 + HT-2 (µg/kg)	*Fusarium* species (%)
44	Campania	Benevento (BN)	n.d.	n.d.	4	n.d.	12	12	
45		Reino (BN)	n.d.	n.d.	n.d.	n.d.	13	13	*1% F. poae*
46		Lacedonia (AV)	n.d.	n.d.	5	n.d.	n.d.	n.d.	
47	Sicilia	Ramacca (CT)	n.d.	n.d.	4	n.d.	n.d.	n.d.	
48		Regalbuto (EN)	n.d.	n.d.	5	15	22	37	
49		Enna (EN)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*8% F. poae*
50		Roccamen (PA)	n.d.	n.d.	n.d.	n.d.	31	31	
51		Valledolmo (PA)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1% F. culmorum; 1% F. equiseti; 1% F. poae*
52		Mussomeli	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	

Supplementary table S.2

Supplementary table S.3 Incidence and levels of mycotoxins in wheat samples and of species within *Fusarium* genus isolates from wheat in different locations of Italy during the growing season 2015-2016. (n.d.= not detectable).

ID	Region	DON (µg/kg)	NIV (µg/kg)	ZEA (µg/kg)	T2 (µg/kg)	HT-2 (µg/kg)	T2 + HT-2 (µg/kg)	*Fusarium* species (%)
1	Marche	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*5%F.poae; 2%F.longipes; 1%F.equiseti; 1%F.tricinctum; 10%F.spp*
2		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1% F. spp*
3		610	n.d.	n.d.	n.d.	n.d.	n.d.	*10%F.acuminatum/tricinctum; 4%F.graminearum; 5%F.spp*
4		605	n.d.	n.d.	n.d.	n.d.	n.d.	*7%F.avenaceum; 3%F.graminearum; 3%F.poae*
5		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*2%F.poae;1%F.graminearum;2%F.spp*
6		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*2%F.poae; 1%F.spp*
7		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*3%F.poae; 1%F.avenaceum; 2%F.spp*
8		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*2%F.acuminatum; 1%F.culmorum*
9		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1%F.poae; 1%F.avenaceum*
10		264	n.d.	n.d.	n.d.	n.d.	n.d.	*11%F.poae; 10%F.graminearum; 2%F.avenaceum; 2%F.fujikuroi complex; 2%F.spp*
11		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*2%F.avenaceum; 1%F.poae*

Supplementary table S.3 *continued*

ID	Region	DON (µg/kg)	NIV (µg/kg)	ZEA (µg/kg)	T2 (µg/kg)	HT-2 (µg/kg)	T2 + HT-2 (µg/kg)	*Fusarium species* (%)
12	Marche	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*3% F.poae; 2%F.scirpi*
13		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*4% F.avenaceum; 1%F.acuminatum; 1%F.poae*
14		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*5% F.acuminatum; 1%F.tricinctum; 1%F.gramineraum*
15		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*4% F.poae; 2%F.scirpi; 1%F.avenaceum*
16		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1% F.acuminatum; 1%F.spp*
17		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1% F.poae; 1%F.avenaceum*
18		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*5% F.poae; 4% F.nivale; 3% F.avenaceum; 1%F.spp*
19	Toscana	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*2% F.poae*
22		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*3% F.poae*
21		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
22		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1% F. graminearum; 1% F. sporotrichioides; 1% F. avenaceum*
23		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1% F. graminearum*
24		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*1% F. avenaceum*

Supplementary table S.3 *continued*

ID	Region	DON (µg/kg)	NIV (µg/kg)	ZEA (µg/kg)	T2 (µg/kg)	HT-2 (µg/kg)	T2 + HT-2 (µg/kg)	*Fusarium species (%)*
25	Toscana	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*3% F. avenaceum*
26		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*2% F. proliferatum; 1% F. tricinctum*
27		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*7% F. poae; 1% F. proliferatum*
28		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*8% F. avenaceum; 3% F. poae; 2% F. equiseti*
29		486	n.d.	n.d.	n.d.	n.d.	n.d.	*3% F. poae; 2% F. oxysporum; 2% F.graminearum*
30		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*2% F. poae; 2% F. proliferatum; 2% F. avenaceum*
31		570	n.d.	n.d.	n.d.	n.d.	n.d.	*2% F. proliferatum; 1% F. poae; 1% F. tricinctum*

Supplementary table S.3 *continued*

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