UNIVERSITY OF PADOVA

DOCTORATE SCHOOL OF CROP SCIENCE

CURRICULUM CROP PROTECTION- CYCLE XXII

Department of Land Use and Agro-Forestry Syestems – Plant Pathology

A molecular approach to biological control of mycotoxigenic fungi

Director of school: Ch.mo Prof. Andrea Battisti

Supervisor : Prof. Roberto Causin

PhD student: Davide Ferrigo

DATE OF THESIS SUBMISSION:

JANUARY 15TH, 2010

Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

15.01.2010

Davide Ferrigo

A copy of the thesis will be available at *http://paduaresearch.cab.unipd.it/*

Table of contents

Chapter 3 – ROS and fumonisins regulation

Changes in fumonisin biosynthesis by F. verticillioides in response to oxidative stress by H2O2

Chapter 4 – Ochratoxin A biosynthesis

A cDNA-AFLP approach to study Ochratoxin A production in Aspergillus carbonarius

Chapter 5 – General conclusions

Riassunto

Capitolo 1

La sicurezza alimentare è oggigiorno considerata una priorità a livello mondiale. Nei paesi sviluppati così come in quelli in via di sviluppo ci si trova sempre più spesso a dover fronteggiare numerose problematiche legate a disordini alimentari e rischi per la salute. Una regolare alimentazione per tutti rappresenta un obiettivo da perseguire con forza ma ciò deve andare di pari passo con una alimentazione sana e priva di rischi.

Il miglioramento delle condizioni di vita nei paesi emergenti è da sempre accompagnato al potenziamento della produzione agricola e risulta di fondamentale importanza per uno sviluppo economico sostenibile. La globalizzazione dei mercati e lo sviluppo di una economia di scambio mondiale hanno notevolmente influenzato ed allargato i mercati di sbocco delle produzioni alimentari ma hanno al contempo aumentato la circolazione di beni possibilmente pericolosi dal punto di vista sanitario.Tra le principali minacce legate al settore alimentare, le micotossine rappresentano una problematica da contenere e risolvere. Le micotossine sono sostanze chimiche prodotte dal metabolismo secondario di alcuni funghi con ospiti e necessità climatiche molto diversi tra loro. Diverse condizioni climatiche portano alla sviluppo di funghi capaci di produrre diverse micotossine e per i paesi affacciati sul bacino mediterraneo, due tra i funghi micotossigeni più presenti risultano essere il *Fusarium verticillioides* in mais e *Aspergillus carbonarius* sull'uva, responsabili dell'accumulo rispettivamente di fumonisine e ocratossina A.

Negli ultimi anni la comunità internazionale ha maturato maggiore coscienza dei rischi correlati alle micotossine e i principali organi di controllo hanno stilato strategie operative di accertamento di salubrità da applicare in ogni pasaggio della filiera produttiva per contenere il diffondersi di malattie e disturbi legati all'assunzione di micotossine. A tal riguardo nel 2006, l'Agenzia Europea per la Sicurezza Alimentare (EFSA) ha fornito le linee guida per la tracciabilità degli alimenti sia in fase produttiva che distributiva ed aggiorna periodicamente i quantitativi limite di micotossine per le diverse matrici in cui possono essere rinvenuti. Mentre le normative finora risultano focalizzate al controllo della salubrità dei prodotti ed alla rimozione di quelli risultanti non conformi, molto poco viene suggerito per limitare l'accumulo delle micotossine nei prodotti alimentari. Le

trasformazioni a cui vengono sottoposte le produzioni cerealicole e agricole in genere sono la fase maggiormente controllabile di tutta la catena produttiva, i punti critici di rischio sono invece legati all'accumulo di micotossine in campo durante la fase di maturazione.
I trattamenti effettuati con composti chimici hanno dato prova di essere parzialmente efficaci nel ridurre i livelli di contaminazione, ciononostante i vari aspetti economici, ecologici e di salubrità dovrebbero condurre allo sviluppo di nuove strategie a basso impatto come il controllo biologico. Gli studi finora sviluppati sulla produzione e la regolazione delle micotossine sono molto diversificate in base all'importanza economica che rivestono, mentre le fumonisine sono ben studiate, le informazioni riguardanti le ocratossine sono lacunose.
Nel presente lavoro approcci molecolari come studi di attività enzimatica, Real-Time PCR e cDNA-AFLP sono stati utilizzati per verificare la capacità di biocontrollo di *Trichoderma harzianum* nei confrionti di *F. verticillioides* in mais, gli effetti di condizioni di stress ossidativo nell'influenzare la produzione di micotossine e fornire importanti informazioni sui geni biosintetici e regolativi coinvolti nella produzione delle ocratossine, la cui biosintesi è ancora parzialmente sconosciuta.

Capitolo 2

La colonizzazione del mais da parte di *F. verticillioides* è spesso accompagnata da un accumulo di fumonisina B1 e B2, entrambe tossiche e cancerogene in seguito ad assunzione da parte dell'uomo o di animali. L'individuazione, la prevenzione ed il controllo delle infezioni causate da *F. verticillioides* è difficile, soprattutto nei casi in cui il fungo vive come endofita e non dà alcun sintomo visibile. La lotta tramite l'utilizzo di agenti di biocontrollo può risultare una strategia promettente per il controllo di funghi fitopatogeni in un ampia varietà di ospiti. I funghi appartenenti al genere *Trichoderma* in particolare colonizzano le radici, producono sostanze antifungine, promuovono lo sviluppo della pianta e sono in grado di stimolare le difese sistemiche nella pianta.
Nel presente lavoro, alcune piante di mais sono state trattate con *T. harzianum* T22 e infettate con *F. verticillioides* per valutare l'efficacia dell'induttore di resistenza nel controllare l'infezione. Le piante trattate con T22 presentavano una diminuzione dei sintomi (in ampiezza e gravità) molto marcata rispetto alle piante non trattate. Le analisi dei

marcatori enzimatici e molecolari per SAR e ISR hanno confermato l'avvenuta induzione. Infine i risultati ottenuti in sera sono stati replicati in prove di campo: il trattamento con T22 ha comportato una generalizzata riduzione dell'inoculo fungino a livello delle sete e della granella, inoltre si è notato un importante abbattimento del contenuto di fumonisine.
In conclusione i nostri risultati confermano l'efficacia di *T. harzianum* come agente di biocontrollo in mais rappresentando un semplice ed efficace metodo di lotta contro *F. verticillioides* e l'accumulo di fumonisine.

Capitolo 3

A risposta di numerosi stimoli e minacce alla propria integrità, le piante hanno sviluppato varie strategie, tra queste vi è il rilascio controllato di specie reattive dell'ossigeno (ROS). A seguito di stress sia biotici che abiotici come strategia di signalling oppure di difesa le piante producono ed accumulano ROS attivando un "burst ossidativo".
Queste variazioni del potenziale ossidativo oltre ad essere funzionali per la pianta, sono in grado di indurre la biosintesi delle micotosine. Da precedenti studi è stato notato che in mais le fumonisine vengono prodotte da *Fusarium verticillioides* solo nelle fasi finali della maturazione e avvengono maggiormente in concomitanza con episodi di stress quali ferite o stress idrico. In *Aspergillus flavus* così come in *F. graminearum* è stato inoltre provato che l'accumulo intracellulare di ROS è un prerequisito fondamentale per la sintesi rispettivamente di aflatossine e tricoteceni, nessuno studo è stato effettuato sulle fumonisine. Accreditata l'ipotesi che il potenziale ossidativo esogeno della pianta sia in grado di influenzare il fungo, risulta pertanto plausibile che i ROS prodotti dalla pianta in situazioni di stress possano influenzare la sintesi di micotossine. Nel presente lavoro sull'efficacia di H_2O_2 nell'indurre l'accumulo di fumonisine e sui cambiamenti trascrizionali a carico dei geni coinvolti nella biosintesi delle fumonisine.
I trattamenti con differenti concentrazioni di H_2O_2 effettivamente inducono la biosintesi di fumonisine e dal punto di vista trascrizionale tutti i geni indagati sono risultati maggiormente espressi sebbene non tutti allo stesso modo.
In conclusione viene confermata anche per le fumonisine l'importanza dei ROS nell'iniziazione e modulazione della biosintesi delle micotossine, supportando l'ipotesi che le situazioni di stress della pianta favoriscono l'accumulo di fumonisine nella granella.

Capitolo 4

Le ocratossine sono metaboliti secondari prodotti da alcune specie di funghi filamentosi appartenenti principalmente ai generi *Aspergillus* e *Penicillium*. L'accumulo di ocratossine e specialmente di ocratossina A (OTA) è fortemente influenzato da fattori quale la temperatura, l'attività dell'acqua, il pH, la composizione del substrato e pertanto sono di particolare rilievo le condizioni climatiche in cui si sviluppa il fungo. Nei paesi del bacino mediterraneo la maggioranza delle contaminazioni da OTA sono a carico di *Aspergillus carbonarius* che infetta le uve in via di maturazione. Il vino prodotto con uve contaminate risulta esso stesso contenere OTA e paesi come Italia, Francia e Spagna come principali produttori ed esportatori si trovano a fronteggiare questa problematica.

La risoluzione dell'emergente problematica OTA necessita una maggiore conoscenza della biosintesi delle tossine così come della loro regolazione al fine di predisporre strategie di controllo e prevenzione della contaminazione in campo. Una strategia finora poco utilizzata potrebbe essere legata all'immissione in campo di ceppi ipotossigeni per limitare lo sviluppo dei ceppi autoctoni di *A. carbonarius* ma un'analisi attenta dovrebbe includere la verifica dei geni legati alla biosintesi delle ocratossine, geni che finora risultano quasi totalmente sconosciuti.

Nel presente lavoro l'utilizzo della tecnica cDNA-AFLP ha permesso di identificare ed isolare geni differentemente espressi tra un ceppo alto produttore ed uno basso produttore di *A. carbonarius*, permettendo di identificare e parzialmente caratterizzare 119 sequenze differenti. In base alla loro funzione tali sequenze hanno permesso di ricostruire un ipotetico modello regolativo influenzato sia dalla disponibilità di molecole precursori sia dallo stadio di sviluppo della cellula, come evidenziato per altre micotossine.

Questo lavoro ha dato un importante contributo alla comprensione della biosintesi delle OTA, finora poco approfondito per *A. carbonarius.*

Summary

Chapter 1

Food safety has become a worldwide priority in public health. In rich as in developing countries, population must face up many problematic related to food able to cause alimentary disorders. While availability of food for everybody is a real conquest to pursue, nevertheless safe and health food is a condition which we can not renounce. With the improvement of life-conditions in emerging countries, agricultural production became once more, fundamental for sustainability of population health and economic development. In addition the occurrence of globalization increase agricultural productions and food industries, turning the products distribution from local to bigger scale, so that localized food-threat can be exported to global market, making easier the people assumption to multiple damaging compounds. Among the most important food threats, contamination by mycotoxin in cereals represent a past, daily and incoming problem to solve. Mycotoxins are chemical compounds produced by a large variety of Fungi with host and climatic needs very different each other. In different European countries, climatic conditions lead to the development of different fungi, consequentially different mycotoxins. Climate conditions of Mediterranean basin, promotes the development, among all, of *Fusarium verticillioides* in maize and *Aspergillus carbonarius* in grape, responsible for production of fumonisins and ochratoxins respectively. In the last years, the international community acquired consciousness of mycotoxigenic risks and is trying to furnish operative strategies to control every step of the food chain, from producers to consumers to avoid the spreading of new diseases and health problems. In particular in 2006 the European Authority for Food Safety (EFSA) gave the guidelines to traceability of foods in every step of productive and distributive chains, while the contamination limits for each mycotoxin were periodically updated. While European regulations are focusing on removal of contaminated and unsafe food, very few is indicated to prevent the mycotoxin occurrence. The different transformations from cereal to derivatives are the stage more verifiable in food chains, anyway the critical time to accumulation of mycotoxins happen in field during grain maturation. Treatments with many synthetic chemical compounds have been proved to reduce the contamination levels but economical, ecological and nutritional aspects in public

opinion, lead to attempt new strategies, as biological control. State of art for mycotoxins production and regulation is much diversified according to importance of economical threat, while fumonisins are well studied, knowledge on ochratoxins presents many gaps. In the present research, molecular approaches as enzymatic activity, sqReal-Time PCR and cDNA-AFLP have been used to evaluate the biocontrol ability of *Trichoderma harzianum* in maize, the ability of stress condition to promote fumonisin production and furnish an important overview on genes involved in ochratoxin biosynthetic pathway, still unknown.

Chapter 2

F. verticillioides colonization of maize kernels results in fumonisin B1 (FB1) and B2 (FB2) contamination, having a notable impact on human and animal health. Detection, prevention and control of the infections by *F. verticillioides* in maize are difficult, especially when infections remain at endophytical stage and kernels appear to be uninfected Control by biological control agents (BCA) can be a promising strategy for managing seed-borne, soil-borne and foliar diseases in a wide range of crops. Fungi belonging to genera *Trichoderma* colonize roots, produce antifungal substances and enhance plant growth and trough secondary metabolites can trigger systemic resistance towards a wide range of pathogen.

In our experiment maize stalks were inoculated with *Fv*S19 to evaluate the ability of T22 root- priming to induce resistance against this endophytic pathogen. Decrease of symptoms was observed with a significant reduction of necrotic areas in T22-primed plants compared to untreated plants. Furthermore, enzymatic and transcriptional profiles on gene resistance-related confirm the activation of a systemic resistance response even if further study will clarify the effective component connected to ISR and SAR. Finally, once verified effectiveness of T22 treatment, an experiment was carried out to evaluate the ability of *T. harzianum* priming to work also in field conditions. T22-coated seeds were sown and at the harvest time kernels reported a lower contamination by *F. verticillioides* and a reduction of both FB1 and FB2 compared to untreated.

In summary, our findings provide further evidences that the biocontrol agent *T. harzianum* T22 was able to decrease infection ratio in maize against *F. verticillioides* inducing systemic resistance. This approach can represent an easy and useful method to control *F. verticillioides* occurrence and fumonisin accumulation in maize.

Chapter 3

In response to different environmental threats, plant developed a broad range of strategies. Plant responses can include rapid and transient releasing of reactive oxygen species (ROS) Both in abiotic stress as part of signalling or in biotic stress to restrict pathogen growth plant trigger an `oxidative burst'. This signalling pathway and response strategy, besides to plant protection, have been noticed to acts as inducer in biosynthesis of some mycotoxin. Fumonisins are biosynthesized by *F. verticillioides* only late in plant development especially as a consequence of tissue damage, stress drought and high temperature. Beyond abiotic stress, it is possible that exogenous compounds, as ROS, can activate signalling pathways in Fungi capable to modulate gene expression. Since in *Aspergillus flavus* and *Fusarium graminearum* intracellular oxidative stress can be considered a "prerequisite" for respectively aflatoxin and trichotecenes production, we may hypothesise that H_2O_2 produced by plant following oxidative burst, could modulate mycotoxin production in host tissues. The mechanism supervising regulation by oxidative stress still remains unknown. In the present work we surveyed the expression of many genes involved in biosynthesis of fumonisins. The objectives of this study were to determine the influence of oxidative stresses on fumonisin production. Treatments with different concentration of H_2O_2 effectively stimulate fumonisin production. Our results showed that under oxidative stress, gene expression seems to be globally enhanced although genes were differentially modulated by H_2O_2 concentration. The present work confirms also for fumonisin the strong importance of reactive oxygen species for initiation and modulation of fumonisin biosynthesis, supporting hypothesis that plant stresses promote fumonisins accumulation in kernels.

Chapter 4

Ochratoxins (OTs) are secondary metabolites produced by several species of filamentous fungi belonging principally to the genera *Aspergillus*. OTA contamination may be affected by different factors such as temperature, water activity, pH and substrate composition. Therefore the toxigenicity of an ochratoxigenic fungus may vary according to the crop and the geographical area. In warm locations, *A. carbonarius* is responsible for the majority of

contaminations in grapes. Among the commodities that can be contaminated by OTA, grapes and wines are those with the major content of toxin, next only to cereals. Wine is an important beverage in the world trade, of which France, Italy and Spain are the main producers and exporters. Due to the actual importance of the wine industry, several studies were carried out to assess the relevance of OTA contamination in wine. Since OTA represents a challenging problem both for food and feed producers and consumers, the full elucidation of the biosynthetic pathway and its regulation would allow the setting up of innovative tools to control and/or prevent the crop contamination. In this view, biocompetition strategies using atoxigenic strains have revealed to be a possible solution of biological control. The OTA biosynthetic pathway has not yet been completely elucidated. In the present research, cDNA-AFLP differential display was used to isolate genes differentially expressed in OTA high- and low-producing strains of *A. carbonarius*. allowing the identification of 119 partially characterized sequences. A possible model for OTA biosynthesis regulation may be hypothesized, according to the up- and down-regulation patterns pointed out by means of cDNA-AFLP differential display. According to this model, OTA biosynthesis may depend both on the availability of precursors and on the developmental stage, as pointed out for several other. The present study gives an important contribution to the understanding of OTA biosynthesis, even though specific studies should be addressed to the functional characterization of the genes herein isolated, either by silencing or knocking-down approaches.

Chapter 1 – General introduction

Mycotoxins and food safety

Food safety has become a worldwide priority in public health. The occurrence of globalization and the will to assure satisfactory nutritional level, lead to an increment of agricultural productions and food industries, turning the goods distribution from local to bigger scale in shorter time. This purpose influences the presence in foods of natural or synthetic chemical compounds able to alter people's health and localized food-threat can be exported to global market, making easier the people's assumption of multiple damaging compounds. An evaluation of potential effects of these molecules brings us to define "safety" as the certainty that human exposure to a particular substance can't provoke health damage. In order to evaluate quality of foods by different perspectives, not only nutritional, hygienic or productive but also toxicological aspects must be taken into account. In rich as in developing countries, population must face up many problematic related to food causing alimentary disorders and risks for human health. Among the main threats, one of the most important is represented by biological risk, and among dangerous microorganisms to human, Fungi are often underestimated. Some Fungi are pathogen to human, animals and plants. Fungi are responsible to direct health damages as mycoses, but they act also in food depletion and finally, some genus can contaminate food with mycotoxins. In the last years, the international community acquired consciousness of mycotoxigenic risks and is trying to furnish operative strategies to control the mycotoxin contamination in every step of the food chain, from producers to consumers, to avoid the spreading of new diseases and health problems. Developing countries are more threaten to mycotoxins contamination, due to undeveloped production system and scarce ability to control toxins occurrence. Cereals food chains are involved in mycotoxin concern in every step of production, just from field where pedoclimatic conditions and uncorrected agricultural practices promote harmful fungal diseases. Other critical points are food and feed storage and finally transformation steps to edible goods.

Incoming needs to dispose normative that regulates food safety led WTO and European Authority for Food Safety (EFSA) to establish measures to apply in food safety, animal, plant health measures (sanitary and phytosanitary or SPS measures) and the guidelines to traceability of foods in every step of productive and distributive chains. Mycotoxins are ruled in European Union by Reg. (CE) N. 1881/2006 and 1126/2007 relatively to food

contaminants and maximal amount of each mycotoxin: maximal amounts are express in μg/kg for different matrix and food typology. While European regulations are focusing on removal of contaminated and unsafe food, very few is indicated to prevent the mycotoxin occurrence. In different European countries, climatic conditions lead to the development of different fungi, consequentially different mycotoxins. Due to their relative stability to physics and chemical treatments, mycotoxins in food represent a practical and economic problem. Prevention of contamination become, therefore, the most effective practice to avoid the harvest of hardly contaminated crops. Not only good agronomical practice can support reduction of fungal disease and consequently mycotoxins control, also synthetic chemical compounds have been proved to reduce the contamination levels (Causin *et al.,* 2008; Folcher *et al.,* 2009). Anyway, economical, ecological and nutritional aspects in public opinion, lead to attempt new environmental friendly strategies, as biological control. The identification and utilization of new biocontrol agents (BCA) towards different fungi in different crops represent a challenging opportunity. These new technologies and practices could prevent losses of agricultural production and promote food safety. Fungal biological control agents (BCA) carry out their "control activity" principally as antagonist, parasites or producing secondary metabolites (Weller, 1988; Adams, 1990). Traditional methods to evaluate biological control ability are often time consuming and cannot explain the complex web of interrelation and regulation between host and pathogen. Nowadays, new techniques use molecular rather than metabolomic approaches to probe new BCA in agriculture. State of art for mycotoxins production and regulation is very diversified according to importance of economical threat, while fumonisins and aflatoxin are well studied, knowledge on ochratoxins presents many gaps. The present work was carried out with the aim to enrich knowledge about mycotoxin production and provide useful elements to point out strategies of biological control of mycotoxigenic Fungi.

Mycotoxins

Mycotoxins are low weight chemical compounds synthesized by filamentous Fungi. These metabolites are chemically and toxicologically very different each other, but are all associated to be harmful to human and animals.

Mycotoxins can be classified according to the injuries that can provoke (Galli *et al.,* 2004):

- Immunotoxins: aflatoxins ochratoxins, fumonisin and trichotecenes interact with immunological defences altering the ability to control pathogenic agents, toxic compounds and carcinogenic cells.
- Hepatotoxins: aflatoxins and ochratoxins act on liver cells provoking necrosis and fat accumulation.
- Nephrotoxins: ochratoxin A damages kidney and alters absorbance of ionic species in blood.
- Neurotoxins: fumonisins are able to damage nervous system cells blocking or altering nervous impulses and signals.
- Hormone-like toxins: zearalenone is a potent estrogenic-like molecule causing infertility, abortion and early child development.

Term mycotoxin was coined in 1962 in U.K. in relation to death of 100.000 turkeys due to feed contaminated by aflatoxin (X-disease). Mycotoxin contamination of food represents a risk for both human and animal health, with consequences of economic losses.

Several economic studies have estimated costs associated with mycotoxins in food and feed crops. A recent estimation of the economic losses by mycotoxin contamination reports a worldwide cost of 2.67 billion dollars. (Robens and Cardwell, 2003; Vardon *et al*., 2003; Wu *et al*., 2008).

The most economically relevant toxins are produced by genus *Aspergillus*, *Fusarium*, *Penicillium* and *Alternaria*. Aflatoxins are produced by *Aspergillus* (Goto *et al.,* 1996) and ochratoxin can be product by *Aspergillus* and *Penicillium* (Miller, 2002; Niessen *et al.,* 2005) Genera *Fusaria* is responsible to production of trichotecenes (DON, NIV, T2, HT2, DAS,) (Maier *et al*., 2006) Zearalenone (Hartmann *et al.,* 2008) and fumonisin (Binder *et al.,* 2007). Although mycotoxin are a well studied focus, very low has been discovered

about their function in fungal physiology (Proctor *et al.,* 2002; Glenn *et al.,* 2008) due to their wide heterogeneity. However, for fumonisin, aflatoxins and trichotecenes several studies provided information about production, biosynthetic pathways (Sweeney and Dobson, 1998; Proctor *et al.,* 2003; Yu *et al.,* 2004; Keller *et al.,* 2005) and regulation (Calvo *et al.,* 2002; Bluhm *et al.,* 2006; O'Callaghan *et al,* 2006; Kim and Woloshuk, 2008; Jurado *et al.,* 2008).

A wide variety of agricultural products can be contaminated by mycotoxins, damages for human consumption and animal feed are also linked to mycotoxins stability to chemical and physical treatments that permit a carry over from raw materials to transformed food products. Climate conditions of Italian regions, promotes the development, among all, of *Fusarium verticillioides* in maize in northern Italy and *Aspergillus carbonarius* in grape in southern Italy, responsible for production of fumonisins and ochratoxins respectively. In this elaborate will be elucidate in particular, elements concerning fumonisins B and ochratoxin A.

Fumonisins

Fumonisins are mycotoxins produced by the maize pathogen *Gibberella moniliformis* Wineland (syn. *G. fujikuroi* mating population A; anamorph *Fusarium verticillioides* (Sacc.) Nirenberg. Among all species of Fusarium, only *F. verticillioides*, *F. proliferatum*, and *F. nygamai* has been proved to produce fumonisins (Rheeder *et al.,* 1992; Thiel *et al.,* 1993) at appreciable level. Fumonisins are structurally similar to the sphingolipid intermediates sphinganine and sphingosine (Fig. 1), and they disrupt sphingolipid metabolism by inhibiting the enzyme ceramide synthase (sphinganine N-acyltransferase) (Wang *et al.,*1991). The discovery of fumonisins came as a result of a decade of search by Bezuidenhout *et al* (1988) for a possible cause of the high incidence of oesophageal cancer in the Transkei region of southern Africa. The most of fumonisin is B1 and was shown to be a diester of propane-l,2,3-tricarboxylic acid and a 2-amino12,16-dimethyl, 3,5,10,14,15-pentahydroxyicosane with both C-14 and C-15 hydroxyl groups esterified with the terminal carboxyl group of the acids. In addition to fumonisin B1 several related fumonisins have been reported: fumonisin B2 and fumonisin B3 are homologs that lack one

of the free hydroxyl groups on the backbone fumonisin while B4 lacks both hydroxyl groups at C-5 and C-l0 (Wentzel *et al.,* 2007).

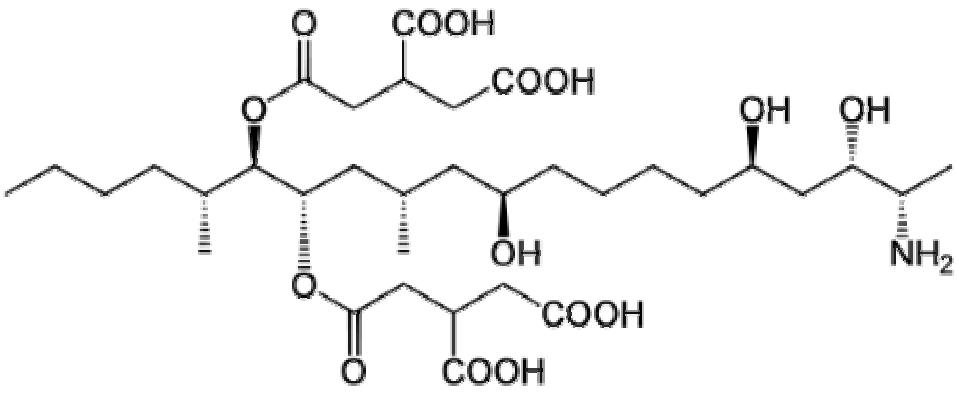


Figure 1: Structure of Fumonisin B1

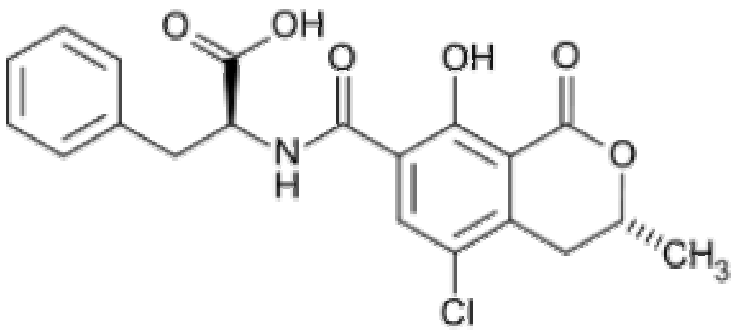
Fumonisin has been reported to be toxic and known to cause pulmonary oedema in swine, leukoencephalomalacia due to the ingestion of mouldy corn in horses (Prelusky *et al.,* 1994) and immunological disorder in ducks (Bennet *et al,.* 2003). As previously reported, human exposure to *F. verticillioides* contaminated maize has been linked to elevated rates of oesophageal cancer and FB1 itself has been evaluated by IARC as a group 2B carcinogen (Vainio *et al.,* 1993) Because of the high potential of toxicity, the European Union has limited the fumonisin content in food to 4000 μg/kg in raw maize, 1000 μg/kg in maize flour and grits, 800 μg/kg for human consumption food and 200 μg/kg in child food. In filamentous ascomycetes, genes involved in the biosynthesis of mycotoxins (e.g., aflatoxins, paxilline, and trichothecenes) and other secondary metabolites (e.g., gibberellins, lovastatin, antibiotics) are frequently organized into gene clusters (Laich *et al.,* 1999; Brown *et al.,* 2001; Young *et al.,* 2001). Evidences proved that gene organization in *Fusarium spp*. is similar to other fungi and, whereas regulatory genes are isolated each other, biosynthetic genes are clustered together. Up to now, several genes involved in fumonisin production were characterized: *Fum1* (Desjardin *et al.,* 1995) *Fum2* and *Fum3* (Desjardin *et al.,* 1996), *Fum4* (Plattner *et al.,* 1996), *Fum5* (Proctor *et al.,* 1999), *areA* (Tudzynski *et al.,* 1999), *Fum6*, *Fum7*, *Fum8*, and *Fum9* (Seo *et al.,* 2001), *Fcc1* (Shim and Woloshuk, 2001) *Fum10-Fum19*, (Proctor *et al.,* 2003) *Pac1* (Flaherty *et al.,* 2003), *Zfr1*

(Flaherty *et al.,* 2004) *Fum20* (Brown *et al.,* 2005) *Fck1* (Bluhm *et al.,* 2006), *Fum21* (Brown *et al.,* 2007), *Fst1* (Bluhm *et al.,* 2008). Attempts to correlate gene expression with fumonisin production has been surveyed (López-Errasquín *et al.,* 2007), these kind of researches could provide promising tools to the prediction of mycotoxin in crops but regulation of biosynthetic pathway is complex and much must be discovered. Worldwide surveys have indicated that fumonisins occur at biologically significant levels in maize and in a variety of maize-based human foodstuffs and animal feed (Marasas *et al.,* 1995; Bolger *et al.,* 2001). Due to severity and importance of maize in worldwide agricultural production, several researches are carrying out to furnish effective practice in fumonisin control. At the present time agronomical practice (GAP) and insecticide treatments against European Corn Borer (ECB) are the better strategy to reduce fumonisin content in grain. GAP application and ECB control have to be integrated with direct, chemical and biological, control strategies of *F. verticillioides* and *F. proliferatum* infections. Among the best strategies, biological control using fungi and bacteria are a promising perspective to investigate.

Ochratoxins

Ochratoxins are secondary metabolites produced by several species of filamentous fungi mainly belonging to the genera *Aspergillus* and *Penicillium* (Perrone *et al.,* 2006). In temperate climate, ochratoxins are prevalently produced by *Penicillium spp*, in warmer and tropical regions are synthesized prevalently by *Aspergillus* species, in particular *A. ochraceus*, *A. niger* and *A. carbonarius* with different host range and ability to contaminate products. *A. ochraceus* in tropical region contaminate several products, such as legumes, coffee and nuts rice (Joonsten *et al.,* 2001; González *et al.,* 2005) whereas *A. niger* and *A. carbonarius* contaminate prevalently grain and grape respectively (Battilani *et al.,* 2003; Atoui *et al.,* 2007; Perrone *et al.,* 2008). Between ochratoxins, the form A is the most potent, chlorinated derivative and was discovered in 1965 as a secondary metabolite of an *Aspergillus ochraceus* strain (Van der Merwe *et al.,* 1965). Chemically, OTA is composed by a phenylalanine chlorinated bound to an isocumarinic group (Fig. 2). OTA is chemically stable, it's persistent and can be easily found in meat and derivates from animals feed with contaminated grains. (Moss, 2002; Pietri *et al.,* 2006) OTA has been reported to be

nephrotoxic, carcinogenic and immunosuppressive (Bennet and Klich, 2003) in animal as in human and a limited amount of OTA content can be transferred to child through human milk. (Breitholtz-Emanuelsson *et al.,* 1993; Micco *et al.,* 1995). Because of the high potential of toxicity, the European Union in 2006 has limited the OTA content to 10 μg/kg in dried wine fruits, 5 μg/kg in raw cereals, 5μg/kg in roasted coffee and 2 μg/kg in wine (Bayman and Baker, 2006). Chemical methods to control *Aspergillus carbonarius* in grapes are not always effective, as their efficacy often are temperature and strain-dependent (Bellì *et al.,* 2006). As a preventive sustainable solution to accumulation of *Aspergillus* mycotoxins, several atoxigenic strains of *Aspergillus spp* are currently used to reduce contamination through competitive exclusion of mycotoxigenic producers strains (Cleveland *et al.,* 2003). This strategy seems to work well against aflatoxin and could be taken into account for OTA-contaminating species. At the present time the OTA biosynthetic pathway has not been completely clarified, only a polyketide synthase (PKS) has been cloned and characterized in *A. carbonarius* (Gallo *et al.,* 2009) Attempts to correlate gene expression with ochratoxins production has been surveyed (Atoui *et al.,* 2007; Selma *et al.,* 2008), these tools could be promising to the prediction of this mycotoxin but these studies must be further widen to read such as prediction character. Regulation of OTA production is yet poorly understood and further studies are needed to better characterize the biosynthesis and factors affecting it.



.

Figure 2: Structure of Ochratoxin A

Systemic resistances in plants

As previously reported the biological control strategies are promising in the control of mycotoxin contamination. Among the possibilities of biological control the use of BCA able to stimulate systemic resistance in plants are of a particular interest. Application of BCA are interesting due to “environmental friendly” action, the ability to trigger resistance only as a consequence of pathogen infection and persistency for a long time after application. Plants hold a large variety of defence mechanisms activated in response to biotic stresses and damages and one of critical factors for the effectiveness of the defence system is represented by time of response. (Choudary *et al.,* 2007) Systemic resistance is a generalized physiological "state of enhanced defensive capacity" elicited by specific environmental stimuli, in which the plant's innate defences are potentiated against subsequent biotic challenges (van Loon *et al.,* 1998). This enhanced state of resistance is effective against a broad range of pathogens and parasites, including fungi, bacteria, viruses, nematodes, parasitic plants, and even insect herbivores (Hammerschmidt and Kuc, 1995; Benhamou and Nicole, 1999; Kessler and Baldwin, 2002). Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are the two forms of systemic resistance; in both plant defences are preconditioned by prior infection (pathogen or specific BCAs) or treatment with chemical substances (BTH, ABA, chitosan) (Pena-Cortes *et al.,* 1995; Morris *et al.,* 1998; Schweizer *et al.,* 1999; Reddy *et al.,* 1999) resulting in enhancement of resistance or tolerance against challenges with a pathogen.

SAR and ISR can be differentiated on the basis of the nature of the elicitor and the regulatory pathways involved (Fig. 3 and 4). Although both produce resistance in plant, the spectrum of diseases against which SAR and ISR are effective overlaps only partially. In *Arabidopsis*, SAR is most effective against biotrophic pathogens, downy and powdery mildews as well as viruses that are sensitive to SA-dependent defences whereas ISR is more active against nectrotrophic pathogens. In SAR, the complex mechanism that triggers resistance is often due to a virulent pathogen able to attack host tissues causing or less an hypersensitive response HR (Compant *et al.,* 2005) in infection site. The main signal recognized by plant to effect systemic resistance is salicylic acid accumulation. (Mauch Mani *et al.,* 1998) but recently, the role of azelaic acid as translocable signal of SAR and promoter of salicylic acid synthesis were ascerted in *A. thaliana* (Jung *et al.,* 2009).

Salicylic acid is synthesized from *trans*-cinnamic acid in turn produced by phenylalanine ammonia lyase activity. In SAR, to an increment of lignification in cell wall and production of antimicrobial compounds (phitoalexines) is always accompanied the induction of pathogenesis-related (PRs) proteins (Mauch Mani *et al.,* 1998) responsible to the prolonged maintenance of resistance (Spoel *et al.,* 2003). In particular, in SAR the PRs accumulation represent the major change in soluble proteins (Broekaert *et al.,* 2006). To control the effective occurrence of SAR, the main molecular markers responsive to SA are PAL, PR1, and PR5. (Farag *et al.,* 2005; Gao *et al.,* 2006; Djonovic *et al.,* 2007). Disease resistance achieved by SAR is associated with fitness costs. Probably that plants evolved the ISR mechanisms because SAR activation it is too costly to be switched on all the time (Brown, 2002; Heil and Baldwin, 2002). The phenotypes of many mutants with scarce fitness showing constitutive *PR* gene expression, accumulation of SA and resistance to pathogens support this idea.

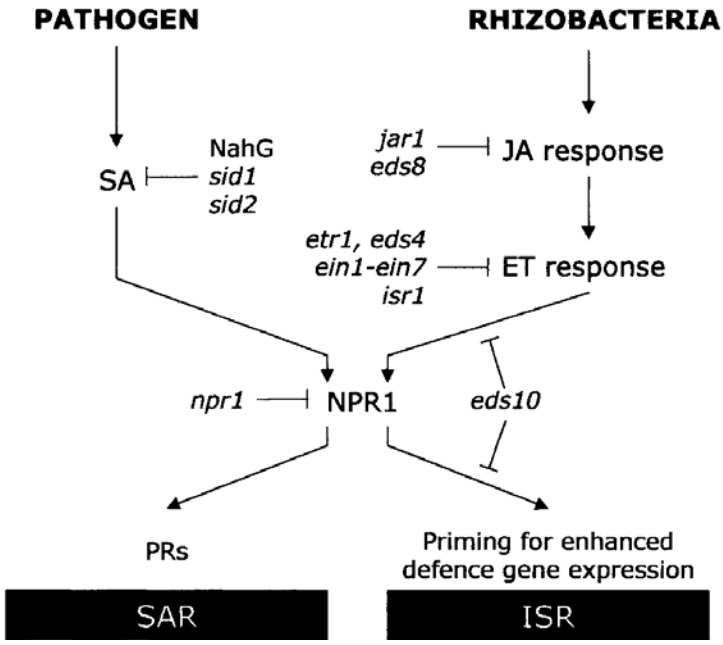


Figure 3: Signalling and regulative systems for systemic acquired resistance and induced systemic resistance in *Arabidopsis thaliana* as reported by Pieterse (Pieterse et al 2002)

In induced systemic resistance defence systems are alerted by some specific stimuli as chemical compounds (BTH) or priming action by plant growth-promoting rhizobacteria or fungi (PGPR or PGPF), of which the best characterized are strains within several species of *Pseudomonas* or *Trichoderma* that cause no visible damage to the plant's root system (van Loon *et al.,* 1998; Harman, 2000). In addition, the ability of PGPR strains to elicit ISR could differ according to plant species and genotypes (van Wees *et al.,* 1997; Yan *et al.,* 2002). Unlike SAR, ISR does not involve the accumulation of pathogenesis-related proteins or salicylic acid, but is regulated by jasmonate and ethylene (Pieterse *et al.,* 1998; Yan *et al.,* 2002). Jasmonic acid is the terminal product of octadecanoic acid cycle and LOXs are important enzymes to production of substrate that in turn, after a sequences of transformation, produce JA. To control the effective occurrence of ISR, the main molecular markers reported in literature responsive to JA are LOX10, AOS, OPR7 and HPL (Farag *et al.,* 2005; Nemchenko *et al.,* 2006; Djonovic *et al,* 2007). There are some common regulators between SAR and ISR. NPR1 is an essential salicylic-dependent regulator containing a nuclear localization and an ankyrin repeat domain near the middle of the protein sequence (Cao *et al.,* 1997; Ryals *et al.,* 1997). NPR1 is a protein that interacts with and influences the activity of SA responsive transcription factors, nonetheless plays a crucial role in resistance activated by JA and ET. (Pieterse *et al.,* 2002) In fact through NPR1, SA interacts antagonistically inhibiting JA-related genes as *LOX2* (Bell *et al.,* 1995), *VSP* (Berger et al., 1995) and *PDF1.2* (Pennickx *et al.,* 1996).

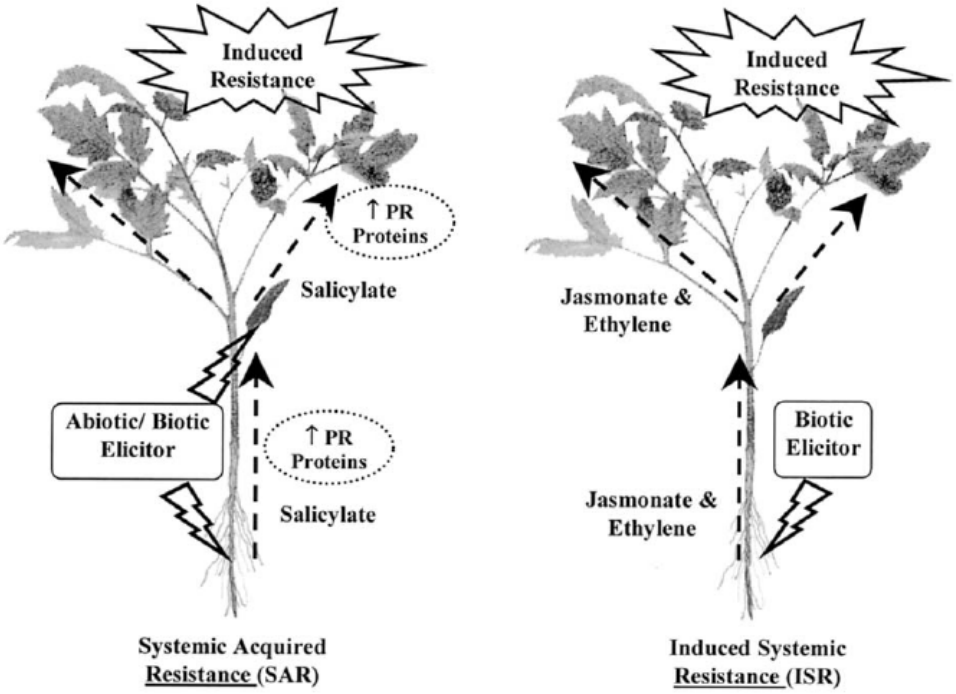


Figure 4: A comparison of the forms of induced resistance in plants as reported by Vallad and Goodman 2004, Systemic acquired resistance, induced by the exposure of root or foliar tissues to abiotic or biotic elicitors, is dependent of salicylic acid, and associated with the accumulation of pathogenesis-related (PR) proteins. Induced systemic resistance, induced by the exposure of roots to specific strains of plant growth-promoting rhizobacteria, is dependent of ethylene and jasmonic acid, independent of salicylate, and is not associated with the accumulation of PR proteins (or transcripts). However, both responses are intertwined molecularly, as demonstrated by their reliance on a functional version of the gene *NPR1* in *Arabidopsis thaliana*.

Plant stresses and reactive oxygen species

A further strategy to control mycotoxin accumulation proceeds from the knowledge of factors affecting it. Among these factors, stress conditions in plant-pathogen interactions are retained one of the main causes favourishing mycotoxin accumulation.

Abiotic stress conditions cause extensive losses to agricultural production worldwide (Boyer, 1982; Bray *et al.,* 2000). While single stress conditions such as drought, salinity or heat have been the subject of intense research (Cushman and Bohnert, 2000) in crops, plants are routinely subjected to a combination of different abiotic stresses which interactions are less known and lead to different consequences than separately activated stresses (Jang and Huang, 2001; Moffat, 2002). In drought-stricken areas, for example, many crops encounter a combination of drought and other stresses, such as heat or salinity (Moffat, 2002). Thus, molecular, biochemical and physiological processes set in motion by a specific stress condition might differ from those activated by a slightly different composition of environmental parameters. In addition to the basic differences that exist between different stress conditions (Cheong *et al.,* 2002; Kreps *et al.,* 2002; Rizhsky *et al.,* 2004) these conditions when combined, could require conflicting or antagonistic responses or can sinergically enhance each others. As far as is concerned to stresses, both in abiotic stress as part of signalling pathways (Zhao *et al.,* 2001; Apel *et al.,* 2004; Torres *et al.,* 2005) or in biotic stress to restrict pathogen growth (Repka *et al.,* 1999, Kachroo *et al.,* 2003, Zhou *et al.,* 2004) plant triggers an "oxidative burst". In *A. thaliana* reactive oxygen species (ROS) are associated with many different biotic or abiotic stress conditions, different genes of the ROS gene network were found to differently respond to different stress treatments (Mittler *et al.,* 2004). In addition, not only the plant cells are affected by changes in redox potential but also the organisms (*e.g.* fungal pathogens) relationed to the host, are exposed to ROS activity (Reverberi *et al.,* 2007; Boutigny *et al.,* 2008).

Also Fungi can be exposed to environmental stresses, changes in pH, temperature, osmosity, oxygen partial pressure, as well as concentrations of substrates, drying, mechanical damages, and other external factors influencing microbial growth and development, induce generation of oxygen radicals within the fungal cell (Sokolovskii *et al.,* 2000; Aguirre *et al.,* 2005; Georgiou *et al.,* 2006). In fungi, H_2O_2 is a signal molecule involved in various processes, such as the change in growth rate, differentiation, and

proliferation (Ivanova *et al.,* 2005; Belozerskaya *et al.,* 2006). As it is demonstrated, ROS induces the differentiation of sclerotia in *S. rolfsii* (Sideri and Georgiou, 2000), the transition to filamentous growth and pathogenicity in *Ustilago maydis* (Leuthner *et al.,* 2005) and activates the keratinogenesis in *N. crassa* (Iigusa *et al.,* 2005).

The ROS generation exceeding the cell ability to neutralize them can cause hyperoxidation, an oxidative stress. Characteristic of the oxidative stress in fungi are a massive protein oxidation with their subsequent degradation, release of iron free radicals during oxidation of [4Fe-4S] dehydrogenase clusters, oxidation of glutathione and excretion of its disulfide into the extracellular medium, changes level of pyridine nucleotides and in the activity of the AOD system (Aguirre *et al.,* 2005; Gessler *et al.,* 2006). The metabolic rearrangement leads to the arrest of growth and the synthesis of secondary metabolites in fungal cells, many of which are antioxidants (Sokolovskii *et al.,* 2000; Bai *et al.,* 2003): reactive oxygen species change the expression profiles of the *A. nidulans* genes encoding the proteins connected with transport, synthesis of amino acids, sexual reproduction, and sporulation. On the one hand, ROS causes cell damages and, on the other, induces differentiation of fungi, the switch between developmental phases occurs through an unstable hyperoxidant state determined by an increase in the cell ROS level, playing the role of secondary messengers regulating fungal ontogenesis (Sigler *et al.,* 1999; Moye-Rowley, 2003; Aguirre *et al.,* 2005; Georgiou *et al.,* 2006). The lifespan of a fungal organism is tightly connected with its stability to the environmental factors, which is determined by the state of the cell AOD components. AOD comprises both enzymes (SOD, catalase, and heme-containing and thiol peroxidases), antioxidants pigments (carotenoids, melanins, etc.), phenolic compounds, and proline. The components of AOD system capable of decreasing the level of primary ROS (O_2^- and H_2O_2) formed directly in the cell are SOD and catalases.

Relatively to secondary metabolites, besides to antioxidant compounds certain filamentous Fungi species produce mycotoxins. Many papers reported that the effect of external factors on mycotoxin biosynthesis is exerted at transcriptional level. In general, growth parameters and substances or conditions that impose stress on the fungus also have an influence on mycotoxin biosynthesis. Abiotic factors such as temperature, *aw* and pH have a strong influence on the expression of mycotoxin biosynthesis genes. This is in agreement with the findings of several other authors (Keller *et al.,* 1997; Geisen, 2004; Price *et al.,* 2005;

O'Callaghan *et al.,* 2006; Jurado *et al.,* 2008). All stresses (*aw*, pH, temperature) similarly activate the mycotoxin biosynthesis genes. The positive influence of oxygenic stress on aflatoxin biosynthesis by *A. parasiticus* was described by Fabbri *et al.* (1983) and Jayashree and Subramanyam (2000) while similar results were reported in trichotecenes produced by *F. graminearum* by Ponts *et al.* (2006).

Taken together, these and other evidences, imply that the production of mycotoxins can be regarded as an adaptation of mycotoxigenic species to imposed abiotic and other stress.

The molecular approaches to biological control

In the present research, molecular approaches as enzymatic activity, sqReal-Time PCR and cDNA-AFLP have been used to evaluate the biocontrol ability of *Trichoderma harzianum* in maize, the ability of stress condition to promote fumonisin production and furnish an important overview on genes involved in ochratoxin biosynthetic pathway, still unknown. Brief descriptions of Real-time PCR and cDNA-AFLP are reported below.

Real-time PCR

While a variety of methods can be used to quantification of gene transcripts, Real-time PCR enclosed the peculiarities of that techniques. PCR is a sensitive method and using specific primers can discriminate closely related mRNAs present in low copy number, on the other hand results are difficulty reliable. Different efficiency of primers and methods of staining are the first problems to face up in a simple RT-PCR, because ethidium bromide binding nucleic acid in a rather insensitive way. With the occurrence of development of Real-time PCR, a lot of problems were overcome giving quantitative results starting from mRNAs. Real-time PCR is a kinetic approach in which reaction is visible in real time since the early stages and the amplification were just started, overtaken the sensitivity of ethidium bromide staining. In this study, the detector used to control the PCR amplification was SYBR Green. SYBR Green is a fluorescent dyes able to intercalate in the minor groove of double strand DNA. Since the SYBR Green binding is independent from the sequence, specificity of used primers is of first importance to avoid aspecific product signals. However, a SYBR Green method is the cheapest and the most adaptable method to different experimental plan. To correctly perform a Real-time PCR, a series of experiments

must be done in advance to the correct set point of reaction. Beyond the primer sequences, the concentration of both primers and starting template has to be optimized before analysis, to check the reliability of reaction in that particular temperature conditions. Once parameters have been adjusted and a Real-time PCR performed, an important step is constituted by analysis of product dissociation. With dissociation stage, it's possible to survey the melting point of PCR products, to assure the presence of only one products. According to the melting curves analysis, the step for fluorescence data collection of the Real-time PCR can be set. The temperature of fluorescence data collection should be chosen just before the beginning of the denaturation curves, so that all non specific amplicons and dimers were completely denaturated. As suggested by Pfaffl (Pfaffl, 2001), the expression data of analyzed genes was adjusted by efficiency coefficient of primers and subsequently normalized according to the expression of a housekeeping gene.

In the present work the housekeeping genes used were glyceraldehyde 3-phosphate *ZmGAPc* for maize and β-tubulin for *F. verticillioides*. *FvTUB2*. Efficiency of primers were obtained by Real-time PCR reactions of serial dilution, data collected (Cycle threshold) were plotted against the dilution coefficient (or cDNA amounts of starting template) and a linear plot were fitted. The slope of the linear plot permits to calculate the efficiency of primers, to achieve an efficiency coefficient of 100%, the slope values should be -3,334. Concluding, compared to traditional RT-PCR the Real-time PCR offers many advantages as increment of range detection, increment of sensitivity and precision of signal, better discrimination of differences between different samples, in addition the Pfaffl devices allows to compare the expression of different genes each other.

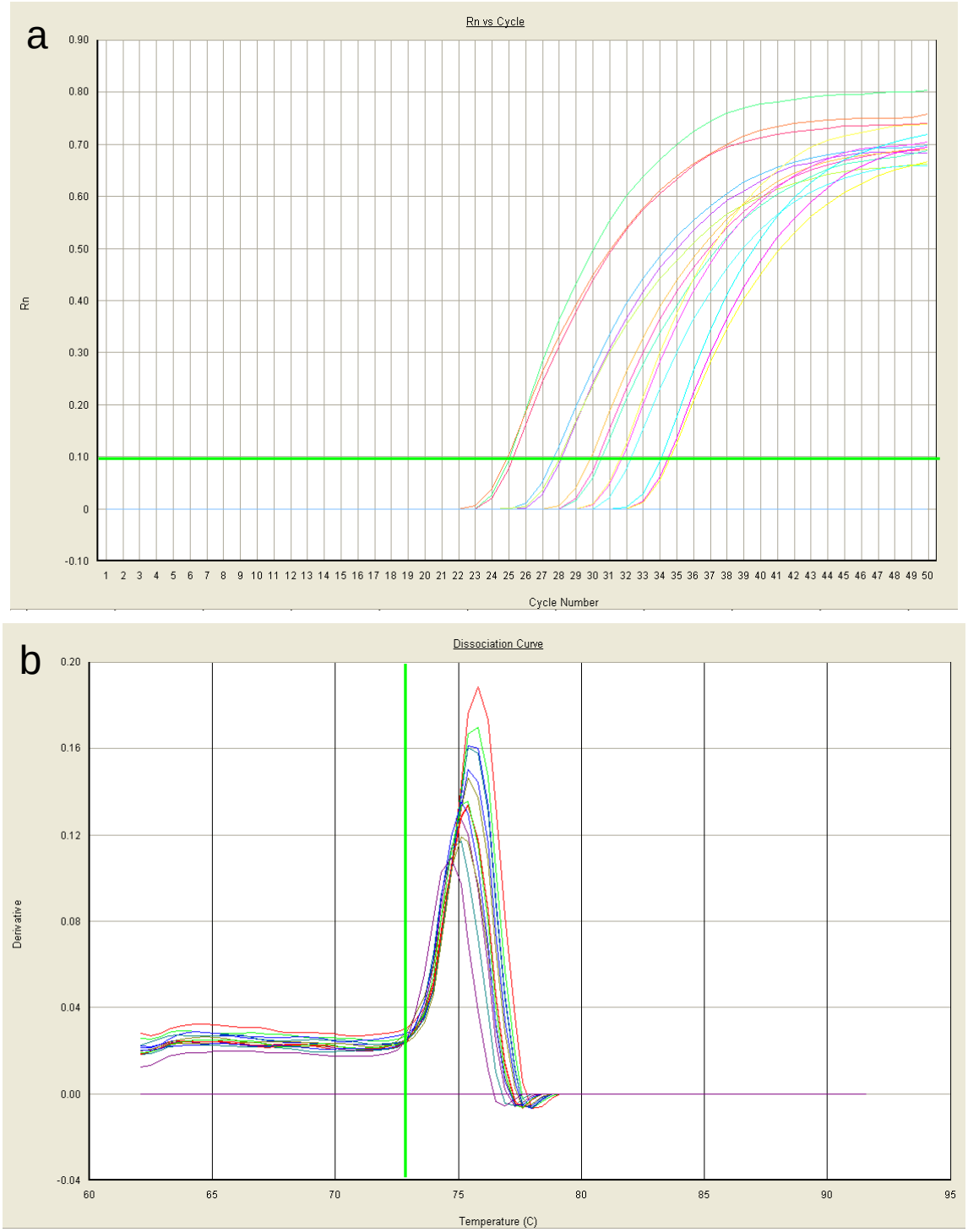


Figure 5a: Serial dilution of template cDNA for the calculation of primer efficiency according to Pfaffl method, the green lines indicates the threshold. The points in which the amplification curves are crossed by threshold determine the Ct values for that sample. **5b**: Dissociation analysis of Real time-PCR products, reported as derivate analysis of fluorescence data. The green line represents the beginning of melting curve products and corresponds to the temperature at which data were collected. The peak is reached when the PCR products are completely denaturated.

cDNA-AFLP

The complementary DNA-Amplified Fragment Length Polymorphism (cDNA-AFLP) is a techniques applied on cDNA double strand fragment and permits to detect differences on expressed genes (Fig. 6). First used to the detection of genomic differences in DNA (Vos *et al.,* 1995; Bachem *et al.,* 1998), this techniques applied to reverse-transcripted RNA is a powerful tool to display several unknown transcript simultaneously and to detect differentially expressed genes. Analysis of transcriptional profiles of genotypes with antagonistic phenotypes (low producer mycotoxigenic strain against an high producer one), is an useful strategy for the identification and isolation of genes differentially expressed. In plant research, this method is reliable to discriminate differences of transcripts from different tissues as from differently treated plants (Albertini *et al.,* 2004; Quaggiotti *et al.,* 2007) whereas in fungal research were applied to identify differentially regulated genes in mycotoxin-producing and non-producing growth stages of *Fusarium proliferatum* (Jeney *et al.,* 2004) or difference in transcriptomic profile in response to fungal infection (Wang *et al.,* 2009) as well to transcripts involved in avirulent interaction. (Guo *et al.,* 2006) cDNA-AFLP approach is able to assays a large set of mRNA-derived fragments and increase the reliability of amplification-based transcriptome analysis. An advantage of cDNA-AFLP is the fact that is not necessary known the studied genome and therefore it is being widely used in less well investigated systems. A further advantage of the present technique is the increase of resolution of expression patterns highlighting the presence of little copy sequences or just starting from small amount of RNA. The latter annotation result of particular interest due to the peculiarity of *Aspergillus carbonarius*, the high production of pigments is often associated to low yields and purity, therefore RNA would result unfit for the large part of methods. In addition the sensitivity of amplification products was proved to be very high, and expression patterns visualized by cDNA-AFLP showed to be well correlated with results obtained with similar techniques.

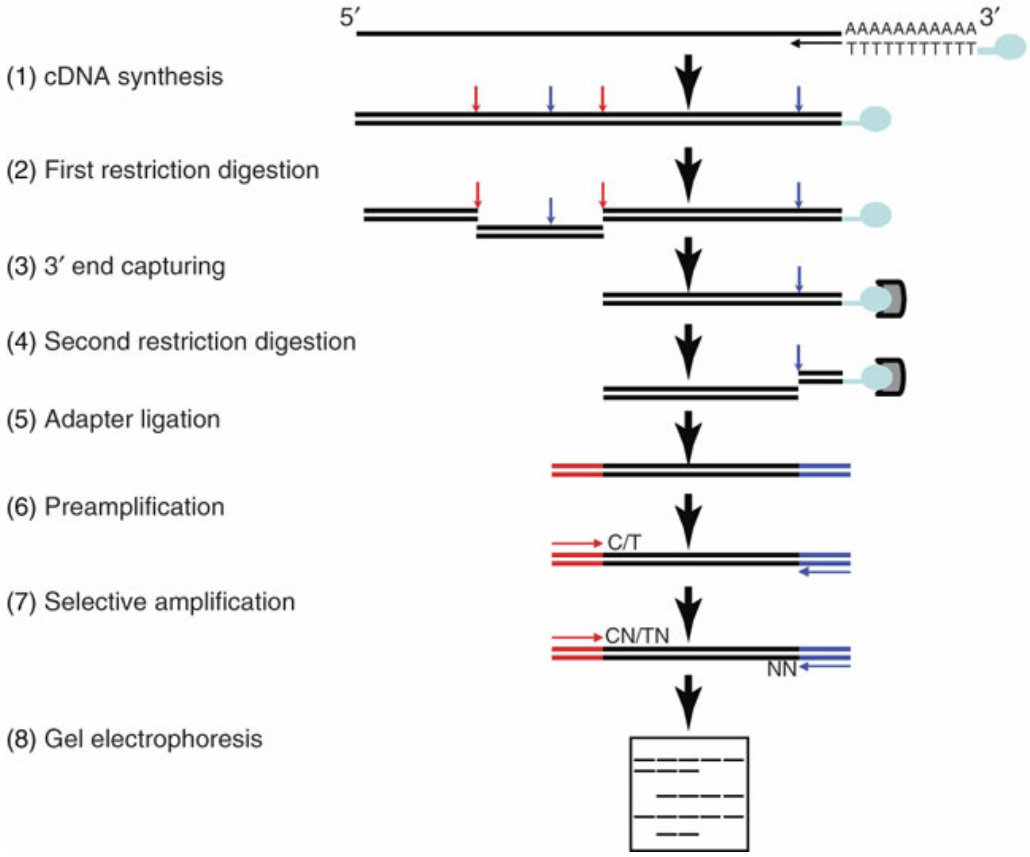


Figure 6: Representation of a generic cDNA-AFLP techniques. 1) mRNA is converted into double-stranded cDNA using a oligo-dT or random primer. Red and blue arrows represent *Eco*RI and *Mse*I restriction enzyme sites, respectively. 2) After a first digestion with *Eco*RI in our operative conditions we immediately succeed 4) a second digestion with *Mse*I (in contrast with the figure); (5) ligation of the double-stranded *Eco*RI(red)- and *Mse*I (blue)-specific adapters to the ends to generate PCR templates; (6) reduction of the template mixture complexity by selective pre-amplification, using either the *EcoR*I and *Mse*I with selective nucleotides; (7) final selective amplification of subsets primers labeled to allow subsequent detection; and (8) electrophoretic size fractionation and display on denaturing polyacrylamide gels of the *EcoRI*/*Mse*I.

References

Adams, P.B. 1990. The Potential of Mycoparasites for Biological Control of Plant Diseases. Annual Review of Phytopathology. 28, 59-72.

Albertini, E., Marconi, G., Barcaccia, G., Raggi, L., Falcinelli, M. 2004. Isolation of candidate genes for apomixis in *Poa pratensis L*. Plant Molecular Biology. 56, 879-894.

Aguirre, J., Ríos-Momberg, M., Hewitt, D., Hansberg, W. 2005. Reactive oxygen species and development in microbial eukaryotes. Trends in Microbiology. 13, 111-118.

Apel, K. and Hirt, H. 2004. REACTIVE OXYGEN SPECIES: Metabolism, Oxidative Stress, and Signal Transduction. Annual Review of Plant Biology. 55, 373-399.

Atoui, A., Mathieu, F., Lebrihi, A., 2007. Targeting a polyketide synthase gene for *Aspergillus carbonarius* quantification and ochratoxin A assessment in grapes using real-time PCR. International Journal of Food Microbiology. 115, 313-318.

Atoui, A., Mitchell, D., Mathieu, F., Magan, N., Lebrihi, A., 2007. Partitioning of ochratoxin A in mycelium and conidia of *Aspergillus carbonarius* and the impact on toxin contamination of grapes and wine. Journal of Applied Microbiology. 103, 961-968.

Bachem, C.W.B., Oomen, R.J.F.J., Visser, R.G.F., 1998. Transcript imaging with cDNA-AFLP: a step-by-step protocol. Plant Molecular Biology Reporter. 16, 157-173.

Bai, Z., Harvey, L., McNell, B. 2003. Oxidative Stress in Submerged Cultures of Fungi. Critical Rev. Biotechnology. 23, 267–302.

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

Bayman, P., Baker, J.L. 2006. Ochratoxins: a global perspective. Mycopathologia. 162, 215-223.

Bell, E., Creelman, R.A. Mullet, J.E. 1995. A chloroplast lipoxygenase is required for wound-induced accumulation of jasmonic acid in *Arabidopsis*. Plant Biology. 92, 8675-8679.

Bellí, N., Marín, S., Sanchis, V., Ramos, A.J. 2006. Impact of fungicides on *Aspergillus carbonarius* growth and ochratoxin A production on synthetic grape-like medium and on grapes. Food Additives and Contaminants. 23, 1021-1029.

Belozerskaya, T.A. and Gessler, N.N. 2007. Reactive oxygen species and the strategy of antioxidant defence in fungi: A review. Applied Biochemistry and Microbiology. 43, 506-515.

Benhamou, N. and Nicole, M. 1999. Cell biology of plant immunization against microbial infection: The potential of induced resistance in controlling plant diseases. Plant Physiology and Biochemistry. 37, 703-719.

Bennett, J.W. and Klich, M. 2003. Mycotoxins. Clinical Microbiology Reviews 16: 497-516.

Berger, S., Bell, E., Sadka, A., Mullet, J.E. 1995. *Arabidopsis thaliana Atvsp* is homologous to soybean *VspA* and *VspB* genes encoding vegetative storage protein acid phosphatases, and is regulated similarly by methyl jasmonate, wounding, sugars light and phosphate. Plant Molecular Biology. 27, 993-942.

Bezuidenhout, S.C., Wentzel, C.A., Gelderblom, C.P., Gorst-Allman,R., Horak, M., Marasas, W.F., Spiteller, G., Vleggaar, R. 1988. Structure Elucidation of the Fumonisins, Mycotoxins from *Fusarium moniliforme*. Journal of the Chemical Society. 743-745.

Binder, E.M., Tan, L.M., Chin, L.J., Handl, J., Richard, J. 2007. Worldwide occurrence of mycotoxins in commodities, feeds and feed ingredients. Animal Feed Science and Technology. 137, 265-282.

Bluhm, B.H., and Woloshuk, C.P. 2006. *Fck1*, a C-type cyclin-dependent kinase, interacts with *Fcc1* to regulate development and secondary metabolism in *Fusarium verticillioides*. Fungal Genetics and Biology. 43, 146-154.

Bluhm, B.H., Kim, H., Butchko, R.A.E., Woloshuk, C.P. 2008. Involvement of *ZFR1* of *Fusarium verticillioides* in kernel colonization and the regulation of *FST1*, a putative sugar transporter gene required for fumonisin biosynthesis on maize kernels. Molecular Plant-Microbe Interactions. 9, 203–211.

Bolger, M., Coker, R.D., Dinovi, M., Gaylor, D., Gelderblom, M.O., Paster, N., Riley, R.T., Shephard, G., Speijers, J.A. 2001. Fumonisins safety evaluation of certain mycotoxins in food and agriculture organization of the United Nations. World Health Organization Food Additives. 47, 103– 279.

Boutigny, A.L., Richard-Forget, F., Barreau, C. 2008. Natural mechanisms for cereal resistance to the accumulation of *Fusarium* trichothecenes. European Journal of Plant Pathology. 121, 411-423.

Boyer, J.S. 1982. Plant productivity and environment. Science. 218, 443-448.

Bray, E.A., Bailey-Serres, J., Weretilnyk, E. 2000. Responses to abiotic stresses. Biochemistry and Molecular Biology of Plants. 1158-1249.

Breitholtz-Emanuelsson, A., Palminger-Hallén, I., Wohli,P., Oskarsson, A., Hult, K., Olsen, M. 1993. Transfer of ochratoxin a from lactating rats to their offspring: A short-term study. Natural Toxins. 1, 347-352.

Broekaert, W.F., Delauré, S.L., De Bolle, M.F.C., Cammue, B.P.A (2006) The role of ethylene in host pathogen interaction. Annu. Rev. Phytopathol. 44, 393–416.

Brown, D.W., McCormick, S.P., Alexander, N.J., Proctor, R.H., Desjardins, A.E. 2001. A Genetic and Biochemical Approach to Study Trichothecene Diversity in *Fusarium sporotrichioides* and *Fusarium graminearum*. Fungal Genetics and Biology. 32, 121-133.

Brown, J.K.M. 2002. Yield penalties of disease resistance in crops. Current Opinion in Plant Biology. 5, 339-344.

Brown, D.W., Cheung, F., Proctor, R.H., Butchko, R.A.E., Zheng, L., Lee, Y., Utterback, T., Smith, S., Feldblyum, T., Glenn, A.E., Plattner, R.D., Kendra, D.F:, Town, C.D., Whitelaw, C.A. 2005. Comparative analysis of 87,000 expressed sequence tags from the fumonisin-producing fungus *Fusarium verticillioides*. Fungal Genetics and Biology. 42, 848-861.

Brown, D.W., Butchko, R.A.E., Busman, M., Proctor, R.H. 2007. The Fusarium verticillioides FUM Gene Cluster Encodes a $Zn(II)_2Cys_6$ Protein That Affects FUM Gene Expression and Fumonisin Production. Eukaryotic Cell. 6, 1210-1218.

Calvo, A.M., Wilson, R.A., Bok, J.W., Keller, N.P. 2002. Relationship between secondary metabolism and fungal development. Microbiology and Molecular Biology Reviews. 66, 447-459.

Cao, H., Glazebrook, J., Clarke, J.D., Volko, S., Dong, X. 1997. The *Arabidopsis NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell. 88, 57-63.

Causin, R., Costa, E., Rasera, R., Rossi, G., Scopel C. 2008. Chemical treatments at silk stage reduce fumonisin contamination of maize kernels. "The World Mycotoxin Forum – the Fifth Conference - The international networking conference for the food and feed industry". 124.

Cleveland, T.E., Dowd, P.F., Desjardins, A.E., Bhatnagar, D., Cotty, P.J. 2003. United States Department of Agriculture-Agricultural Research Service research on pre-harvest prevention of mycotoxins and mycotoxigenic fungi in US crops. Pest management science. 59, 629-642.

Cheong, Y.H., Chang, H.S., Gupta, R., Wang, X., Zhu, T., Luan, S. 2002. Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal responses in *Arabidopsis*. Plant Physiology. 129, 661-677.

Choudhary, D.K., Prakash, A., Johri, B.N. 2007. Induced systemic resistance (ISR) in plants: mechanism of action. Indian J. Microbiol. 47, 289-297.

Compant, S., Duffy, B., Nowak, J., Clément, C., Barka, E.A. 2005. Use of Plant Growth-Promoting Bacteria for Biocontrol of Plant Diseases: Principles, Mechanisms of Action, and Future Prospects. Applied and Environmental Microbiology. 71, 4951-4959.

Cushman, J.C. and Bohnert, H.J. 2000. Genomic approaches to plant stress tolerance. Current Opinions in Plant Biology. 3, 117-124.

Desjardins, A.E., Plattner, R.D., Nelsen, T.C., Leslie J.F. 1995. Genetic analysis of fumonisin production and virulence of *Gibberella fujikuroi* mating population A (*Fusarium moniliforme*) on maize (*Zea mays*) seedlings. Appl. Environ. Microbiol. 61, 79-86.

Desjardins, A.E., Plattner, R.D., Proctor R.H. 1996. Linkage among genes responsible for fumonisin biosynthesis in *Gibberella fujikuroi* mating population A. Appl. Environ. Microbiol. 62, 2571-2576.

Djonović S., Vargas W.A., Kolomiets M.V., Horndeski M., Wiest A., Kenerley C.M. 2007. A proteinaceous elicitor Sm1 from the beneficial fungus *Trichoderma virens* is required for induced systemic resistance in maize. Plant Physiology. 145: 875-889.

Farag M.A., Fokar M., Zhang H.A., Allen R.D., Pare P.W 2005. (*Z*)-3-Hexenol induces defence genes and downstream metabolites in maize. Planta. 220, 900–909.

Färber, P. and Geisen, R. 2004. Analysis of Differentially-Expressed Ochratoxin A Biosynthesis Genes of *Penicillium nordicum*. European Journal of Plant Pathology. 110, 661-669.

Flaherty, J.E., Pirttilä, A.M., Bluhm, B.H., Woloshuk, C.P. 2003. *PAC1*, a pH-Regulatory Gene from *Fusarium verticillioides*. Applied and Environmental Microbiology. 69, 5222-5227.

Flaherty, J.E., Woloshuk, C.P. 2004. Regulation of Fumonisin Biosynthesis in *Fusarium verticillioides* by a Zinc Binuclear Cluster-Type Gene, *ZFR1*. Applied and Environmental Microbiology. 70, 2653-2659.

Folcher, L., Jarry, M., Weissenberger, A., Gérault F., Eychenne, N., Delos, M., Regnault-Roger, C. 2009. Comparative activity of agrochemical treatments on mycotoxin levels with regard to corn borers and *Fusarium* mycoflora in maize (*Zea mays* L.) fields. Crop Protection. 28, 302-308.

Galli, C.L., Corsini, E., Marinovich, M. 2004. Tossicologia. Piccin Editore.

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

Gao L., Smith F.A., Smith S.E. 2006. The *rmc* locus does not affect plant interactions or defence-related gene expression when tomato (*Solanum lycopersicum*) is infected with the root fungal parasite *Rhizoctonia*. Functional Plant Biology. 33, 289-296.

Gelderblom, W.C.A., Sewram, V., Shephard, G.S., Snijman, P.W., Tenza, K., van der Westhuizen, L., Vleggaar R. 2007. Structure and Natural Occurrence of Stereoisomers of the Fumonisin B Series Mycotoxins . Agric. Food Chem. 55, 4388–4394.

Georgiou, C., Patsoukis, N., Papapostolou, I., Zervoudakis, G. 2006. Sclerotial metamorphosis in filamentous fungi is induced by oxidative stress. Integrative and Comparative Biology. 46, 691-712.

Gessler, N.N., Leonovich, O.A., Rabinovich, Y.M., Rudchenko, M.N., and Belozerskaya, T.A. 2006. A comparative study of the components of the antioxidant defence system during growth of the mycelium of a wild-type *Neurospora crassa* strain and mutants, *white collar-1* and *white collar-2*. Applied Biochemistry and Microbiology. 42, 354–358.

Glenn, A.E., Zitomer, N.C., Zimeri, M.A., Williams, L.D., Riley, R.T., Proctor, R.H. 2008. Transformation-Mediated Complementation of a FUM Gene Cluster Deletion in *Fusarium verticillioides* Restores both Fumonisin Production and Pathogenicity on Maize Seedlings. Molecular Plant-Microbe Interactions. 21, 87-97.

González, G., Hinojo, M.J., Mateo, R., Medina, A., Jiménez, M. 2005. Occurrence of mycotoxin producing fungi in bee pollen. International Journal of Food Microbiology. 105, 1-9.

Goto, T., Wicklow, D.T., Ito, Y. 1996. Aflatoxin and cyclopiazonic acid production by a sclerotium-producing *Aspergillus tamarii* strain. Applied. Environmental Microbiology. 62, 4036-4038.

Guo, J., Jiang, R.H.Y., Kamphuis, L.G., Govers, F. 2006. A cDNA-AFLP based strategy to identify transcripts associated with avirulence in *Phytophthora infestans*. Fungal Genetics and Biology. 43, 111-123.

Hammerschmidt, R. and Kuć, J. 1995. Induced resistance to disease in plants. Published by Hammerschmidt and Kuć.

Hansberg, W. and Aguirre, J. 1990. Hyperoxidant states cause microbial cell differentiation by cell isolation from dioxygen. Journal of Theoretical Biology. 142, 287-293.

Harman, G. 2000. Myths and Dogmas of Biocontrol Changes in Perceptions Derived from Research on *Trichoderma harzianum* T-22. Plant Disease. 84, 377-393.

Hartmann, N., Erbs, M., Forrer, H.R., Vogelgsang, S., Wettstein, F.R., Schwarzenbach, R.P., Bucheli, T. 2008.Occurrence of Zearalenone on *Fusarium graminearum* Infected Wheat and Maize Fields in Crop Organs, Soil, and Drainage Water. Environ. Sci. Technol. 42, 5455–5460.

Heil, M. and Baldwin, I.T. 2002. Fitness costs of induced resistance: emerging experimental support for a slippery concept. Trends in Plant Science. 7, 61-67.

Iigusa, H., Yoshida, Y., and Hasunuma, K. 2005. Oxygen and hydrogen peroxide enhance light-induced carotenoid synthesis in *Neurospora crassa*. FEBS Letters. 579, 4012-4016.

Ivanova, A.E., Aslanidi, K.B., Karpenko, Y.V., Belozerskaya, T.A. 2005. The Effect of Hydrogen Peroxide on the Growth of Microscopic Mycelial Fungi Isolated from Habitats with Different Levels of Radioactive Contamination. Microbiology. 74, 655-663.

Jayashree, T. and Subramanyam, C. 2000. Oxidative stress as a prerequisite for aflatoxin production by *Aspergillus parasiticus*. Free Radical Biology and Medicine. 29, 981-985.

Jeney, A., Béki, E., Mule, G., Hornok, L. 2004. Identification of Growth Stage Specific Transcript Profiles in *Fusarium proliferatum* (*Gibberella fujikuroi*, Mating Population D) by cDNA-AFLP Analysis. European Journal of Plant Pathology. 110, 619-625.

Jiang, Y. and Huang, B. 2001. Drought and heat stress injury to two cool season turf grasses in relation to antioxidant metabolism and lipid peroxidation. Crop Science. 41, 436-442.

Joosten, H.M.L.J., Goetz, J., Pittet, A., Schellenberg, M., Bucheli, P. 2001. Production of ochratoxin A by *Aspergillus carbonarius* on coffee cherries. International Journal of Food Microbiology. 65, 39-44.

Jung,H.W., Tschaplinski, T.J., Wang, L., Glazebrook, J., Greenberg, J.T. 2009. Priming in Systemic Plant Immunity. Science. 324, 89-91.

Jurado, M., Marın, P., Magan,N., Gonzàlez-Jaén, M.T. 2008. Relationship between Solute and Matric Potential Stress, Temperature, Growth, and *FUM1* Gene Expression in Two *Fusarium verticillioides* Strains from Spain. Applied and Environmental Microbiology. 74, 2032–2036.

Kachroo, A. He, Z., Patkar, R., Zhu, Q., Zhong, J., Li, D., Ronald, P., Lamb, C., Chattoo B.B. 2003 Induction of H_2O_2 in Transgenic Rice Leads to Cell Death and Enhanced Resistance to Both Bacterial and Fungal Pathogens. Transgenic Research. 12, 577-586.

Keller, N.P. and Hohn, T.M. 1997. Metabolic Pathway Gene Clusters in Filamentous Fungi. Fungal Genetics and Biology. 21, 17-29.

Keller, N.P. Turner, G., Bennet, J.W. 2005. Fungal secondary metabolism. Nature reviews Microbiology. 3, 937-947.

Kessler, A. and Baldwin, I.T. 2002. PLANT RESPONSES TO INSECT HERBIVORY: The Emerging Molecular Analysis. Annual Review of Plant Biology. 53, 299-328.

Kim, H. and Woloshuk, C.P. 2008 Role of *AREA*, a regulator of nitrogen metabolism, during colonization of maize kernels and fumonisin biosynthesis in *Fusarium verticillioides.* Fungal Genetics and Biology. 45, 947-953.

Kreps, J.A., Wu, Y., Chang, H.S., Zhu, T., Wang, X., Harper, J.F. 2002. Transcriptome changes for *Arabidopsis* in response to salt, osmotic, and cold stress. Plant Physiology. 130, 2129-2141.

Laich, F., Fierro, F., Cardoza, R.E., Martin, J.F. 1999. Organization of the Gene Cluster for Biosynthesis of Penicillin in *Penicillium nalgiovense* and Antibiotic Production in Cured Dry Sausages. Applied and Environmental Microbiology. 65, 1236-1240.

Leuthner, B., Aichinger, C., Oechmen, E., Koopmann, E., Muller, O., Muller, P., Kahmann, R., Bolker, M., Schreier, P.H. 2005. A H_2O_2-producing glyoxal oxidase is required for filamentous growth and pathogenicity in *Ustilago maydis* . Mol. Genet. Genomics. 272, 639–650.

López-Errasquín, E., Vázquez, C., Jiménez, M., González-Jaén, M.T. 2007. Real-Time RT-PCR assay to quantify the expression of *fum1* and *fum19* genes from the Fumonisin-producing *Fusarium verticillioides*. Journal of Microbiological Methods. 68, 312-317.

Maier, F.J., Miedaner, T., Hadeler, B., Felk, A., Salomon, S., Lemmens, M., Kassner, H., Schäfer, W. 2006. Involvement of trichothecenes in fusarioses of wheat, barley and maize evaluated by gene disruption of the trichodiene synthase (*Tri5*) gene in three field isolates of different chemotype and virulence. Molecular Plant Pathology. 7, 449-461.

Marasas, W.F. 1995. Fumonisins: Their implications for human and animal health. Natural Toxins. 3, 193-198.

Mauch-Mani, B. and Métraux, J.P. 1998. Salicylic Acid and Systemic Acquired Resistance to Pathogen Attack. Annals of Botany. 82, 535-540.

Micco, C., Miraglia, M., Brera, C., Corneli, S., Ambruzzi, A. 1995. Evaluation of ochratoxin A level in human milk in Italy. Food additives and contaminants. 12, 351-354.

Miller, J.D. 2002. Aspects of the ecology of *Fusarium* toxins in cereals. Advances in experimental medicine and biology. 504, 19-27.

Mittler, R., Vanderauwera, S., Gollery, M., Van Breusegem, F. 2004. The reactive oxygen gene network of plants. Trends in Plant Science. 9, 490-498.

Moffat, A.S. 2002. Finding new ways to protect drought-stricken plants. Science. 296, 1226-1229.

Morgavi, D.P. and Riley, R.T. 2007. Fusarium and their toxins: Mycology, occurrence, toxicity, control and economic impact. Animal Feed Science and Technology. 137, 199-200.

Morris, S.W., Vernooij,B., Titatarn, S., Starrett, M., Thomas, S., Wiltse, C.C., Frederiksen, R.A., Bhandhufalck, A., Hulbert, S., Uknes, S. 1998. Induced Resistance Responses in Maize. Molecular Plant Microbe Interaction. 11, 643-658.

Moss, M.O. 2002. Mycotoxin review - *Aspergillus* and *Penicillium*. Mycologist. 16, 116-119.

Moye-Rowley, W.S. 2003. Regulation of the Transcriptional Response to Oxidative Stress in Fungi: Similarities and Differences. Eukaryotic Cell. 2, 381–389.

Niessen, L., Schmidt, H., Muhlencoert, E., Färber, P., Karolewiez, A., Geisen, R., 2005. Advances in the molecular diagnosis of ochratoxin A-producing fungi. Food Additives and Contaminants. 22, 324-334.

O'Callaghan, J., Caddick, M.X., Dobson, A.D.W., 2003. A polyketide synthase gene required for ochratoxin A biosynthesis in *Aspergillus ochraceus*. Microbiology. 149, 3485-3491.

O'Callaghan, J., Stapleton, P.C., Dobson, A.D.W. 2006. Ochratoxin A biosynthetic genes in *Aspergillus ochraceus* are differentially regulated by pH and nutritional stimuli. Fungal Genetics and Biology. 43, 213-221.

Pena-Cortes, H., Fisahn, J., Willmitzer, L. 1995. Signals involved in wound-induced proteinase inhibitor II gene expression in tomato and potato plants. Proc. Natl. Acad. Sci. 92, 4106–4113

Penninckx, I.A.M.A., Eggermont, K, Terras, F.R.G, Thomma, B.P.H.J., De Samblanx, G.W., Buchala, A., Metraux, J.P., Manners, J.M, Broekaert, W.F. 1996. Pathogen induced systemic activation of plant defencis gene in *Arabidopsis* follows a salicylic acid-independent pathway. The Plant Cell. 8, 2309-2323.

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

Perrone, G., Varga, J., Susca, A., Frisvad, J.C., Stea, G., Kocsubé, S., Tóth, B., Kozakiewicz, Z., Samson, R.A. 2008. *Aspergillus uvarum sp. nov*., an uniseriate black *Aspergillus* species isolated from grapes in Europe. Int J Syst Evol Microbiol. 58, 1032-1039.

Pfaffl. M. W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic acid reserch. 29, 2000-2007.

Pieterse, C.M.J., Van Wees S.C.M., Van Pelt J.A., Knoester L.R., Gerrits H., Weisbeek P.J, Van Loon L.C. (1998) A novel signaling pathway controlling induced systemic resistance. The Plant Cell.. 10, 1571-1580.

Pieterse, C.M.J., Van Wees, S.C.M., Ton, J., Van Pelt, J., Van Loon, L.C. 2002 Signaling in Rhizobacteria-Induced Systemic Resistance in *Arabidopsis thaliana*. Plant biology. 4, 535-544.

Pietri, A., Bertuzzi, T., Gualla; A., Piva, G. 2006. Occurrence of ochratoxin a in raw ham muscles and in pork products from Northern Italy. Italian journal of food science. 18, 99-106.

Plattner, R.D., Desjardins, A.E., Leslie, J.F. 1996. Identification and characterization of strains of *Gibberella fujikuroi* mating population A with rare fumonisin production phenotypes. Mycologia. 88, 416-424.

Prelusky D.B., Rotter B.A., Rotter R.G., 1994. Mycotoxins in grain. Compounds other than Aflatoxin. Toxicology of mycotoxins. 359-403.

Price, M.S. Conners, S.B., Tachdjian, S., Kelly, R.M., Payne, G.A. 2005. Aflatoxin conducive and non-conducive growth conditions reveal new gene associations with aflatoxin production. Fungal Genetics and Biology. 42, 506-518.

Proctor, R.H., Desjardins, A.E., Plattner, R.D., Hohn, T.M. 1999. A Polyketide Synthase Gene Required for Biosynthesis of Fumonisin Mycotoxins in *Gibberella fujikuroi* Mating Population A. Fungal Genetics and Biology. 27, 100-112

Proctor, R.H., Brown, D.W., Plattner, R.D., Desjardins, A.E. 2003. Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in *Gibberella moniliformis*. Fungal Genetics and Biology. 38, 237-249.

Quaggiotti, S., Barcaccia, G., Schiavon, M., Nicolé, S., Galla, G., Rossignolo, V., Soattin, M., Malagoli, M. 2007. Phytoremediation of chromium using *Salix* species: Cloning ESTs and candidate genes involved in the Cr response. Gene. 402, 68-80.

Reddy, M.V., Arul, J., Angers, P., Couture, L. 1999. Chitosan Treatment of Wheat Seeds Induces Resistance to Fusarium graminearum and Improves Seed Quality. J. Agric. Food Chem. 47, 1208–1216.

Repka, V. 1999. Improved Histochemical Test for In Situ Detection of Hydrogen Peroxide in Cells Undergoing Oxidative Burst or Lignification. Biologia Plantarum. 42, 599-607.

Reverberi, M., Punelli, M., Smith, C., Punelli, F., Zjalic, S., Ricelli, A., Payne, G., Fabbri, A.A., Fanelli, C. 2007. Cell redox balance, lipid metabolism and aflatoxin biosynthesis in *Aspergillus sect. flavi*. Journal of Plant Pathology. 89.

Rheeder, J.P., Marasas, W.F.O., Thiel, P.G., Sydenham, E.W., Shephard, G.S., Van Schalkwyk, D.J. 1992. *Fusarium moniliforme* and fumonisins in corn in relation to human oesophageal cancer in Transkei. Phytopathology. 82, 353-357.

Rizhsky, L., Liang, H., Shuman, J., Shulaev, V., Davletova, S., Mittler, R. 2004. When defense pathways collide: the response of *Arabidopsis* to a combination of drought and heat stress. Plant Physiology. 134, 1683-1696.

Robens, J., and Cardwell, K. 2003. The costs of mycotoxin management to the USA: management of aflatoxins in the United States. J. Toxicol. Toxin. Rev. 22, 143–156.

Schweizer, P., Schlagenhauf, E., Schaffrath, U., Dudler, R. 1999. Different Patterns of Host Genes are Induced in Rice by *Pseudomonas syringae*, a Biological Inducer of Resistance, and the Chemical Inducer Benzothiadiazole (BTH). European Journal of Plant Pathology. 105, 659-665.

Selma, M.V., Martínez-Culebras, P.V., Aznar, R. 2008. Real-time PCR based procedures for detection and quantification of Aspergillus carbonarius in wine grapes. International Journal of Food Microbiology. 122, 126-134.

Seo, J.A., Proctor, R.H., Plattner, R.D. 2001. Characterization of four clustered and coregulated genes associated with fumonisin biosynthesis in *Fusarium verticillioides*. Fungal Genetics and Biology. 34, 155–165.

Ryals, J., Weymann, K., Lawton, K., Friedrich, L., Ellis, D., Steiner, H.Y., Johnson, J., Delaney, T.P., Jesse, T., Vos, P., Uknes, S. 1997. The *Arabidopsis* NIM1 protein shows homology to the mammalian transcription factor inhibitor *IkB*. The Plant Cell. 9, 425-439.

Shim, W.B. and Woloshuk, C.P. 2001. Regulation of Fumonisin B1 Biosynthesis and Conidiation in *Fusarium verticillioides* by a Cyclin-Like (C-Type) Gene, *FCC1*. Applied Environmental Microbiology. 67, 1607–1612.

Sideri, M. and Georgiou, C.D. 2000. Differentiation and hydrogen peroxide production in *Sclerotium rolfsii* are induced by the oxidizing growth factors, light and iron. Mycologia. 92, 1033–1042.

Sigler, K., Chaloupka, J., Brozmanova, J., Stadler, N., Hofer, M. 1999. Oxidative stress in microorganisms. Folia Microbiologica. 44, 587–624.

Sokolovskii, V., and Belozerskaya, T.A. 2000. Effect of Stressors on Gene Differential Expression in the course of *Neurospora crassa*. Usp. Biol. Khim. 40, 85-152.

Spoel, S.H., Koornneef, A., Claessens, S.M.C., Korzelius, J.P., Van Pelt, J.A., Mueller, M.J., Buchala, A.J., Metraux, J.P., Brown, R., Kazan, K., Van Loon, L.C., Dong, X., Pieterse, C.M.J. 2003. NPR1 Modulates cross-talk between salicylate and jasmonate dependent defence pathways through a novel function in the cytosol. The Plant Cell. 15, 760–770.

Sweeney, M.J. and Dobson, A.D.W. 1999. Molecular production by *Aspergillus*, *Fusarium* and *Penicillium* species. International Journal of Food Microbiology. 175, 149-163.

Thiel, P.G., Sydeham, E.W.; Shepard, G.S., Van Schalkwyk, D.J. 1993. Study of the reproducibility characteristics of a liquid chromatographic method for the determination of fumonisins B1 and B2 in corn : IUPAC collaborative study. Journal of AOAC International. 76, 361-366.

Torres, M.A. and Dangl, J.L. 2005. Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Current Opinion in Plant Biology. 8, 397-403.

Tudzynski, B., Homann, V., Feng, B., Marzluf, G.A. 1999. Isolation, characterization and disruption of the *areA* nitrogen regulatory gene of *Gibberella fujikuroi*. Molecular and General Genetics. 261.

Vainio H., Heseltine E., Wilbourn J. 1993. Report on an IARC working group meeting on some naturally occurring substances. International Journal of Cancer. 53, 535-537

Vallad, G.E. and Goodman, R.M. 2004. Systemic Acquired Resistance and Induced Systemic Resistance in conventional agriculture. Crop Science Society of America. 44, 1920-1934.

Van der Merwe, K.J., Steyn, P.S., Fourie, L., Scott, D.B., Theron, J.J. 1965. Ochratoxin A, a Toxic Metabolite produced by *Aspergillus ochraceus*. Nature. 205, 1112- 1113.

Van Loon, L.C., Bakker P.A.H.M., Pieterse C.M.J. 1998. Systemic resistance induced by rhizosphere bacteria. Annual Review of Phytopathology. 36, 453-483.

Van Wees, S.C.M., Pieterse, C.M.J., Trissenaar A., Van't Westende Y.A.M., Hartog F. and Van Loon L.C. 1997. Differential induction of systemic resistance in *Arabidopsis* by biocontrol bacteria. Mol. Plant-Microbe Interactions. 10, 716-724.

Vardon, P., McLaughlin, C., Nardinelli, C. 2003. Potential economic costs of mycotoxins in the United States. In Council for Agricultural Science and Technology (CAST): Mycotoxins: risks in plant, animal, and human systems. Task force report no. 139. Ames, IA, CAST.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research. 23, 4407-4414.

Wang, E., Norred, W.P., Bacon, C.W., Riley R.T., Merril A.H. 1991. Inhibition of sphingolipid biosynthesis by fumonisins. Implications for diseases associated with Fusarium moniliforme. The Journal of Biological Chemistry. 266, 14486-14490.

Wang, X., Tang, C., Zhang, G., Li, Y., Wang, C., Liu, Bo., Qu, Z., Zhao, J., Han, Q., Huang, L., Chen, X., Kang, Z. 2009. cDNA-AFLP analysis reveals differential gene expression in compatible reaction of wheat challenged with *Puccinia striiformis* f. sp. *Tritici*. Biomed Central (BMC) Genomics. 10, 289.

Weller, D.M. 1988. Biological Control of Soilborne Plant Pathogens in the Rhizosphere with Bacteria. Annual Review of Phytopathology. 26, 379-407.

Wu, F., Liu, Y., Bhatnagar, D. 2008. Cost-effectiveness of aflatoxin control methods: Economic incentives. Toxin Reviews. 27, 203–225.

Young, C., McMillan, L., Telfer, E., Scott, B. 2001. Molecular cloning and genetic analysis of an indolediterpene gene cluster from *Penicillium paxilli*. Molecular Microbiology. 39, 754-764.

Yan, J., Wang, J., Zhang, H. 2002. An ankyrin repeat-containing protein plays a role in both disease resistance and antioxidation metabolism. The Plant Journal. 29, 193-202.

Yu, J., Chang, P.K., Ehrlich, K.C., Cary, J.W., Bhatnagar, D., Cleveland,T.E., Payne, G.A., Linz, J.E., Woloshuk, C.P., Bennett, J.W. 2004. Clustered Pathway Genes in Aflatoxin Biosynthesis. Applied and Environmental Microbiology. 70, 1253-1262.

Zhao, Z., Chen, G., Zhang, C. 2001. Interaction between reactive oxygen species and nitric oxide in drought-induced abscisic acid synthesis in root tips of wheat seedlings. Australian Journal Plant Physiology. 28, 1055–1061.

Zhou, F., Menke, F.L.H., Yoshioka, K., Moder, W., Shirano, Y., Klessig, D.F. 2004. High humidity suppresses *ssi4*-mediated cell death and disease resistance upstream of MAP kinase activation, H_2O_2 production and defense gene expression. The Plant Journal. 39, 920–932.

Chapter 2 – Resistance induction in maize

Trichoderma harzianum strain T22 induces in maize systemic resistance against *Fusarium verticillioides*

Davide Ferrigo, Elisabetta Piccolo, Cristina Scopel, Riccardo Rasera, Roberto Causin*

T.E.S.A.F.– Department of Land Use and Agro-Forestry Systems – Plant Pathology, University of Padova, Campus of Agripolis - Viale dell'Università 16, 35020 Legnaro (Padova).

*Corresponding author, Roberto Causin, Department of Land Use and Agro-Forestry Systems – Plant Pathology; University of Padova, Campus of Agripolis - Viale dell'Università 16, 35020 Legnaro (Padova), Italy. Fax +39 049 8272885. E-mail, roberto.causin@unipd.it

Abstract

Fusarium verticillioides is one of the most common plant pathogen fungi affecting maize and the nearly totality of strains are able to produce fumonisins at very different levels. The *Trichoderma harzianum T22* ability to induce resistance was verified in maize plant against the pathogen *F. verticillioides*. Plants grown from T22-treated seed had reduced symptoms following inoculation of stems with *Fusarium verticillioides*, which confirm that root colonization by T22 induces systemic resistance in maize. Enzymatic and transcriptional markers for ISR and SAR were surveyed and the kinetics of response of primed plants towards *F. verticillioides* were shown. Field trials evidenced once more the effectiveness of *Trichoderma harzianum* inoculation reducing both pathogen and fumonisins occurrences in maize kernels.

Keywords, *Trichoderma harzianum*; *Fusarium verticillioides*; biocontrol; maize; fumonisins; induced systemic resistance.

Introduction

Fusarium verticillioides (Saccardo) Nirenberg (teleomorph: *Gibberella moniliformis* Wineland, = *G. fujikuroi* mating population A, sect. Liseola) is one of the most common plant pathogen fungi affecting maize (Leslie, 1991; Nelson, 1992; Sharma *et al.,* 1993) and the prevalent one isolated in Italian harvested maize (Rossi *et al.,* 2004). This pathogen can attack roots, stems and ear causing reductions in crop yields that can vary between 10% and 30%. Most of *F. verticillioides* infection come from infected seeds (Bacon *et al.* 1996), soil inoculum through roots colonization (Kedera *et al.,* 1992; Lumsden *et al.,* 1995), environmental damages (Wicklow *et al.,* 1988), corn borer lesions (Sobek *et al.,* 1999; Placinta *et al.,* 1999; Miller *et al.,* 2001), and silks (Munkvold *et al.,* 1997). Despite the inoculation pathways, the pathogen spreads to the whole plant and kernels before yielding. *F. verticillioides* colonization of maize kernels results in fumonisin B_1 (FB_1) and B_2 (FB_2) contamination. Surveys have indicated that fumonisins occur at biologically significant levels in maize and a variety of maize-based human foodstuffs and animal feed produced worldwide (Marasas *et al.,* 1995; Bolger *et al.,* 2001). FB1 and FB2 are the most common mycotoxins in maize kernels (Placinta *et al.,* 1999) having a notable impact on human and animal health (Logrieco *et al.,* 2002), they are toxic and known to cause pulmonary oedema in swine, encephalomalacia in horses (Prelusky *et al.,* 1994) and immunological disorder in ducks (Bennet *et al.,* 2003). Human exposure to *F. verticillioides* contaminated maize has been linked to elevated rates of oesophageal cancer and FB1 itself has been evaluated by *IARC* as a Group *2B* carcinogen "possibly carcinogen to humans" (Vainio *et al.,* 1993).

Maize is one of the most important cereal grown worldwide. Detection, prevention and control of the infections by *F. verticillioides* in maize are difficult, especially when infection remain at endophytical stage and kernels appear to be uninfected (Bacon *et al.,* 1995). All these evidences make necessary the identification of effective control strategies to *F. verticillioides* infections in maize. To date, the principal strategies consisted in indirect control as Good Agriculture Practice or limitation of European corn borer occurrence in fields, however very few is known on the possibility to use agrochemicals or biological agents. The opportunities to employ fungicides against *F. verticillioides* has been referred by (Causin *et al.,* 2008; Folcher *et al.,* 2009), while potentiality of biological control are yet not well broaden (Cavaglieri *et al.,* 2005; Nayaka *et al.,* 2008; Nayaka *et al.,*

2009). Control by biological control agents (BCA) can be a promising strategy for managing seed-borne, soil-borne and foliar diseases in a wide range of crops and could be considered also to control fumonisin contamination in maize. An exciting perspective could be the resistance induction through seed treatments with BCA.

Trichoderma harzianum appears as one of the more promising BCA: it is able to colonize roots and compete for space and nutrient with plant-pathogenic fungi (Elad, 1996), it produces antifungal substances (Sivasithamparam *et al.,* 1998; Vinale *et al.,* 2008) and enhance plant growth improving nutrient uptake and the efficiency of nitrogen use (Yedidia *et al.,* 2001; Harman *et al.,* 2000; Harman *et al.,* 2004[a]). Furthermore avirulent symbiotic interaction (Harman *et al.,* 2004[a]) with releasing of elicitors (Woo *et al.,* 2006) and secondary metabolites auxin-like (Vinale *et al.,* 2008) can trigger, through jasmonic acid and ethylene signals pathway (Shoresh *et al.,* 2005; Djonović *et al.,* 2007), inducing systemic resistance (ISR) in more than 10 different dicots and monocots, including graminaceae, solanaceae, and cucurbitaceae to infection by fungi (*R. solani, B. cinerea, Colletotrichum spp., Magnaporthe grisea, Phytophthora spp., Alternaria spp*., etc.), bacteria (*Xanthomonas spp., Pseudomonas syringae*, etc.), and even viruses (*Cucumber mosaic virus*) (Lumsden *et al.,* 1989; Zimand *et al.,* 1996; Mao *et al.,* 1998; Howell, 2002; Harman *et al.,* 2004[a]; Chet *et al.,* 2006).

Among commercially available Trichoderma strains, T22 is the most useful, characterized by a good rhizosphere competence is able to colonize plants root in different type of soil and pH (Harman and Björkman, 1998). Up to date, several works have been published about interaction between *T. harzianum*, maize and a variety of pathogens but *F. verticillioides* (Chen *et al.,* 2004; Harman *et al.,* 2004[b]; Rojo *et al.,* 2007; Shoresh and Harman, 2008) or about the effect of a range of BCA against *F. verticillioides* but *T. harzianum* (Bacon *et al.,* 2001; Cavaglieri *et al.,* 2005; Nayaka *et al.,* 2009) but very few has been investigated on *F. verticillioides*-maize-*T. harzianum* interactions and their dynamics (Sobowale *et al.,* 2007; Nayaka *et al.,* 2008). This work was conducted to determine 1) the ability of *T. harzianum* T22 to induce resistance in maize against *F. verticillioides;* 2) the involvement of two enzymatic activities and four molecular markers in induced resistance; 3) the ability of *T. harzianum* T22 to decrease endophytic *Fusarium* colonization in maize; 4) the effects of seed biopriming with *T. harzianum* T22 to decrease

in field trials the *F. verticillioides* kernel colonization and the consequent fumonisin B1 and B2 content.

Materials and Methods

Plant and fungal material

Subjects utilized in this work were *T. harzianum* Rifai strain T22, KERMESS hybrid maize and *F. verticillioides* strain 19 (*Fv*S19). Uncoated Kermess (FAO 600 KWS) seeds were used in all experiments in the present work. Seed treatment with *Trichoderma harzianum* T22 was carried out using commercial formulation (ROOTSHIELD® Intrachem Italia - BioWorks, Inc., Geneva, NY, USA) containing 10^7 CFU g^{-1} (propagules and conidia) of that strain. *F. verticillioides* strain used in the present work was chosen after morphological identification (Leslie and Summerell, 2006), among some *Fusaria* strains isolated from maize kernels grown in North East of Italy. From these *F. verticillioides* isolates were obtained monoconidial cultures as reported by Tuite (Tuite, 1969). Monoconidial isolates were subcultured in PDB (Difco. Detroit, MI, USA) at 25°C for 7 days, mycelium was extracted and genomic DNA used to molecular identification with primers VER1 and VER2 coding calmodulin (Mulè *et al.,* 2005). All the monoconidial strains were inoculated in maize plant, 15 days post inoculation the pathogenicity was assessed by measurement of necrotic areas and the strain 19 (*Fv*S19) was selected as the most aggressive towards KERMESS hybrid.

Resistance induction assays

The effect of T22 in inducing resistance in maize was preliminarily studied in planta as reduction of necrotic lesions produced by *F. verticillioides* as a consequence of infection. Experiments were carried-out under gnotobiotic conditions: kernels were subjected to double treatment with surface disinfection in H_2O_2 10% for 15 min and a heat treatment (65°C for 5 minutes) to remove both external and internal bacteria and fungi (Bacon *et al.,* 1994). Five seeds per pot (24 cm diameter) were sown in aseptic soil in separated and sterilized greenhouses box, and grown in standard conditions (25 ± 1°C, 16h photoperiod and 75 ± 5% RH). After 15 days, germinated seeds were inoculated with T22 and/or *Fv*S19

as briefly summarized in Table 1. T22 inoculation were performed with 50 ml per pot of 10^6 CFU/ml conidial suspension. For each repetition, plant roots were analyzed to assess colonization by T22 and leaves were also analyzed to confirm absence of T22 inoculum. At the same time the inoculation with *Fv*S19 was carried out. A pocket-like lesion was produced in the stalks through a gentle cut, a 25 mm^2 mycelium plug of PDA actively colonized by *Fv*S19 was inserted in the pocket and lesions were protected with Parafilm (Pechiney Plastic Packaging Company, Inc. Chicago, ILL, USA). Control plants (T-F-) had the same treatment but the plug inserted was sterile. Fifteen days after pathogen challenging, plants were collected and the necrotic area of lesions was digitally calculated with Autocad® Autodesk after digitalization of areas. Disease areas were collected from 75 plant per thesis. The same experiment was carried out to comparison using as biocontrol agent *T. harzianum* strain T39 (TRICHODEX, Intrachem Italia - BioWorks, Inc., Geneva, NY, USA). Furthermore, to score each sample, we categorized the severity of symptoms in classes from1 to 6, from "no yellowing" to "deep stalk necrosis" as reported in Fig. 2.

Thesis	**Treatments inoculation**	
	To germinated seeds	**To plants**
T-F-	-	-
T-F+	-	*F. verticillioides* strain 19
T+F-	*T. harzianum* T22	-
T+F+	*T. harzianum* T22	*F. verticillioides* strain 19

Table 1: Description of different treatments among the thesis.

Enzyme extraction and activity assays

For enzymatic assays seeds were grown and inoculated as previously described. After the inoculation of T22 and *Fv*S19, roots and leaves were collected every 12 hours starting from 0 h to 84 hpi and stored at -80 C° until extraction and analysis. All reagents utilized in the following experiments were purchased by Sigma Aldrich (Sigma Aldrich. St. Louis, MI, USA). Crude enzymes for PAL activity were extracted from 0.5 g of fresh tissues in a potassium borate buffer 100 mM pH 8.8 added of β–mercaptoethanol 14 mM and then centrifuged for 35 min at 13000 g, 4°C. (Ke and Saltveit, 1986) To a substrate solution containing 950 µl potassium borate buffer 100 mM and 0.2% L-phenylalanine , 50 µl of

crude extract were added and incubated at 35 °C for 30 min. The use of 50 µl of HCl 5M stopped the enzymatic reaction. PAL activity was determined as formation from substrate of trans-cinnamic acid and monitored by spectrophotometer (Biomate 3, Thermo Scientific, Waltman, WA, USA) as the increase in absorbance at 290 nm. Amount of *trans*-cinnamic acid synthesized was calculated using molar extinction coefficient 9630 (Dickerson *et al.,* 1984; Shi *et al.,* 2007). Crude enzymes for LOX activity were extracted from 0.5 g of fresh tissues in a sodium phosphate buffer 50 mM pH 6.5 added to 1% PVPP, 1mM PMSF and then centrifuged for 35 min at 13000 g, 4°C (Vieira *et al.,* 2001). The substrate solution with a 25 mM final concentration of linoleic acid was obtained by mixing 155 µl of pure linoleic acid, 157 µl of Tween-20 and 4.59 ml of deionized water (Anthon and Barret, 2001). To measure LOX activity, to 860 µl of a sodium phosphate buffer 50 mM, 40 µl of substrate solution and 100 µl of crude enzyme extract were added and incubated at 30°C for 5 min. The use of 0.2 M NaOH both stopped the enzymatic reaction and ensured the optical clarity. LOX activity was determined as formation of hydroperoxide was monitored as the increase in absorbance at 234 nm due to the presence of a conjugated hydroperoxydiene moiety (Gökmen *et al.,* 2002). The amount of hydroperoxydiene synthesized was calculated using its extinction coefficient of 23000 (Anthon and Barret, 2001). Enzymatic activity data were obtained from 5 samples of roots and leaves per thesis per hpi both for PAL and LOX.

Protein quantification

Protein determinations were carried out using the dye-binding method of Bradford (Bradford, 1976). To Bradford Reagent ,50 µl of crude extract in sodium phosphate buffer 50 mM pH 6.5 were added as described in manufacturer's instructions. A standard curve was constructed using BSA in the concentration range 5–25 µg/ml. The dye-binding was monitored as the increase in absorbance at 595 nm.

RNA isolation

To study the expression of defence related genes, plants inoculated and grown as previously described condition were harvested seven days after T22 treatment, at 0, 24, 48 and 72 hours from *Fv*S19 inoculation. Daily sampling and collection was chosen to limit effects of circadian rhythm on genes (Linthorst *et al.,* 1993; Epple *et al.,* 1995; Rogers *et al.,* 2005; Nemchenko *et al.,* 2006). Stalks were stored at -80 C° until extraction and analysis. Total RNA was extracted using the Illustra Rna Mini Spin Kit (GE Healthcare. Chalfont St. Giles, UK) following the manufacturer instructions. Total RNA was extracted from 50 mg of maize stalks challenged with *Fv*S19, quantified with a spectrophotometer (Biomate 3, Thermo Scientific, Waltman, WA, USA), and run on 1% agarose gel to check its integrity. Ten μg of total RNA were treated with 10 U of RQ1 RNase free DNase (Promega. Madison, WI, USA) and 2 Unit of RNasin® Ribonuclease Inhibitor (Promega. Madison, WI, USA) for 30 min, then purified by phenol–chloroform extraction and precipitated in isopropyl-alcohol as described by Sambrook (Sambrook and Russel, 2001). cDNA was synthesized from 2 μg of DNA-free total RNA using the Reverse Transcription System (Promega. Madison, WI, USA) according to the manufacturer's instructions.

Real Time-PCR

One tenth of cDNA was used to optimize PCR amplification and primers efficiency. Sequences for gene-specific primer pairs for *ZmPal*, *ZmHpl* (Farag *et al.,* 2005) and *ZmLox10* (Nemchenko *et al.,* 2006) were obtained from previous work. Gene-specific primer pairs for *ZmGAPc* (Accession EU953063; forward 5'-CTG GTT TCT ACC GAC TTC CTT G-3' and reverse 5'-CGG CAT ACA CAA GCA GCA AC-3') and *ZmPr1* (Accession U82200; forward 5'-CTA GCA CCA CGA CAC CAA CA-3' and reverse 5'-ACA AAT CGC CTG CAT GGT-3') were designed using Primer3 v0.4.0 software available at http://frodo.wi.mit.edu/primer3/ (Rozen and Skaletsky, 2000). Specific primers were checked with PerlPrimer software (available at http://perlprimer.sourceforge.net/download.html) to confirm absence of dimerization. *ZmGAPc* was used as internal control (Farag *et al.,* 2005). Reaction condition were optimized as follows: 40 cycles composed by 95°C for 15s, annealing for 30 sec, 72°C for 35 s. Annealing temperatures were 50°C for *ZmLox10*, 55° C for *ZmPr1*, 58°C for *ZmPal*,

ZmHpl and *ZmGAPc*. A final extension step of 72°C for 4 min was employed. All reactions were carried out in triplicate. The absence of dimer formation was checked in no-template samples by product dissociation analysis and electrophoretic separation in agarose gel. Transcript expressions were analyzed with ΔC_t (cycle threshold) method according to Pfaffl adaptation (Pfaffl, 2001) using Q-Gene software (Muller *et al.*, 2002). *ZmGAPc* was used as housekeeping gene to normalize raw expression data. The main parameters of primers used are listed in Table 2.

Primer	Sequence	T_m primer °C	T_m product °C
*ZmGAPc*_F	5'-CTG GTT TCT ACC GAC TTC CTT G-3'	66	84,2
*ZmGAPc*_R	5'-CGG CAT ACA CAA GCA GCA AC-3'	66	
*ZmPal*_F	5'-CGA GGT CAA CTC CGT GAA CG-3'	64	86,2
*ZmPal*_R	5'-GCT CTG CAC GTG GTT GGT GA-3'	64	
*ZmHpl*_F	5'-TAC GAG ATG CTG CGG ATG-3'	56	83,6
*ZmHpl*_R	5'-CTC GAA GTC GTC GTA GCG-3'	58	
*ZmLox10*_F	5'-ATC CTC AGC ATG CAT TAG TCC A-3'	64	75,8
*ZmLox10*_R	5'-AGT CTC AAA CGT GCCTCT T-3'	56	
*ZmPr1*_F	5'-CTA GCA CCA CGA CAC CAA CA-3'	62	80,6
*ZmPr1*_R	5'-ACA AAT CGC CTG CAT GGT-3'	58	

Table 2: Sequences and melting temperature of primer of surveyed genes and melting temperature of PCR products .

DNA isolation and quantification of endophytic content

To analyze *F. verticillioides* endophytic content, maize seeds were only externally disinfected, sowed and grown as previously described. Seven days after T22 inoculation leaves were wounded with a toothpick previously submerged in 10^8 CFU/ml *Fv*S19 conidial suspension (water and 0.01% Tween-20), to simulate European corn borer damage. At 15 dpi from pathogen inoculation, stalks were collected and immediately frozen in liquid nitrogen. For analysis of endophytic content, 10 samples from 10 different plants per thesis were processed. Genomic DNA from maize stalk was extracted using Wizard® Genomic DNA. Purification Kit (Promega Corporation. Madison, WI, USA). Absolute quantification was performed by Real Time-PCR using Power SYBR® Green PCR Master Mix (Applied

Biosystems. Foster City, CA, USA) with primer coding *F. verticillioides* calmodulin gene VER1: 5'-CTTCCTGCGATGTTTCTCC-3' and VER2: 5'-AATTGGCCATTGGTATTAT ATATCTA-3' (Mulè *et al.,* 2005). Real Time-PCR condition were optimized to 35 cycles with 95°C for 15s, 56°C for 50 sec, 72°C for 60 s. A final extension step of 72°C for 4 min was employed. Genomic DNA of *Fv*S19 was used to fit standard curve.

Field experiment

Commercial product ROOTSHIELD® and moistened seeds (30 min in sterile water) were put in sterile bags (in ratio of 1g of commercial product per 10g of seeds) and shacked for ten minutes. The treated seeds were placed on sterile paper towels and allowed to dry overnight in a laminar flow hood. The *T. harzianum* colonization was checked on a sample of 100 seeds placed (5 seeds per plate) on PDA plates (Difco. Detroit, MI, USA) added with streptomycin sulfate (Sigma Aldrich. St. Louis, MI, USA). The plates were incubated in the dark at 20°C. After 5 days the kernels showing *Trichoderma* mycelia growth were counted. After the seeds had dried they were sowed. The thesis were arranged in a complete randomized block designed with 3 replications (300 m^2 each). Ten days after germination and at silking, 30 maize plant from each thesis, 10 per replication, were uprooted and a samples of roots were collected. The roots were rinsed with tap water and 100 fragments for theses were placed on PDA plates with streptomycin sulfate (5 fragments per plate) and incubated in the dark at 20°C; after 5 days fragments developing *Fusarium* or *Trichoderma* colonies were checked. Seven days after silking and at the end of female flowering, ten samples of silk per repetition were collected. At waxy and at harvest time, for each plot, 100 ears were collected by hand and shelled. Silk and kernel samples were milled, homogenized and a serial dilution with sterile distilled water were made starting from 10^{-2} to 10^{-6} concentration. For every dilution, aliquots of 0,5 ml were spread on PDA plates (5 replicates per dilution). Plates were incubated 7 days at 25°C and total count and count per colony type were done. The colonies were isolated and subcultured for fungal species identification. *F. verticillioides* colonies were morphologically identified (Leslie and Summerell, 2006). Samples collected at harvest time were also analysed for fumonisin B1 and B2 content using certified HPLC methods (NEOTRON. Modena, Italy)

Statistical analysis

Data were analyzed using Statistica 6.0 software (StatSoft, Tulsa, USA) The normal distribution and homogeneity of variances were verified by performing the Wilk-Shapiro test and the Levene test, respectively. Excluding field trials, all biological experiments were conducted twice. Mean values for disease areas were analyzed by ANOVA test with α = 0.001. Mean values for enzymatic activity and gene expression were analyzed by ANOVA test with α = 0.05. For dynamic analysis of *Fusarium* spp. CFU in samples, dataset were compared to 95% confidence limits estimated for the mean. For field trials, the mean values for *F. verticillioides* presence and fumonisin content were analyzed by ANOVA test with α = 0.05. Multiple mean values comparison were performed according to the Student–Newman–Keuls test.

Results

Effect of T22 on disease symptoms reduction

Maize plants were grown in greenhouse in sterile conditions to avoid microorganisms able to interfere with experiments. Sterile conditions were maintained also to prevent the cross-contaminations during the inoculation steps, cultivation operations and sampling activities. As a consequence of inoculation, *T. harzianum* colonized 92% of root fragments belonging to plants of thesis T+F+ and T+F-, while it was not detectable in roots of uninoculated maize plants. A significant reduction of 65% in the size necrotic area was observed in T+F+ plants compared to the T-F+ ones (Fig. 1). The same result was obtained using *T. harzianum* T39 but with a lower reduction (59%) of the necrotic areas (Fig. 1). Severity of lesions produced by *Fv*S19 resulted strongly reduced in plant treated with T22 in comparison to those untreated with the BCA, the reduction for the higher severity classes was of 31%, 10% and 27% for classes 4, 5 and 6 respectively (Fig. 2)

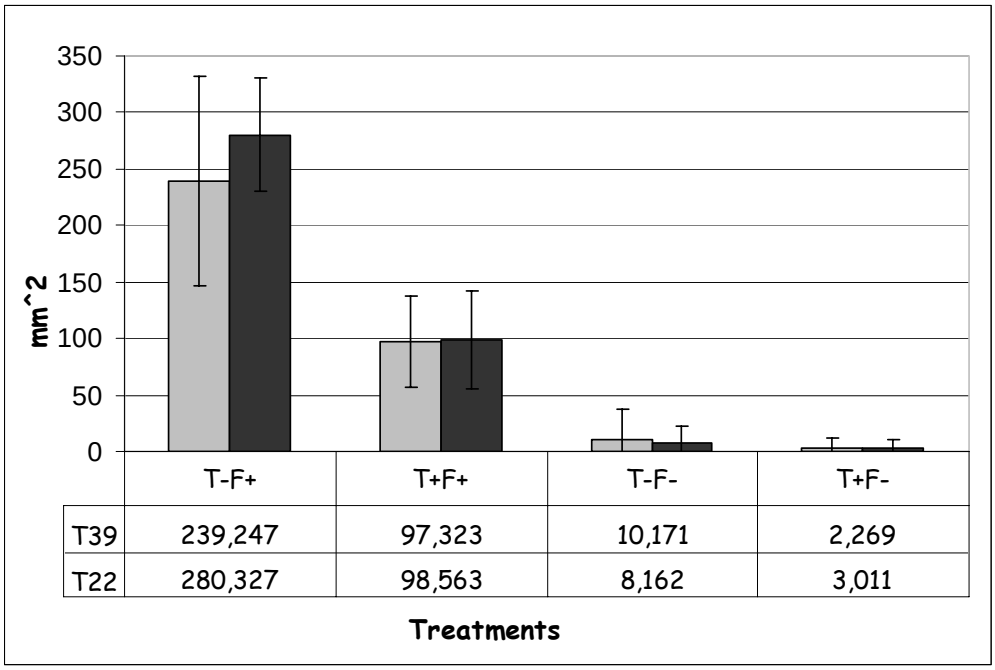


Figure 1 Effect of T22 and T39 treatment on necrotic lesion by *Fv*S19. The graph illustrates the mean lesion area (in mm^2) in maize stalks, collected after 15 days from infection. The lighter gray corresponds to T22 treatment, the darker gray corresponds to T39 treatment. Each bar represent mean lesion area of 75 plants in two independent experiments with SE bar. From the left: *Trichoderma* uninoculated-*Fv*S19 infected (T-F+); *Trichoderma* inoculated-*Fv*S19 infected (T+F+); *Trichoderma* inoculated- *Fv*S19 uninfected (T-F-) and *Trichoderma* inoculated- FvS19 uninfected (T+F-)

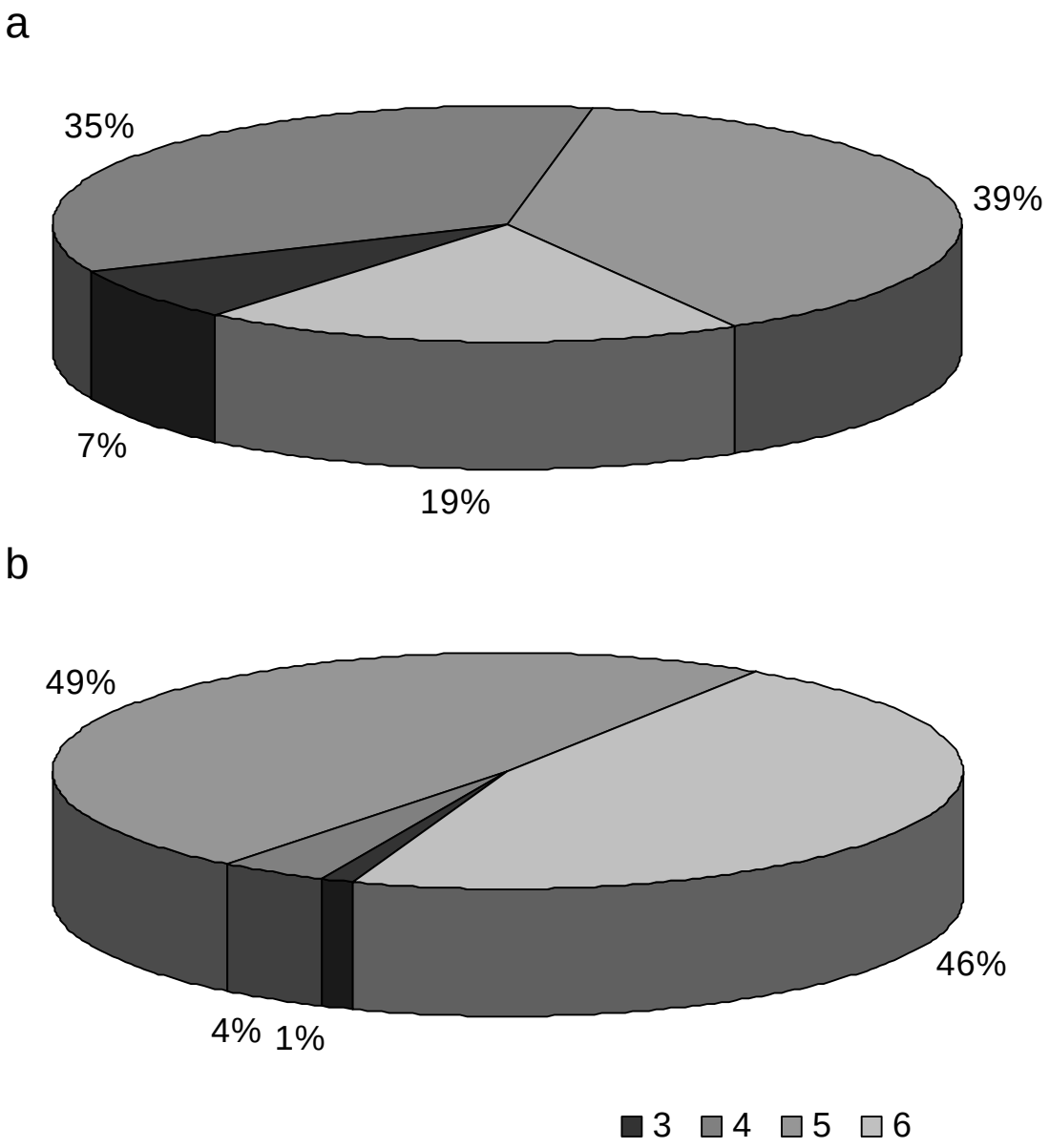


Figure 2: Effect of T22 treatment on severity of disease produced by *Fv*S19 on maize stalks in treated plants (**2a:** T+F+) and untreated (**2b:** T-F+). Lesions were classified according to severity of disease on necrotic areas. Class 1: "no yellowing"; Class 2: "Yellowing of inoculum site"; Class 3: "Localized necrosis of inoculum site"; Class 4: "Localized necrosis with aerial fungal mycelium"; Class 5: "Extended necrosis over inoculum site"; Class 6: "Extended necrosis to deeper tissues of stalk" The cake graphs illustrate the component of each class relative to treatment. **Upper graph**: 19% Class 6; 39% Class 5; 35% Class 4 and 7% Class 3. **Lower Graph**: 46% Class 6; 49% Class 5; 4% Class 4; 1% Class 3.

Effects of T22 inoculation on *F. verticillioides* endophytic content

The endophytic presence of *F. verticillioides* in plants treated or less with T22 was quantified a quantitative Real-Time PCR from a sample containing total genomic maize DNA. The effect of T22 inoculation was remarkable on the endophytic content only in artificially infected plant (T+F+). In fact, these plants showed a 45% reduction of *F. verticillioides* DNA compared to the untreated ones. Furthermore, T22 treatment resulted in a reduction of 50% of *F. verticillioides* DNA in plants where only natural infection was present (T+F-) but for the high variability among replications these data lack of statistical significance. (Fig. 3)

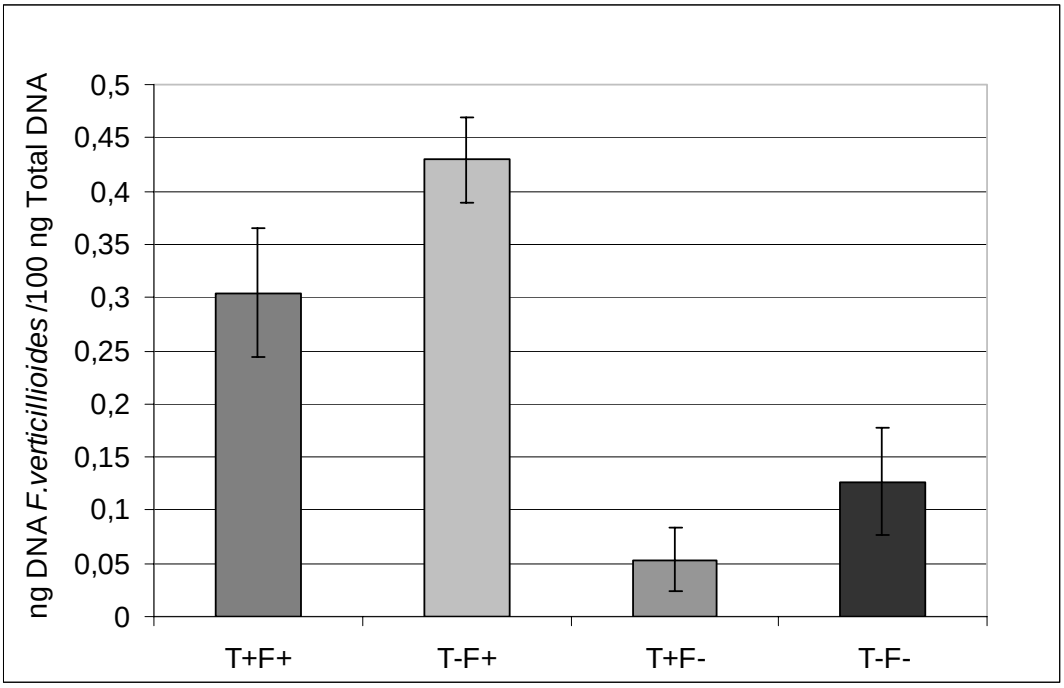


Figure 3: Endophytic content of Fusarium verticillioides on maize. Total DNA extracted from maize stalk were submitted to quantitative Real-Time PCR to quantify *F. verticillioides* endophytic contamination. T22 inoculation was performed on two-week-old maize plants, 15 days after, plants were infected with *Fv*S19. Samples were collected from infection site. From the left: T22 inoculated-FvS19 infected (T+F+); T22 uninoculated-FvS19 infected (T-F+); T22 uninoculated- *Fv*S19 infected (T+F-) and T22 uninoculated- FvS19 uninfected (T-F-)

Effects of T22 inoculation on PAL and LOX enzymatic activity in maize

The enzymatic activity of PAL and LOX in roots and leaves was quantified. In general we found in leaves, two-fold phenylalanine ammonia-lyase levels than in roots. Results showed that both in roots and leaves, the plants inoculated with T22 increased PAL activity at 36 hpi. In particular the T22-treated plants compared to the untreated ones, increased the PAL activity of 2.5 fold and 3 fold higher in roots and leaves respectively (Fig. 4a and 4b). In addition, although trend of PAL activity were higher in inoculated plants compared to non-inoculated ones, T22-treated plants were statistically different only at 36 hpi for roots and leaves and at 60 hpi in leaves. Regard to LOX enzyme, activity in leaves was detected as 4-fold higher compared to roots. Data analysis of LOX activity showed significance only for inoculated plants at 36 hpi in roots and from 48 to 60 hpi in leaves (Fig. 4c and 4d)

Effects of *F. verticillioides* infection and T22 inoculation on enzymatic activity in maize.

Maize plant inoculated with T22 and challenged with *Fv*S19 were collected to analyze enzymatic activity changes respect to *Trichoderma* inoculation and *F. verticillioides* infection. The PAL activity in roots was constant in non-infected plants, while decreased in *Fv*S19-challenged plants since 12 hpi and rapidly raised up to highly significant amounts. At 60 hpi PAL activity in T+F+ plants resulted 1.5 fold higher compared to not inoculated T-F+ plants (Fig. 5a). In leaves, PAL activity trend was very similar to roots, with a reduction in infected plants that reached its minimum at 36 hpi before to rise up. At 48 hpi plants treated with T22 and *Fv*S19 showed a PAL activity of 1,5 to 2 fold higher compared to all others thesis (Fig. 5b). Quantification of lipoxygenase activity in roots gave data highly variable and no reliable consideration can be advanced, (data not shown), in leaves results were more clear and a trend very similar to PAL activity in leaves was observed (Fig. 5c).

Effects of F. verticillioides infection on resistance related gene expression in T22 primed maize

The expression of *ZmPal* and *ZmPR1* genes known as markers for acquired resistance and *ZmLox10* and *ZmHpl* genes known as markers for induced resistance, were examined in the four thesis listed in Table 1 (T-F-, T+F-, T-F+ and T+F+ plants). In Fig. 6 data were normalized according to T-F- values to remove physical damage component in gene expression. PAL transcript in T+F+ plants at 24 hpi seemed to be non-induced, anyway it increase rapidly since 48 hpi to surpass other thesis from 72 hpi. In particular, when T22 and *Fv*S19 separately colonized plants, effect of T22 on *ZmPal* expression resulted higher in those treated only with T22 rather than those infected only with pathogen. Further, in comparison to the respective enzyme, reduction of transcript in T+F+ at 24 hpi coincided with enzymatic results.

As far as it concern expression of *ZmPr1*, the plants inoculated with *Fv*S19 (T-F+ and T+F+) reported an increase of transcript over time. Also in plants primed with T22 (T+F-) it was possible to observe a slight increment in transcription fold, however at lower level compared to infected plants. Expression of *ZmLox10* showed a trend without large differences both overtime and among thesis. At 72 hpi this gene was expressed in the same way in plants primed or not with T22 and inoculated or not with *Fv*S19. On the contrary, the expression of *ZmHpl* grown over time. The plants inoculated with the pathogen presented an induction stronger compared to the uninfected just from 24 hpi despite T22 treatment. The priming with T22 without *Fv*S19 inoculation resulted in a similar trend to other thesis but induction occurred 24 hours later. The highest values in *ZmHpl* gene expression was reached in T22 primed and pathogen infected (T+F+), in particular at 72 hpi the induction of *ZmHpl* in double treated plants (T+F+) was twice compared to the infected but unprimed thesis (T-F+). Finally, the confrontation among thesis at 72 hpi was highlighted to better explain resistance response in advanced infection. In *ZmPal* and *ZmHpl* expression the infection with *Fv*S19 brought in T22 primed plants (T+F+) an induction of 3-fold and 2-fold respectively in comparison to the uninoculated ones (T-F+). Further, while *ZmLox10* expression was comparable and not different between theses, PR1 expression was stimulated above all by infection. In Figure 7 data were normalized by the highest value and expressed as arbitrary unit.

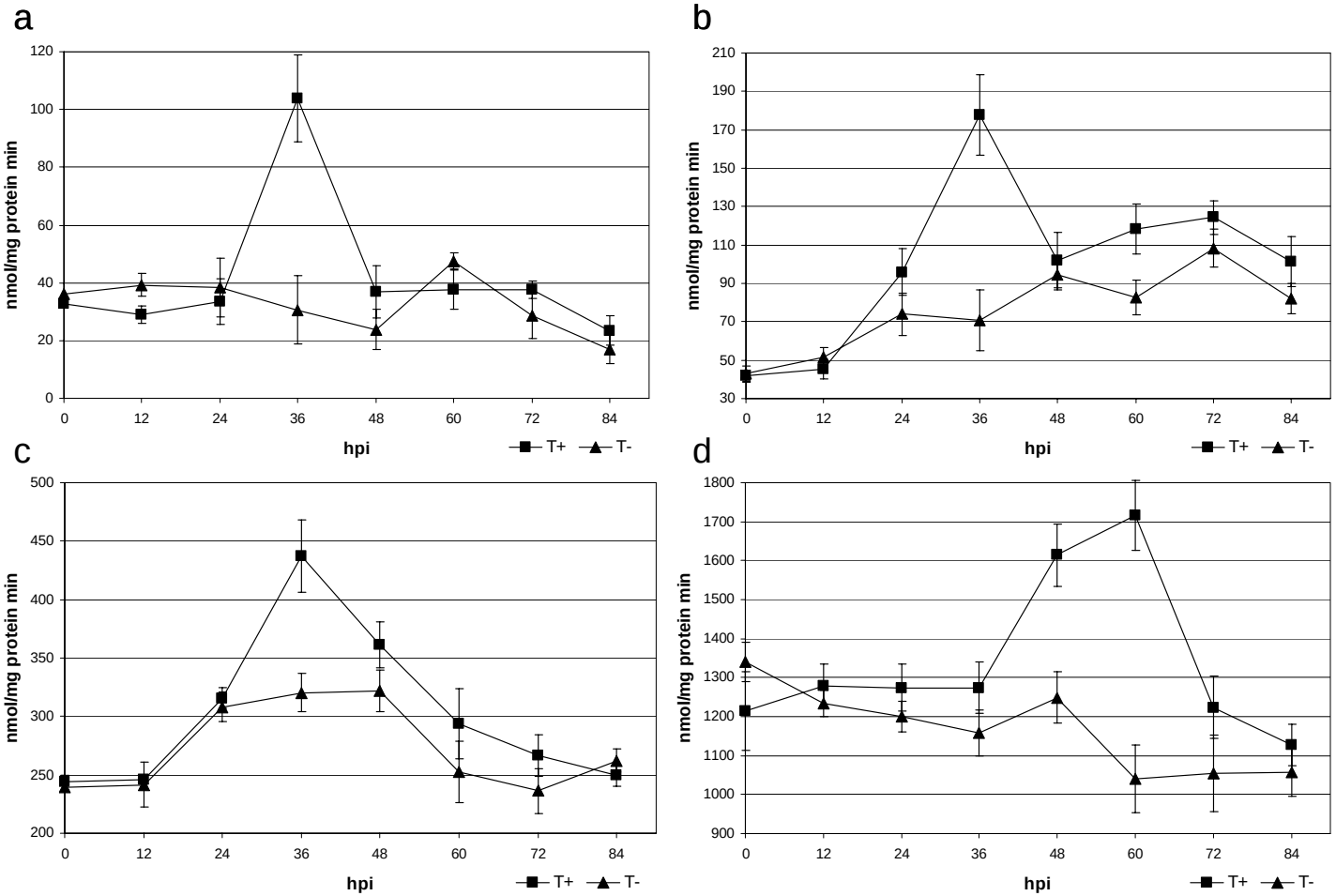


Figure 4: Enzymatic activity of phenylalanine ammonia lyase in roots (**a**) and leaves (**b**) of plants inoculated with T22. In graphs (**c**) and (**d**) LOX activity in roots and leaves respectively were showed. T22 inoculation was performed on two-week-old maize plants. Activity were surveyed each 12 h from 0 hpi (before T22 inoculation) to 84 hpi. Activity were measured as formation of product of enzymatic activity in nmol/mg protein per minute.

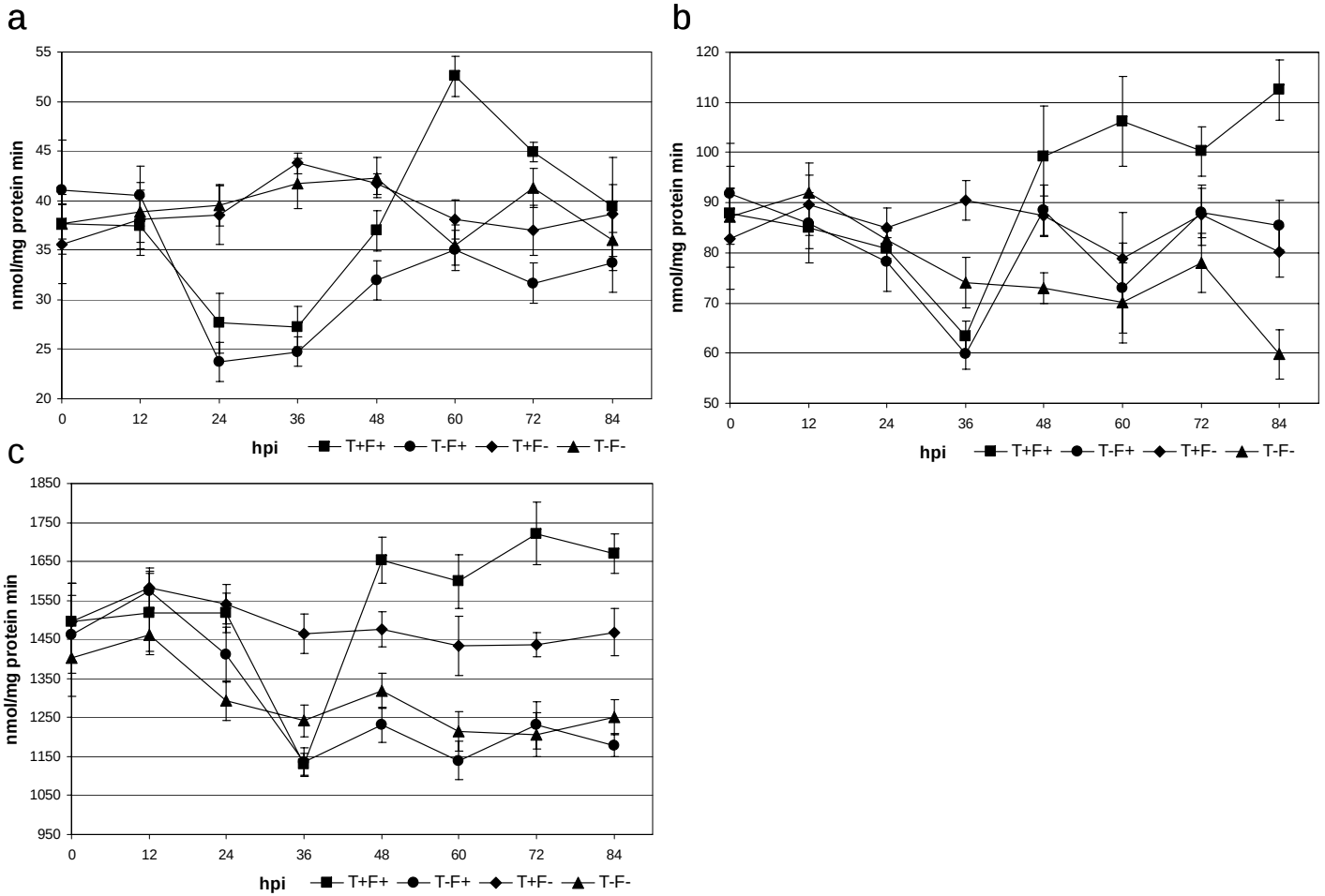


Figure 5: Enzymatic activity of lipoxygenase in roots (**a**) and leaves (**b**) of plants inoculated with T22 and infected with the pathogen FvS19. In graphs (**c**) LOX activity in leaves respectively was showed. T22 inoculation was performed on two-week-old maize plants, 15 days after, plants were infected with *Fv*S19. Activity were surveyed each 12 h from 0 hpi (before pathogen challenge) to 84 hpi. Activity were measured as formation of product of enzymatic activity in nmol/mg protein per minute.

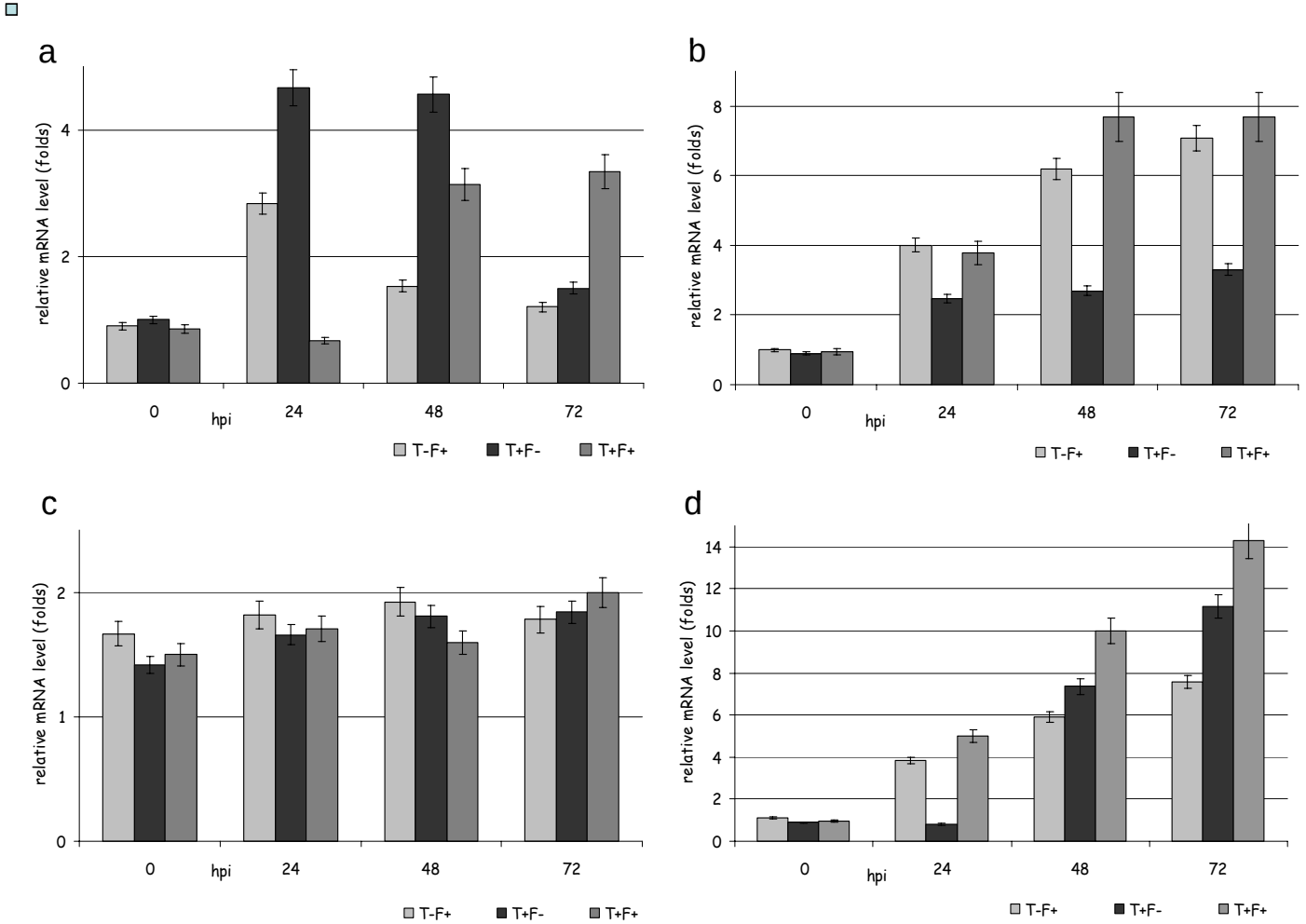


Figure 6: Expression of defence-related genes in plants induced by *T. harzianum* T22 and challenged with *F. verticillioides* strain 19. SAR related genes: (**a**) *ZmPal* and (**b**) *ZmPR1*. ISR related genes: (**c**) *ZmLOX10* and (**d**) *ZmHpl*. Expression of *ZmGapC* was used as reference gene expression. One week-old maize plants were inoculated with *T. harzianum* T22, after 7 days stalks were infected with *F. verticillioides* strain 19. Samples were collected from infection site each 12 h from 0 hpi (before pathogen challenge) to 72 hpi. Values are the average of two independent replica with SE bar.

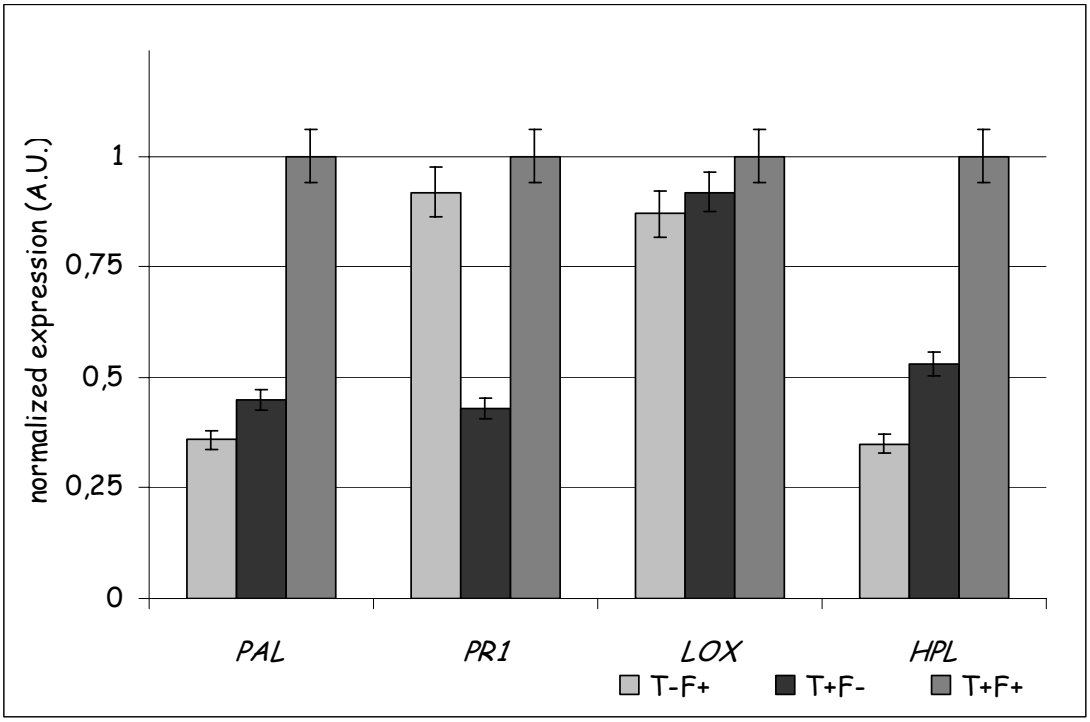


Figure 7: Expression of defence-related genes in plants induced by *T. harzianum* T22 and challenged with *F. verticillioides* strain 19 in advanced infection (72.hpi) SAR related genes: *ZmPal* and *ZmPR1*. ISR related genes: *ZmLOX10* and *ZmHpl*. Expression of *ZmGapC* was used as reference gene expression.

Effect of T22 seed treatment on *F.verticillioides* colonization and fumonisin contamination of maize kernels.

In preliminary assays carried out before sowing, treated seeds resulted 100% colonized by *T. harzianum* Ten days after germination, 82% of maize roots belonging to T22 treated seeds were colonized by *Trichoderma* and 30% by *F.verticillioides* while in untreated plants, 22% of roots were colonized by *Trichoderma* and 40% by *F. verticillioides*. At silking in treated thesis, the roots colonized by *Trichoderma* were still higher than in untreated ones, 50% and 18% respectively while level of *F.verticillioides* root colonization remained stable (29% and 42%). Silks colonization by *F. verticillioides* seven days after silking was undetectable in treated thesis, while reached values of ~10^2 CFU at the end of flowering when the silks were senescent and the colonization levels were rather similar. During kernel ripening the treated thesis were less colonized than the untreated ones, but at harvest time the levels were rather similar (Fig. 8). At the harvest time the fumonisin B1

and B2 contamination of kernels was higher in the untreated thesis compared to those primed with T22. (Fig. 9).

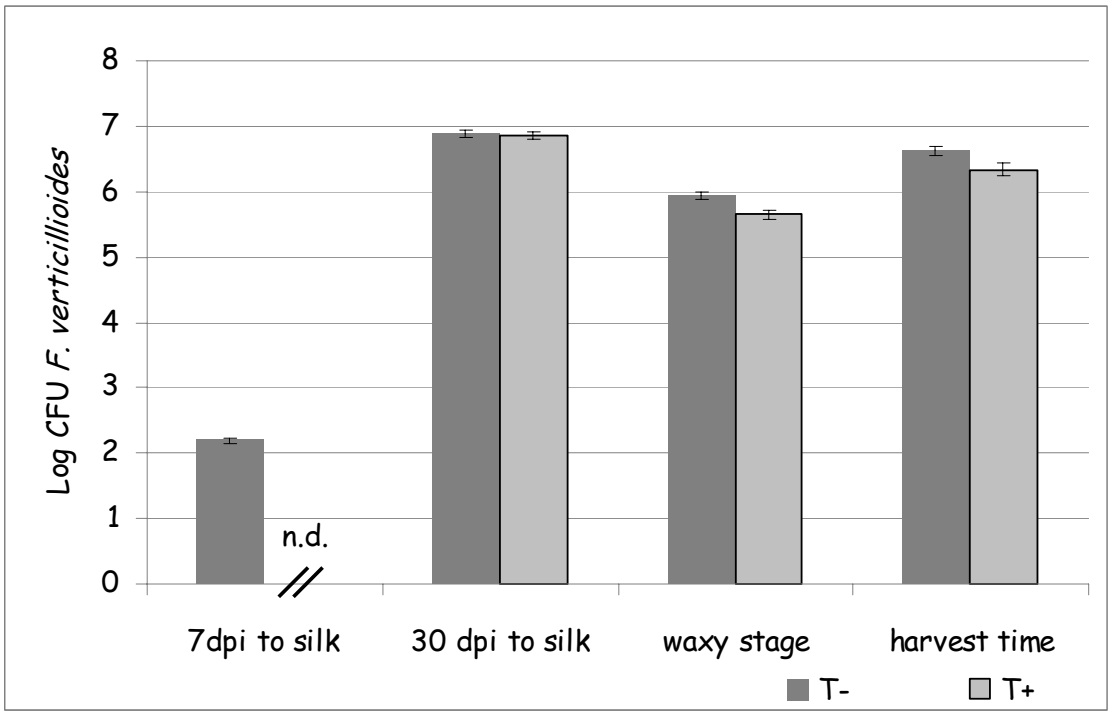


Figure 8: Graphic represent F.verticillioides colonization level as logarithm of CFU values. Samples were collected at the beginning of silking (7 days after) and at the end (30 days after), at the waxy stage and at the harvest time. *Fusarium verticillioides* CFU in treated sample 7 days after silk, were under detection limits. Bars are the 95% confidence limits estimated for the means.

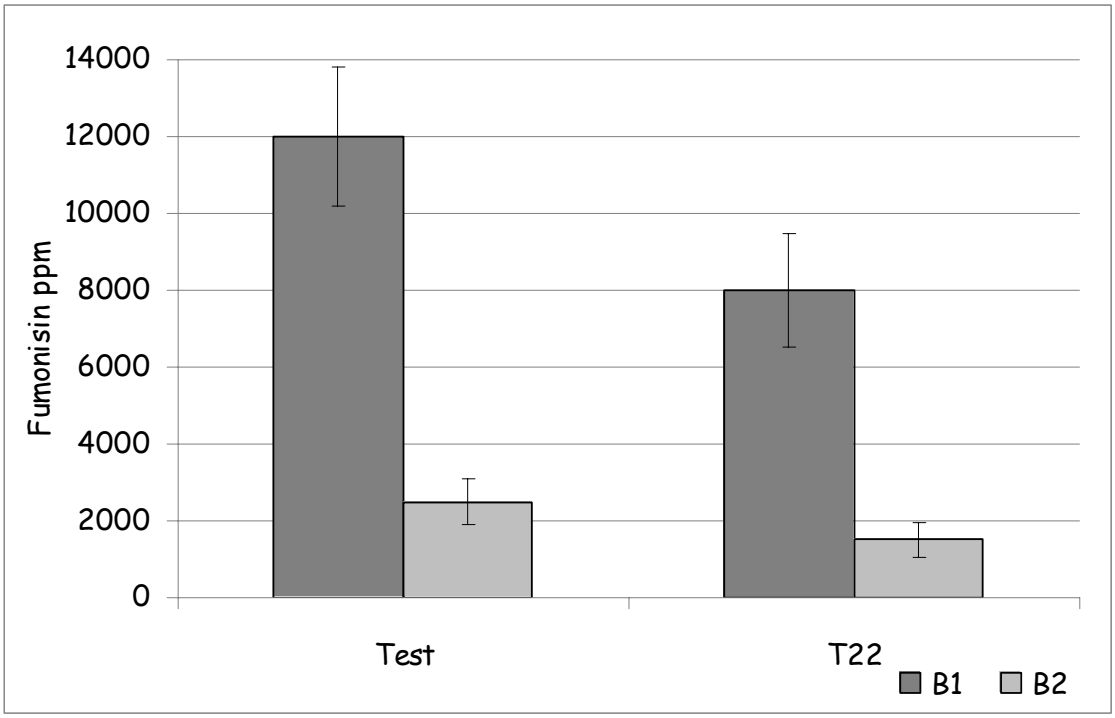


Figure 9: HPLC quantification of fumonisins content in kernels non-primed with T22 or primed. First bar (darker grey) for each histogram correspond to fumonisin B1 content, the latter bar (lighter grey) correspond to fumonisin B2 content. Values are the average of two independent replicates with SE bar.

Discussion and Conclusions

Species belonging to genera *Trichoderma* are known to be able to induce a variety of beneficial responses in plants. Their efficacy vary from increasing in seed germination (Harman and Björkman, 1998) or leaf greenness (Harman and Dozelli 2001; Harman *et al.,* 2004[b]) to improvement in nitrogen uptake and utilisation (Harman, 2000). Furthermore, some *Trichoderma spp* are proposed as potential biocontrol agents able to decrease plant pathogen infection by triggering systemic resistance (Harman *et al.,* 2004[a] ; Harman, 2006; Djonović *et al.,* 2007) or negatively affecting growth or penetration of the pathogen into plants (Elad *et al.,* 1999; Elad and Kapat, 1999). Once induced, plants may remain protected for a considerable part of their lifetime, indicating that when the resistance state has been triggered in the plant, it is rather stable (Van Loon *et al.,* 1998). Although fungi of genera *Trichoderma* have been reported to induce resistance, this activity depends on different *Trichoderma* species and strains, pathogenic fungus and host plant. Defence reactions induced by one particular BCA strain can show a strong heterogeneity depending on plant species: the same BCA could have a strong effect against a pathogen infecting an host but it can lose this ability when used on a different host or against a different pathogen (Benhamou and Chet, 1996; Van Loon *et al.,* 1998). In dicotyledons ISR and SAR pathways are supported by several works carried out both in model plants and in cultivated ones, whereas the triggering of resistance in monocotyledons have not been well documented and not yet completely clarified (Yedidia *et al.,* 2003; Shoresh *et al.,* 2005).

In maize systemic resistance response may be triggered both by defence related signal as SA (Morris *et al.,* 1998) or by modulation of JA/ET-responsive genes, as reported by Djonović *et al*. (2007). Recent studies shown that SAR pathways can play (Gao *et al.,* 2006) or not (Djonović *et al.,* 2006) an important role in *Trichoderma spp*. mediated resistance, nonetheless, global expression profiling in many plant-pathogen interaction primed with *Trichoderma* revealed several crosstalk between ISR and SAR signalling pathway (Glazebrook *et al.,* 2003).

In our work the *T. harzianum* T22 ability to decrease the severity of symptoms of *F. verticillioides* disease in maize was investigated. In the experiment carried out to evaluate the ability of priming to induce resistance against this pathogen, the maize roots were

primed with T22 and stalks were inoculated with *Fv*S19. Roots of primed plant resulted well colonized by *Trichoderma*. In T22-primed plants, compared to the untreated ones, a significant abatement (65%) of surface extension of necrotic areas was observed. Protective effects of T22 can also be appreciable by the reduction of necrotic area depth. Compared to T22 primed plants, lesions in T22 non-inoculated ones were deeper and hosted actively growing mycelium. According to other authors (Harman et al., 2004[b]; Shoresh *et al.*, 2005, Shoresh and Harman, 2008), the root colonization by *Trichoderma spp.* combined to its absence in shoots, evidenced that in the observed interactions among maize, T22 and *Fv*S19 a resistance phenomenon could be involved.

This hypothesis was supported also by the effect of T22 root priming in lowering the endophytic *F. verticillioides* content in stem. *Trichoderma* treated plants presented a significant reduction (31%) of endophytic pathogen density in consequence of artificial inoculation. The asymptomatic plants instead, characterized only by natural *F. verticillioides* seed-borne inoculum, presented an highly variability in contamination values. Variability among data could be due to typically observed differences in *F. verticillioides* natural infections, but another interesting hypotheses could be done. *F. verticillioides* can colonize the maize both as pathogen and as endophyte (Schulz *et al.*, 1999), since resistance response depends to specificity among BCA, host and pathogen, changes of *F. verticillioides* behaviour from pathogenicity to endophytic state could led changes also in resistance responses. As demonstrated in *A. thaliana* (Pieterse *et al.*, 2001), ISR is related to enhanced sensitivity to precise signal molecules produced after infection, lacking of this kind of signals in endophytic *F. verticillioides* could unable plants to trigger systemic defence systems at effective level.

To verify the hypothesis that T22 can stimulate resistance phenomenon and considering that there is a strong maize genetic component in plant response to *Trichoderma spp* (Harman, 2006), two enzymatic markers known to be involved in systemic defence responses of plants, PAL and LOX, were surveyed. PAL and LOX are the first enzymes involved in biosynthesis of salicylic and jasmonic acid respectively. PAL is a key regulatory enzyme in SAR pathway and his expression is reported to be activated by JA/Et signalling pathway (Diallinas and Kanellis 1994; Kato *et al.*, 2000; Shoresh *et al.*, 2005). As the first enzyme in the phenylpropanoids pathway, PAL lead to the formation of several

antimicrobial compound like phytoalexins (Hahlbrock and Scheel, 1989) and also it is the rate-limiting enzyme of lignin formation (Li *et al.,* 1991). A further involvement of PAL in induced systemic resistance triggered by PGPF may be correlated to the deposition of phenolic compounds like lignin that reinforce the cell wall structure as evidenced in cucumber (Meera *et al.,* 1995; Koike *et al.,* 2001) and other plants (Saldajenom *et al.,* 2008). Lipoxygenases (LOX) belonging to the first step of biosynthetic signalling in ISR pathway. LOX are ubiquitous enzymes converting poly unsaturated fatty acids to 9- and 13-hydroperoxides. LOX biosynthetic pathway produce a large variety of metabolites involved in ISR signalling as jasmonic acid and oxylipins (Pieterse *et al.,* 2006) as well as other oxygenated fatty acids used as substrate by other enzymes as HPL.

In our experiments, application of T22 to maize roots gave a transient elevation of PAL activity in roots and leaves at 36 hpi. Elevated activity in leaves, where T22 was absent, was the evidence that signal was translocated from roots. Initially the activity in infected plants decreased despite T22 treatment, then the same rapidly increased with a positive and synergic effect of T22 priming and *Fv*S19 infection (T+F+) while the untreated plant risen to basal level (T-F+). In leaves, PAL behaviour was the same of roots except that the decrease appeared later, and the subsequent increase in T+F+ was stronger. Results on LOX activity showed that, in absence of pathogen infection, roots treatment with T22 induces an increase in LOX activity in roots and similar effects can be visible also in leaves. In plants infected with *Fv*S19 and primed with T22 (T+F+), a clear trend was noticed only in leaves where, as observed for PAL, following an initial decrement the activity of lipoxygenase incremented.

The observed increment in PAL and LOX activity induced by T22, agreed with results obtained in cucumber with *T. asperellum* T203 (Shoresh *et al.,* 2005). One point of disagreement compared to Shoresh's work was the initial decrease of PAL and LOX activity in maize plants infected by *F. verticillioides*, despite T22 priming. This result represent an anomalous instance compared to current knowledge (Kato *et al.,* 2000; Shoresh *et al.,* 2005) that never reported such a decrement. A possible elucidation involves reactive oxygen species (ROS) that can be produced following the pathogenic infection. As reported in tomato, ROS formation promoted by low level of UV-C radiation induced a transient decrement both in PAL and LOX activity (Britt, 1995; Barka, 2001). However,

our results on enzymatic activity in T22-maize-FvS19 interaction cannot be considered definitive and further analysis must be carried out to confirm and widen our surveys.

To better elucidate the effects of T22 maize priming in resistance response, transcription of *ZmPal* and *ZmPR1*, known as SAR markers, and *ZmLox10* and *ZmHpl,* known as ISR markers, were quantified.

Transcription analysis of *ZmPal* overlaps the results of PAL enzymatic activity; this enzyme in later phases of infection process appear to be stimulated in synergy by priming and infection, according to other authors (van Wees *et al.,* 1999; Guo *et al.,* 2000) that reported a *Pal* mRNA increment during pathogen infection resistance. Moreover, in maize primed with *T. virens* and infected by *Colletotrichum graminicola,* the consistent upregulated *ZmPal* expression suggested once more the strong importance of this gene in *Trichoderma* mediated defence responses in maize (Djonović *et al.,* 2007). While for *ZmPal* the kinetic of expression resulted relatively simple to argue, *ZmLox10* didn't show a clear transcriptional profile associable to resistance induction and pathogen infection. Lacking of response of *ZmLox10* to pathogen infection is apparently in contrast with Nemchenko *et al.* (2006) findings. These authors found that *ZmLox10* transcription, a 13-LOX, was induced in response to JA, SA, wounding and *Cochliobolus carbonum* infection suggesting that this gene play an important role in both systemic acquired and induced defence reactions. Our findings about lipoxygenase transcription fully agree Djonović *et al.* (2007) in maize and Gallou *et al.* (2009) on potato. These authors working on different plants primed with *Trichoderma* and infected by pathogen found that *Lox* expression was not different between treatments.

In our work we used a different pathogen, *F. verticillioides*, and this fungus could not induce *ZmLox10* transcription. This hypothesis was supported by the works of Gao *et al.* (2007; 2009) that found that the inactivation of *ZmLox3* increases maize susceptibility to *Aspergillus* infection, while disruption of the same gene enhances resistance towards *F. verticillioides* and decreases the fumonisin production. On the basis of that findings the authors suggested that LOX-governed host-pathogen interactions are pathogen-specific.

Discrepancy in our results between LOX enzymatic activity and *ZmLox10* transcription analysis, that didn't revealed the same dynamics, could be related to the large variety of subfamily in lipoxygenase enzymes or to yet unknown regulatory effectors. In fact while

highly similar and clustered together different lipoxygenases may be induced by different "*stimuli*" (Majerana *et al.,* 2005), Weichert *et al.* (1999) working on a barley chloroplast-targeted 13-LOX reported its induction both by methyl jasmonate and SA and the same was reported by Nemchenko *et al.* (2006) in maize for *ZmLox10* transcription. This can be surprisingly since many papers reported that generally JA and SA act antagonistically in defence reactions (Pena-Cortes *et al.,* 1993; Feys and Parker, 2000; Cipollini *et al.,* 2004) but in monocot there is evidences suggesting that both ISR and SAR signal pathways simultaneously contributes to resistance mechanisms (Djonović *et al.,* 2007)

Known as ISR marker connected to LOX activity, hydroperoxide lyase (HPL) is part of the octadecanoic pathway and utilize some lipoxygenase products to produces antimicrobial and wound-related molecules. HPL products include several volatile C-6 aldehydes and alcohols called green leaf volatiles (GLVs). In defence responses to pathogens, GLVs are reported to act as signalling molecule (Bate and Rothstein, 1998; Matsui, 2006; Shiojiri *et al.,* 2006) and to have antifungal activity toward several microorganisms (Croft *et al.,* 1993; Nakamura and Hatanaka 2002; Prost *et al.,* 2005). Dynamics of transcript show that *ZmHpl* was more expressed in challenged plants and that the T22 priming was able to promote its transcription, enhancing the resistance to pathogen. A similar observation on hydroperoxide lyase gene were reported also by other authors (Yedidia *et al.,* 2003; Djonović *et al.,* 2007; Choudhary *et al.,* 2008; Shah, 2009).

Transcripts analysis of *ZmPal*, *ZmLox10* and *ZmHpl* resulted overlapping Djonović 's data found in maize-*T. virens*-*C. graminicola* interaction. In agreement with this author it's possible to suggest that also in maize-*T. harzianum* T22-*F. verticillioides* a ISR-like resistance mechanism could be involved. This hypothesis was further confirmed by *ZmPR1* transcription analysis. PR proteins, known as SAR markers, are produced in leaves in response to a pathogen and are accumulated in the intercellular fluid. While in dicotyledonous PR proteins were induced by SA, in monocotyledonous PR genes can be promoted also by JA as seen by wounding, enzymatic and hormonal treatment in rice (Agrawal *et al.,* 2000). Moreover, also in dicot an overlapping activation of PR genes by MetJA was noticed as reported in tobacco and tomato (Ding *et al.,* 2002). PRs family in maize was well characterized and *ZmPR1* was found to be induced by SA (Morris *et al.,* 1998) and involved in maize resistance response towards *F. verticillioides* infection

(Casacuberta *et al.,* 1992). In a further elucidation, Djonović *et al.* (2006) showed that in maize infected with *C. graminicola*, PR1 and PR5 level didn't change with or without *T. virens* treatment. Present work shows that, despite T22 treatments, infected plants showed an overexpression in *ZmPr1* compared to non-infected plants and then changes in *ZmPR1* expression can be related to *F. verticillioides* infection rather than T22 priming.

Our findings provide further evidences that the biocontrol agent *T. harzianum* T22 was able to induce resistance and decrease infection ratio in maize against *F. verticillioides*. We observed an activation of systemic resistance and, according to previous works, resistance was mediated by ISR pathway (De Meyer *et al.,* 1998; Shoresh *et al.,* 2005; Horst *et al.,* 2005; Ruocco *et al.,* 2007; Di Stefano *et al.,* 2008). Dynamics of gene expression suggest that the early resistance response provided an effective defence since the second day post infection, as previously reported in cucumber by application of *T. harzianum* and *T. asperellum* (Yedidia *et al.,* 1999; Yedidia *et al.,* 2003). The quickness of resistance activation against *F. verticillioides* could be useful also in field cultivation, but not ever greenhouses results can be applied to standard farming condition, so open field tests were carried out.

The well known ability of T22 to colonize maize rhizosphere (Harman *et al.,* 2004) was confirmed also in fields' condition. The effect of T22 on roots resulted in a decrement of colonization by *F. verticillioides* both in roots, silks and kernels of treated plants compared to the untreated. Only in senescent silks the abatement of inoculum was not detected, in facts senescent silks are reasonably not viable and thus defence reactions were not present. The decrement of *F. verticillioides* presence reflected on fumonisin content in kernels; T22 primed plants compared to the untreated ones showed lower 40% less concentration for both FB1 and FB2. Although this decrement could be explained with the reduction of pathogen inoculum, it is well known that plant stresses can enhance fumonisin accumulation (Bacon *et al.,* 1994; Bacon *et al.,* 2001; Yates *et al.,* 2007; Abbas *et al.,* 2006), therefore also the ability of *Trichoderma spp*. to mitigate the effects of biotic and abiotic stresses, (Dana *et al.,* 2006; Montero Barrientos *et al.,* 2008; Shoresh and Harman, 2008; Bae *et al.,* 2009), could be involved in lowering fumonisin concentration.

At our best knowledge, this is the first work investigating the temporal dynamics of early activity of defence enzyme and the expression gene responses in maize challenged by *F.*

verticillioides in presence of *T. harzianum* T22. Seed biopriming with *T. harzianum* was demonstrated to be interesting due to low environmental impact, low doses needed to seed treatments and the possibility to readily enhance this practice with integrated strategies of fumonisin biocontrol. Our results demonstrate that in maize T22 confers resistance against *F. verticillioides* and this ability can be useful exploited in field to control of fumonisins accumulation cultivated in operative farm conditions.

References

Abbas, H.K., Cartwright, R.D., Xie, W., Shier W.T.. 2006. Aflatoxin and fumonisin contamination of corn (maize, *Zea mays*) hybrids in Arkansas. Crop Protection. 25, 1-9

Agrawal G.K., Jwa N.S., Rakwal R. 2000. A Novel Rice (Oryza sativa L.) Acidic PR1 gene highly responsive to cut, phytohormones, and protein phosphatase inhibitors. Biochemical and Biophysical. 274, 157-165.

Anthon G.E. and Barrett D.M. 2001. Colorimetric method for the determination of lipoxygenase activity. J. Agric. Food Chem. 49, 32–37.

Bacon C.W., Hinton D.M., Richardson M.D. 1994. A corn seedling assay for resistance to *Fusarium moniliforme.* Plant Disease. 78, 302-305 .

Bacon W. and Hinton D. M. 1996. Symptomless endophytic colonization of maize by *Fusarium moniliforme*. Canadian Journal of Botany. 74, 1195–1202.

Bacon C.W., Yates I.E., Hinton D.M., Filmore M. 2001. Biological control of *Fusarium moniliforme* in Maize. Environmental Health Perspectives. 109, 325-332.

Bae H., Sicher R.C., Kim M.S., Kim S.H., Strem M.D., Melnick R.L., Bailey B.A. 2009. The beneficial endophyte *Trichoderma hamatum* isolate DIS 219b promotes growth and delays the onset of the drought response in *Theobroma cacao*. Journal of Experimental Botany. 60, 3279–3295.

Barka E.A. 2001. Protective enzymes against reactive oxygen species during ripening of tomato *(Lycopersicon esculentum*) fruits in response to low amounts of UV-C. Australian Journal of Plant Physiology. 28, 785–791.

Bate N.J., Rothstein S.J. 1998. C-6-volatiles derived from the lipoxygenase pathway induce a subset of defense-related genes. Plant Journal. 16, 561–69.

Benhamou N. and Chet I. 1996. Parasitism of sclerotia of *Sclerotium rolfsii* by *Trichoderma harzianum*, ultrastructural and cytochemicals aspects of the interaction. Phytopathology. 86, 405-416.

Bennett J.W.; Klich M. 2003. Mycotoxins. Clinical Microbiology Reviews 16, 497-516.

Bolger M., Coker R.D., Dinovi M., Gaylor D., Gelderblom M.O., Paster N., Riley R.T., Shephard G., Speijers J.A. 2001. Fumonisins safety evaluation of certain mycotoxins in food and agriculture organization of the United Nations. World Health Organization Food Additives. 47, 103– 279.

Bradford M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 72, 248-254.

Britt A.B. 1995. Repair of DNA damage induced by ultraviolet radiation. Plant Physiology. 108, 891-896.

Casacuberta J.M., 2, Raventós D., Puigdoménech P., San Segundo B. 1992. Expression of the gene encoding the PR-like protein PRms in germinating maize embryos. Molecular and General Genetics. 234, 97-104.

Causin, R., Costa, E., Rasera, R., Rossi, G., Scopel C. 2008. Chemical treatments at silk stage reduce fumonisin contamination of maize kernels. "The World Mycotoxin Forum – the Fifth Conference - The international networking conference for the food and feed industry". 124.

Cavaglieri L., Orlando J., Rodríguez M.I., Chulze S., Etcheverry M. 2005. Biocontrol of *Bacillus subtilis* against *Fusarium verticillioides* in vitro and at the maize root level. Research in Microbiology. 156, 748-754.

Chen J., Harman G.E., Comis A., Cheng G. 2004. Proteins related to the biocontrol of *Pythium* damping-off in maize with *Trichoderma harzianum* Rifai. Journal of Integrative Plant Biology 47, 988-997.

Chet I., Viterbo A., Brotman Y. 2006. Enhancement of plant disease resistance by the biocontrol agent *Trichoderma***.** Life Science Open Day. Weizmann Institute of Science.

Choudhary, D.K., Johri, B.N., Prakash, A. 2008. Volatiles as priming agents that initiate plant growth and defence responses. CURRENT SCIENCE. 94, 595-604.

Cipollini D., Enright S., Traw M.B., Bergelson J. 2004. Salicylic acid inhibits jasmonic acid-induced resistance of *Arabidopsis thaliana* to *Spodoptera exigua*. Molecular Ecology. 13, 1643–1653.

Croft K.P.C., Juttner F., Slusarenko A.J. 1993 Volatile products of the lipoxygenase pathway evolved from *Phaseolus vulgaris* (L.) leaves inoculated with *Pseudomonas syringae pv phaseolicola*. PLANT PHYSIOLOGY. 101, 13-24.

Dana, M.M., Pintor-Toro, J.A., Cubero, B. 2006. Transgenic Tobacco Plants Overexpressing Chitinases of Fungal Origin Show Enhanced Resistance to Biotic and Abiotic Stress Agents. Plant Physiology. 142, 722-730

De Meyer, G., Bigirimana, J., Elad, Y, Höfte, M. 1998. Induced systemic resistance in *Trichoderma harzianum* T39 biocontrol of *Botrytis cinerea*. European Journal of Plant Pathology. 104, 279-286

Diallinas G. and Kanellis A.K. 1994. A phenylalanine ammonia-lyase gene from melon fruit, cDNA cloning, sequence and expression in response to development and wounding. Plant Molecular Biology. 26, 473-479.

Dickerson D.P., Pascholati S.F., Hagerman A.E., Butler L.G., Nicholson R.L. 1984. Phenylalanine ammonia-lyase and hydroxycinnamate, CoA ligase in maize mesocotyls inoculated with *Helminthosporium maydis* or *Helminthosporium carbonum*. Physiology Plant Pathology. 25, 111-123.

Ding C.K., Wang C., Gross K.C., Smith D.L. 2002. Jasmonate and salicylate induce the expression of pathogenesis-related-protein genes and increase resistance to chilling injury in tomato fruit. Planta. 214, 895-901.

Distefano, G., La Malfa, S., Vitale, A., Lorito, M., Deng, Z., Gentile A. 2008. Defence-related gene expression in transgenic lemon plants producing an antimicrobial *Trichoderma harzianum* endochitinase during fungal infection. Transgenic Research. 17, 873-879.

Djonović S., Pozo M.J., Dangott L.J., Howell C.R., Kenerley C.M. 2006. *Sm1*, a proteinaceous elicitor secreted by the biocontrol fungus *Trichoderma virens* induces plant defense responses and systemic resistance. Molecular Plant Microbe Interactions. 19, 838–853.

Djonović S., Vargas W.A., Kolomiets M.V., Horndeski M., Wiest A., Kenerley C.M. 2007. A proteinaceous elicitor *Sm1* from the beneficial fungus *Trichoderma virens* is required for induced systemic resistance in maize. Plant Physiology. 145, 875–889.

Elad J. 1996. Mechanisms involved in the biological control of *Botrytis cinerea* incited diseases. European Journal of Plant Pathology. 102, 719-732.

Elad, Y., David, D.R., Levi, T., Kapat, A., Kirshner, B., Guvrin, E. 1999. Modern Fungicides and Antifungal in Compounds II . Intercept Ltd. 459–467.

Elad, Y. and Kapat, A. 1999. The role of *Trichoderma harzianum* protease in the biocontrol of *Botrytis cinerea*. European Journal Plant Pathology. 105, 177–189.

Epple, P., Apel, K., Bohlmann, H. 1995. An *Arabidopsis thaliana* Thionin Gene is Inducible via a Signal Transduction Pathway Different from That for Pathogenesis-Related Proteins. Plant Physiology. 109, 813-820

Farag M.A., Fokar M., Zhang H.A., Allen R.D., Pare P.W 2005. (Z)-3-Hexenol induces defense genes and downstream metabolites in maize. Planta. 220, 900–909.

Feys B.J., Parker J.E. 2000 Interplay of signaling pathways in plant disease resistance. Trends in Genetics. 16, 449-455.

Folcher, L., Jarry, M., Weissenberger, A., Gérault F., Eychenne, N., Delos, M., Regnault-Roger, C. 2009. Comparative activity of agrochemical treatments on mycotoxin levels with regard to corn borers and *Fusarium* mycoflora in maize (*Zea mays* L.) fields. Crop Protection. 28, 302-308.

Gallou A., Cranenbrouck S., Declerck S. 2009. *Trichoderma harzianum* elicits defence response genes in roots of potato plantlets challenged by Rhizoctonia solani. European Journal of Plant Pathology. 124, 219-230.

Gao L., Smith F.A., Smith S.E. 2006. The *rmc* locus does not affect plant interactions or defence-related gene expression when tomato *(Solanum lycopersicum)* is infected with the root fungal parasite Rhizoctonia. Functional Plant Biology. 33, 289–296.

Gao, X., Shim, W., Göbel, C., Kunze, S., Feussner, I., Meeley, R., Balint-Kurti, P., Kolomiets, M. 2007. Disruption of a Maize 9-Lipoxygenase Results in Increased Resistance to Fungal Pathogens and Reduced Levels of Contamination with Mycotoxin Fumonisin. Molecular Plant-Microbe Interactions. 20, 922-933

Gao X., Brodhagen M., Isakeit T., Horowitz Brown S., Göbel C., Betran J., Feussner I., Keller N.P., Kolomiets M.V. 2009. Inactivation of the Lipoxygenase *ZmLOX3* Increases Susceptibility of Maize to *Aspergillus spp*. Molecular Plant Microbe Interactions. 22, 222-231.

Glazebrook, J., Chen W., Estes B., Chang H.S., Nawrath C., Metraux J.P., Zhu T., Katagiri F. 2003. Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. Plant Journal. 34, 217-228.

Gökmen V., Bahçeci S., Acar J. 2002. Characterization of crude lipoxygenase extract from green pea using a modified spectrophotometric method. European Food Research and Technology. 215, 42-45.

Green R. and Fluhr R. 1992. UVB-induced PR1 accumulation is mediated by active oxygen species. The Plant Cell. 7, 203-212.

Guo A.; Salih G., Klessig D. 2000 Activation of a diverse set of genes during the tobacco resistance response to TMV is independent of salicylic acid; induction of a subset is also ethylene independent. Plant Journal 21, 409-418

Hahlbrock K. and Scheel D. 1989. Physiology and molecular biology of phenylpropanoid metabolism. Annual Review of Plant Physiology and Plant Molecular Biology. 40, 347-369 .

Harman G. E. and Björkman T. 1998. Potential and existing uses of *Trichoderma* and *Gliocladium* for plant disease control and plant growth enhancement. *Trichoderma* and *Gliocladium*, Vol. 2. 229-265.

Harman G.E. 2000. Myths and dogmas of biocontrol changes in perceptions derived from research on *Trichoderma harzianum* T-22. Plant Disease. 84, 377-393.

Harman G.E., Dozelli B.G.G. 2001. Enhancing crop performance and pest resistance with genes from biocontrol agents. Enhancing Biocontrol Agents and Handling Risks. IOS Press. 114-128.

Harman G.E., Howell C.R., Viterbo A., Chet I., Lorito M. 2004[a] *Trichoderma* species -opportunistic, avirulent plant symbionts. Nature Reviews Microbiology. 2, 43-56.

Harman G.E. , Petzoldt R., Comis A., Chen J. 2004[b]. Interactions between *Trichoderma harzianum* strain T22 and maize inbred line Mo17 and effects of these interactions on diseases caused by *Pythium ultimum* and *Colletotrichum graminicola*. Biological Control. 94, 147-153.

Harman G.E. 2006. Overview of mechanisms and uses of *Trichoderma spp*. Phytopathology. 96, 190-194.

Horst, L.E., Locke, J., Krause, C.R. 2005. Suppression of *Botrytis* Blight of Begonia by *Trichoderma hamatum* 382 in Peat and Compost-Amended Potting Mixes. Plant Disease. 89, 1195-1200

Howell C.R. 2002. Cotton seedling preemergence damping-off incited by *Rhizopus oryzae* and *Pythium spp*. and its biological control with *Trichoderma spp*.. Biological Control. 92, 177-180.

Inbar J., Abramsky M., Cohen D., Chet I. 1994. Plant growth enhancement and disease control by *Trichoderma harzianum* in vegetable seedlings grown under commercial conditions. European Journal of Plant Pathology. 100, 337-346.

Kato M., Hayakawa Y., Hyodo H., Ikoma Y., Yano M. 2000. Wound-induced ethylene synthesis and expression and formation of 1-Aminocyclopropane-l-Carboxylate (ACC) synthase, ACC Oxidase, Phenylalanine Ammonia-Lyase, and Peroxidase in wounded mesocarp tissue of *Cucurbita maxima.* Plant Cell Physiology. 41, 440-447.

Ke D., Saltveit ME. 1986. Effects of calcium and auxin on russet spotting and phenylalanine ammonia-lyase activity in iceberg lettuce. Horticulture 21, 1169-1171.

Kedera C.J., Leslie J.F., Clattin L.E. 1992. Systemic infection of corn by *Fusarium moniliforme*. Phytopathology 84, 603-607.

Koike N., Hyakumachi M., Kageyama K., Tsuyumu S., Doke N. 2001. Induction of Systemic Resistance in Cucumber against Several Diseases by Plant Growth-promoting Fungi, lignification and Superoxide Generation. European Journal of Plant Pathology. 107, 523-533.

Leslie, J.F. 1991. Mating populations in *Gibberella fujikuroi* (*Fusarium* section Liseola). Phytopathology. 81, 1058-1060.

Leslie J.F., Summerell B.A. 2006. The Fusarium laboratory manual-Blackwell Publishing

Li J., Li R.Q., Yuan W.J. 1991 On the change of enzyme activities of cucumber leaf infected by *Pseudoperonospora cubensis rosws*. Acta Phytapathologica Sin. 21, 277–283 (in Chinese).

Linthorst, H.J.M., van der Does, C., Brederode, F.T., Bol, J.F. 1993. Circadian expression and induction by wounding of tobacco genes for cysteine proteinase . Plant Molecular Biology. 21, 685-694

Logrieco A., Mulè G., Moretti A., Bottalico A. 2002. Toxigenic *Fusarium* species and mycotoxins associated with maize ear rot in Europe. European Journal of Plant Pathology. 108, 597-609.

Loon, van L.C., Bakker P.A.H.M., Pieterse C.M.J. 1998. Systemic resistance induced by rhizosphere bacteria. Annual Review of Phytopathology. 36, 453-483 .

Lumsden R.D. and Locke J. C. 1989 Biological control of damping-off caused by *Pythium ultimum* and *Rhizoctonia solani* with *Gliocladium virens* in soilless mix. Phytopathology. 79, 361-366.

Lumsden R.D., Lewis J.A., Fravel D.R. 1995. Formulation and delivery of biocontrol agents for use against soilborne plant pathogens. Biorational Pest Control Agents. 166-182.

Marasas W.F. 1995. Fumonisins, Their implications for human and animal health. Natural Toxins. 3, 193-198.

Majerana W., Cai Y., Sun Q., van Wijka K.J. 2005. Functional differentiation of bundle sheath and mesophyll maize chloroplasts determined by comparative proteomics. The Plant Cell 17, 3111-3140.

Mao W. , Lumsden R.D. , Lewis J.A. , Hebbar P.K. 1998. Seed treatment using pre-infiltration and biocontrol agents to reduce damping-off of corn caused by species of *Pythium* and *Fusarium*. Plant Disease. 82, 294-299.

Matsui K. 2006 Green leaf volatiles, hydroperoxide lyase pathway of oxylipin metabolism. Current Opinion in Plant Biology. 9, 274–280.

Meera M.S., Shivanna M.B., Kageyama K., Hyakumachi M. 1995. Persistence of induced systemic resistance in cucumber in relation to root colonization by plant growth promoting fungal isolates. Crop Protection. 14, 123-130.

Miller J.D. 2001. Factors that affect the occurrence of fumonisin. Environmental Health Perspectives. 109, 321-324.

Montero-Barrientos, M., Hermosa, R., Nicolás, C., Cardoza, R.E., Gutiérrez, S., Monte, E. 2008. Overexpression of a *Trichoderma* HSP70 gene increases fungal resistance to heat and other abiotic stresses. Fungal Genetics and Biology. 45, 1506-1513.

Morris S.W., Vernooij B., Titatarn S., Starrett M., Thomas S., Wiltse C.C., Frederiksen R.A., Bhandhufalck A., Hulbert S., Uknes S. 1998. Induced resistance responses in maize. Molecular Plant Microbe Interactions 11, 643-658.

Mulè G., Bailey J.A., Cooke B.M., Logrieco A. 2005. Molecular diversity and PCR-detection of toxigenic *Fusarium* species and ochratoxigenic fungi. Mycopathologia. 160, 95-96.

Muller P.Y., Janovjak H., Miserez A.R., Dobbie Z. 2002. Processing of gene expression data generated by quantitative real-time RT-PCR. BioTechniques. 32, 1372-1379 .

Munkvold, G.P., McGee, D.C., Carlton, W.M. 1997. Importance of different pathways for maize kernek infection by *Fusarium moniliforme*. Phytopathology. 87, 209-217

Nakamura S., Hatanaka A. 2002. Green-leaf-derived C6-aroma compounds with potent antibacterial action that act on both Gram-negative and Gram-positive bacteria. Journal Agricolture Food Chemistry. 50, 7639-7644.

Nayaka S.C., Niranjana N.R. , Shankar A.C.U. 2008. Seed biopriming with novel strain of *Trichoderma harzianum* for the control of toxigenic *Fusarium verticillioides* and fumonisins in maize. Archives Of Phytopathology And Plant Protection. 1-19.

Nayaka, C., Shankar U., , Arakere C, Munagala, S., Siddapura, R., Harishchandra, S., Hunthrike, S., Carmen, N. 2009. Control of *Fusarium verticillioides*, cause of ear rot of maize, by *Pseudomonas fluorescens.* Pest Management Science. 65, 769-775.

Nelson, P.E. 1992. Taxonomy and biology of *Fusarium moniliforme*. Mycopathologia. 117, 29-36.

Nemchenko A., Kunze S., Feussner I., Kolomiets M. 2006. Duplicate maize 13-lipossigenase gene are differentially regulated by circadian rhythm, cold stress, wounding, pathogen infection, and hormonal treatments. Journal of Experimental Botany. 57, 3767–3779.

Ousley M.A., Lynch J.M., Whipps J.M. 1994. Potential of *Trichoderma spp*. as consistent plant growth stimulators. Biology and Fertility of Soils. 17, 85-90.

Pena-Cortés H., Albrecht T., Prat S., Weiler E.W., Willmitzer L. 1993. Aspirin prevents wound-induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis. Planta. 191, 123-128.

Pfaffl. M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic acid reserch. 29, 2000-2007.

Pieterse C.M.J., Van Pelt J.A.,. Van Wees C.M., Ton J., Léon-Kloosterziel K.M., Keurentjes J.B., Verhagen W.M., Knoester M., Van der Sluis I., Bakker A.H.M., Van Loon L.C. 2001. Rhizobacteria-mediated Induced Systemic Resistance, Triggering, Signalling and Expression. European Journal of Plant Pathology. 107, 51-61.

Pieterse C.M.J., Schaller A., Mauch-Mani B., Conrath U. 2006. Signaling in plant resistance responses, divergence and cross-talk of defense pathways. Multigenic and induced systemic resistance in plants. 166-196.

Placinta C.M., D'Mello J.P.F., Macdonald A.M.C. 1999 A review of worldwide contamination of cereal grains and animal feed with *Fusarium* mycotoxins. Animal Feed Science and Technology. 78, 21-37.

Prelusky D.B., Rotter B.A., Rotter R.G., 1994. Mycotoxins in grain. Compounds other than Aflatoxin. Toxicology of mycotoxins. 359-403.

Prost I., Dhondt S., Rothe G., Vicente J., Rodriguez M.J., Kift N., Carbonne F., Griffiths G., Esquerré-Tugayé M.T., Rosahl S., Castresana C., Hamberg M., Fournier J. 2005. Evaluation of the antimicrobial activities of plant oxylipins supports their involvement in defense against pathogens. Plant Physiology. 139, 1902-1913.

Rogers, L.A., Dubos, C., Cullis, I.F., Surman, C., Poole, M., Willment, J., Mansfield, S.D., Campbell, M.M. 2005. Light, the circadian clock, and sugar perception in the control of lignin biosynthesis. Journal of Experimental Botany. 56, 1651-1663.

Rojo F., Ferez M., Reynoso M., Torres A., Chulze S. 2007. Effect of *Trichoderma* species on growth of *Fusarium proliferatiom* and production of fumonisins, fusaproliferin and beauvericin. Mycotoxin Research. 23, 173-179.

Rozen S., Skaletsky H. 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol. Biol. 132, 365-386.

Rossi V., Battilani P., Battisti D., Scandolara A. 2004. Epidemiological aspects of *Fusarium verticillioides*, the causal agent of maize pink ear rot. Journal of Plant Pathology. 86, 331.

Ruocco, M., De Masi, L., Soriente, I., De Palma, M., D'Amore, R., Lorito, M., Tucci, M. 2007. *Trichoderma*–Plant interactions are modulated by the plant genotype. XIII IS MP-MI Congress, Sorrento. 21–27

Saldajenom.G.B., Chandanie W.A., Kubota M., Hyakumachi M. 2008. Effects of interactions of arbuscular mycorrhizal fungi and beneficial saprophytic mycoflora on plant growth and disease protection. Mycorrhizae, Sustainable Agriculture and Forestry. 211–226.

Sambrook, J., Russell, W.D. 2001. Molecular Cloning, a Laboratory Manual. CSHL Press, Woodbury NY, USA.

Schulz B., Rommert A.K., Dammann U., Aust H.J., Strack D. 1999. The endophyte-host interaction, a balanced antagonism?. Mycological Research. 103, 1275-1283.

Sivasithamparam K., Ghisalberti E.L., 1998. Secondary metabolism in *Trichoderma* and *Gliocladium*. *Trichoderma* and *Gliocladium*.

Shah, J. 2009. Plants under attack: systemic signals in defence. Current Opinion in Plant Biology. 12, 459-464.

Sharma, R.C., De Leon, C., Payak, M.M. 1993. Diseases of maize in South and South-East Asia: problems and progress. Crop Protection. 12, 414-422

Shi Z., Wang F., Zhou W., Zhang P., Yong J.F. 2007. Application of osthol induces a resistance response against powdery mildew in pumpkin leaves. International Journal of Molecular Sciences. 8, 1001-1012.

Shiojiri K., Kishimoto K., Ozawa R., Kugimiya S., Urashimo S., Arimura G., Horiuchi J., Nishioka T., Matsui K., Takabayashi J. 2006. Changing green leaf volatile biosynthesis in plants, An approach for improving plant resistance against both herbivores and pathogens. PNAS 103, 16672-16676.

Shoresh M., Yedidia I., Chet I. 2005. Involvement of jasmonic acid/ethylene signaling pathway in the systemic resistance induced in cucumber by *Trichoderma asperellum* T203. Biological Control. 95,76-84.

Shoresh M. and Harman G.E. 2008. The molecular basis of shoot responses of maize seedlings to *Trichoderma harzianum* T22 inoculation of the root, a proteomic approach. Plant Physiology. 147, 2147-2163.

Sivan A. and Harman G.E. 1991. Improved rhizosphere competence in a protoplast fusion progeny of *Trichoderma harzianum*. Journal of General Microbiology. 137, 23-29.

Sobek E.A., Munkvold G.P. 1999. European corn borer (Lepidoptera, Pyralidae) larvae as vectors of *Fusarium moniliforme*, causing kernel rot and symptomless infection of maize kernels. Journal of economic entomology 92, 503-509.

Sobowale A.A., Cardwell K.F., Odebode A.C. 2007. Persistence of *Trichoderma* species within maize stem against *Fusarium verticillioides*. Archives of Phytopathology and Plant Protection. 40.

Tuite, J., 1969. Plant Pathological Methods, Fungi and Bacteria. Burgess Publishing Company, Minneapolis, MN, USA.

Vainio H., Heseltine E., Wilbourn J. 1993. Report on an IARC working group meeting on some naturally occurring substances. International Journal of Cancer. 53,535 – 537.

Vieira A., Goreti M. , Chamel I.J., Piovesan N.D., De Rezende S.T., Moreira M.A., De Barros E. G., 2001. Biochemical evalutation of lipoxygenase pathway of soybean plants submitted to wounding. Revista Brasileira de Fisiologia Vegetal. 13, 4-12.

Vinale F., Sivasithamparam K., Ghisalberti E.L., Marra R., Barbetti M.J., Li H., Woo S.L., Lorito M. 2008. A novel role for *Trichoderma* secondary metabolites in the interactions with plants. Physiological and Molecular Plant Pathology. 72, 80-86.

Wees, Van S.C.M., Luijendijk M., Smoorenburg I., Van Loon L.C. and Pieterse C.M.J. 1999 Rhizobacteria-mediated induced systemic reistance (ISR) in *Arabidopsis* is not associated with a direct effect expression of know defence-related genes but stimulates the expression of the jasmonate-inducibile genes *Atvsp* upon challenge. Plant Molecular Biology. 41, 537-549.

Weichert H., Stenzel I., Berndt E., Wasternack C., Feussner I. 1999 Metabolic profiling of oxylipins upon salicylate treatment in barley leaves — preferential induction of the reductase pathway by salicylate. FEBS. 464, 133-137 .

Wicklow D.T., Horn B.W., Shotwell O.L., Hesseltine C.W., Caldwell. R.W. 1988. Fungal interference with *Aspergillus flavus* infection and aflatoxin contamination of maize grown in a controlled environment. Phytopathology. 78, 68-74.

Windham M.T., Elad Y., Baker R. 1986. A mechanism for increased plant growth induced by *Trichoderma spp.* Phytopathology. 76, 518-521.

Woo, S.L., Scala, F., Ruocco, M., Lorito, M. 2005. The Molecular Biology of the Interactions Between *Trichoderma* spp., Phytopathogenic Fungi, and Plants. Symposium The Nature and Application of Biocontrol Microbes II: Trichoderma spp.

Yates, I., Sparks, D., Glenn, A. 2007. Natural and introduced *Fusarium verticillioides* populations in ears of field-grown corn plants. Phytopathology. 97, 127.

Yates I.E. and Sparks D. 2008. *Fusarium verticillioides* dissemination among maize ears of field-grown plants. Crop Protection. 27, 606-613.

Yedidia I., Benhamou N., Chet I. 1999. Induction of defense responses in cucumber plants (*Cucumis sativus L.*) by the biocontrol agent *Trichoderma harzianum*. Applied and Environmental Microbiology. 65, 1061-1070.

Yedidia I., Srivastva A.K., Kapulnik Y., Chet I. 2001. Effect of *Trichoderma harzianum* on microelement concentrations and increased growth of cucumber plants. Plant and Soil. 235, 235-242.

Yedidia I., Shoresh M., Kerem Z., Benhamou N., Kapulnik Y., Chet I. 2003. Concomitant Induction of Systemic Resistance to *Pseudomonas syringae pv. lachrymans* in Cucumber by *Trichoderma asperellum* (T-203) and Accumulation of Phytoalexins. Applied and Environmental Microbiology. 69, 7343-7353.

Zimand G., Elad Y., Chet I. 1996. Effect of *Trichoderma harzianum* on *Botrytis cinerea* pathogenicity. Phytopathology. 86, 1255 1260.

Chapter 3 – ROS and fumonisins regulation

Changes in fumonisin biosynthesis by *F. verticillioides* in response to oxidative stress by H_2O_2

Davide Ferrigo[1], Alessandro Botton[2], Cristina Scopel[1], Amedeo Pietri[3], Roberto Causin[1]*

[1]T.E.S.A.F.– Department of Land Use and Agro-Forestry Syestems – Plant Pathology, University of Padova, Campus of Agripolis - Viale dell'Università 16, 35020 Legnaro (Padova).

[2]Department of Environmental Agronomy and Crop Science, University of Padova, Campus of Agripolis - Viale dell'Università 16, 35020 Legnaro (Padova), Italy.

[3] Istituto di Scienze degli Alimenti e Nutrizione, Università Cattolica del Sacro Cuore, Via E. Parmense 84, 29100 Piacenza, Italy

*Corresponding author, Roberto Causin, Department of Land Use and Agro-Forestry Syestems – Plant Pathology; University of Padova, Campus of Agripolis - Viale dell'Università 16, 35020 Legnaro (Padova), Italy. Fax +39 049 8272885. E-mail, roberto.causin@unipd.it

Abstract

Plant responses to both biotic and abiotic stresses are characterized by the rapid and transient releasing of reactive oxygen species. Since oxidative stress can be considered a "prerequisite" for mycotoxins production, it can be hypothesise that ROS produced by plant can modulate mycotoxin production in host tissues. With the aim to clarify the impact of oxidative stresses on fumonisin production, in vitro cultures of Fusarium verticillioides were supplemented with different concentrations of hydrogen peroxide (H_2O_2). Differential fumonisins production in response to H2O2 were noticed. A 0,5 mM concentration enhance 2,5 fold fumonisin accumulation while stronger oxidative stresses partially inhibit fumonisin production. Furthermore, transcriptional analysis revealed a general up regulation of genes involved in regulation and biosynthesis of fumonisins according to mycotoxin accumulation. Our data confirm the strong importance of oxidative stresses in modulation of fumonisin pathways.

Introduction

The most common fungus associated with seed is *Fusarium verticillioides* (Saccardo) Nirenberg, this can be found in association with a large number of kernels but rarely cause problems with seed germination, however it has been shown to cause severe stalk rot in plants and accumulation of fumonisins in kernels (White, 1999). Fumonisins are known to cause pulmonary oedema, and neural disorders in swine and horses (Prelusky *et al.,* 1994), and is carcinogenic in experimental rodents, (Gelderblom *et al.,*1988; Howard *et al.,* 2001; Bennett and Klich, 2003). Human consumption of fumonisin contaminated maize has been linked to elevated rates of oesophageal cancer and fumonisin B1 itself (Fig. 1) has been evaluated as a group 2B by IARC (Vainio *et al.,* 1993). *F. verticillioides* develops in maize in different way: as saprophyte in senescent tissues and debris (Bullerman, 1996), as endophyte, or in particular climatic conditions as pathogen.

F. verticillioides is a pathogen characterized by low virulence and whereas is not endophytically seed-borne (Bacon and Hinton, 1996), it can penetrate in plants through roots (Kedera *et al.,* 1992; Lumsden *et al.,* 1995), corn borer lesions (Placinta *et al.,* 1992; Sobek and Munkvold, 1999; Miller, 2001) or environmental damage (Wicklow *et al.,* 1988). Attacks by phytophagous and environmental damages not only promote *F. verticillioides* infection but can also cause several stress reactions in plant.

In response to biotic and abiotic stresses these different threats, plants developed a broad range of strategies to protect themselves. Plant responses can include the rapid and transient releasing of reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (HO^-) and lipoperoxides (LOOH), these latter are formed from unsaturated fatty acids or during metabolic processes (Kappus, 1985; Sies, 1985; Belozerskaya and Gessler, 2007). High intracellular levels of H_2O_2 lead to the activation of plant cell death and of defence mechanisms against pathogenic organisms (Takahashi *et al.,* 1997). Both in biotic stress to restrict pathogen growth (Repka, 1999; Kachroo *et al.,* 2003, Zhou *et al.,* 2004) and in abiotic stress as part of signalling pathway (Zhao *et al.,* 2001; Apel and Hirt, 2004; Torres and Dangl, 2005) plant triggers an `oxidative burst'. Reactive oxygen species formation works in plant as signalling pathway and response strategy but

have been noticed to act also as inducer in biosynthesis of some mycotoxin. (Jayashree and Subramanyam, 2000; Ponts *et al.,* 2006)

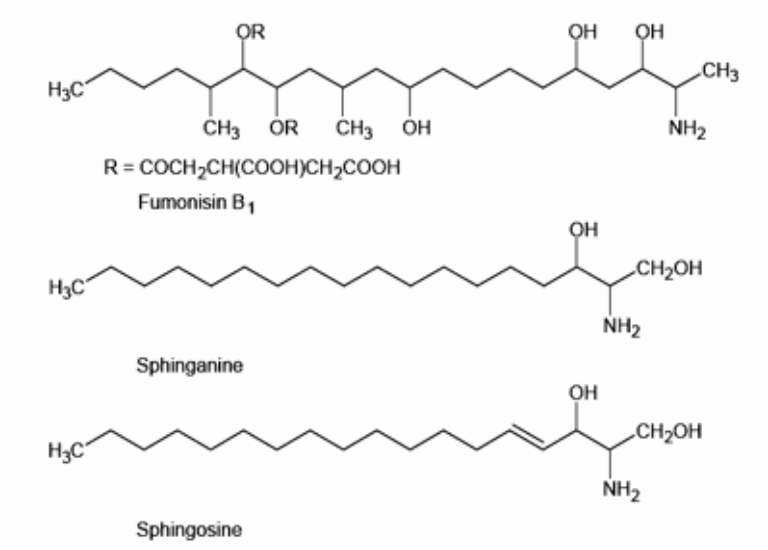


Figure 1: Structural form of fumonisin B1 and analogous molecules constituent of the sphingolipids. FB1 explicates its toxic activity interfering on sphinganine and sphingosine metabolism blocking ceramide synthase enzyme.

Also in mycotoxigenic fungi in consequence to abiotic stress due to unfavourable temperature, *aw* and pH, a strong influence on expression of mycotoxin biosynthesis genes was observed (Schmidt-Heydt *et al.,* 2008). This influence have been hypothesised to be related to compounds as ROS able to activate and modulate gene expression in mycotoxin biosynthesis (Aguirre *et al.,* 2005). *A. parasiticus* strains indicated a positive correlation between aflatoxin production and reactive oxygen species accumulation (Reverberi *et al.,* 2007, Pont *et al.,* 2006). Differential modulation of toxin biosynthesis have been speculated to be linked to the fungal ability to protect themselves from oxidative damage (Ponts *et al.,* 2007). While for *A. flavus* (Jayashree and Subramanyam, 2000), and *F. graminearum* (Boutigny *et al.,* 2008) intracellular oxidative stress can be considered a "prerequisite" for respectively aflatoxin and trichotecenes production, the same author hypothesize that plant compounds involved in plant–fungi interactions as neo-formed ROS are able to interfere with mycotoxin production in host tissues (Boutigny *et al.,* 2008). To our knowledge no studies have been conducted on fumonisin production to verify a similar behaviour than

trichotecenes. The objectives of this study were to determine the *in vitro* influence of oxidative stresses produced by H_2O_2 supplementation on fumonisins accumulation. In particular were surveyed the genes *fcc1* (cyclin like C-type), *zfr1* (transcription factor), *fum1* (polyketide synthase), *fum6* and *fum15* (cytochrome P450 monooxygenases), *fum10* and *fum16* (acyl-CoA synthetase), *fum14* (peptide synthase) and *fum19* (ABC-transporter) involved in both regulatory and biosynthetic pathway.

Material and methods

Strains and culture media

The *F. verticillioides* strains used for the present work, belong to a pool of *Fusaria* isolated in maize kernels grown in Veneto Region. After morphological identification (Leslie and Summerel, 2006), from the *F. verticillioides* isolates monoconidial cultures were obtained as reported by Tuite *et al.* (1969). Monoconidial isolates were subcultured in PDB liquid medium at 25° C for 7 days, the mycelium was extracted and the genomic DNA was used for molecular identification with primers VER1 and VER2 coding calmodulin (Mulè *et al.,* 2004) to confirm the morphological classification. After molecular identification the monoconidial strains were grown on GYAM liquid media (8 mM *L*-asparagine, 1.7 mM NaCl, 4.4 mM K_2HPO_4, 2 mM $MgSO_4$, 8.8 mM $CaCl_2$, 0.05% yeast extract, 0.24 M glucose and 5.0 mM malic acid; pH 5.0) (Seo *et al.,* 2001) and their ability to produce fumonisin was quantified by Competitive I.P.S Fumonisin kit for quantitative ELISA (Generon, Modena, Italy) following the manufacturer's instructions. Among the characterized strains for fumonisin production, a medium-producer as the 8B strain (named *Fv*8B) was selected. For the experiments, inoculum was prepared. *Fv*8B strain was grown on a Petri dish with PDA at 25 °C in dark condition for eight days. Spores suspension was prepared by adding sterile distilled water and gently shaken. Spore concentration was determined by count with Thoma cell and adjusted to 10^8 cfu/ml. Experiments were performed in PDB as non fumonisin permissive medium and in GYAM as permissive medium. The fungal strain was grown in PDB to evaluate the constitutive gene expression in non-inducing condition, instead the experiments of H_2O_2 supplementation were performed on GYAM liquid medium.

Growth conditions and H_2O_2 supplementation

Seventy ml of GYAM or PDB medium in a 250 ml-Erlenmeyer were inoculated with 10 µl of a conidial solution 10^8 cfu/ml of Fv8B. Flasks were incubated at 25 +-0.1 °C in dark condition with shaking at 150 rpm for 24 days. At the time of inoculation (T_0) and daily, cultures in GYAM medium were supplemented with H_2O (control) or H_2O_2 at the concentrations of 0.5mM, 2.0mM and 5mM. Cultures grown in both GYAM and PDB were collected at days 3, 4, 5, 6 and every three days from 6dpi to 24 dpi. Solid and liquid phase separation were obtained with a centrifugation cycle at 3000 rpm for 5 min. Culture broths were filtered under vacuum through Whatman paper no. 4, (Ponts *et al.,* 2006) and stored at -20° C until analysis. Mycelium were immediately frozen in liquid nitrogen and stored at -80° C before mRNA extraction. Replicates of fungal mycelium were dried and weighted to check if treatments affected biomass growth. Broths were analyzed for their pH values to verify if H_2O_2 supplementation did not modify the pH values compared to which of the untreated samples. Each experiment was done in triplicate.

FBs extraction and HPLC analysis.

HPLC grade acetonitrile and acetic acid were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q apparatus from Millipore (Milford, MA, USA). FB1 and FB2 standards were obtained from Sigma-Aldrich (St. Louis, MO, USA); FB3 solution standard (50 µg ml-1) was purchased from Biopure (Tulln, Austria). Fumonisins (FBs) were separately dissolved in 10 ml acetonitrile:water (1:1 v/v); the concentration was calculated using the weight indicated by the manufacturer. The solutions of FBs were diluted to obtain HPLC calibrant solutions in acetonitrile:water (30:70 v/v) at individual concentrations between 2.5 and 50 µg l^{-1}

Samples at 3, 6, 9, 12, 15 and 18 dpi were centrifuged at 3000 g for 5 min., then diluted (0.1 ml brought to 1 ml) with acetonitrile:water (30+70 v/v). Analysis was carried out using a HPLC-MS/MS system, consisting of a LC 1.4 Surveyor pump, a Quantum Discovery Max triple-quadrupole mass spectrometer (Thermo-Fisher Scientific, San Jose, CA, USA) and a PAL 1.3.1 sampling system (CTC Analitycs AG, Zwingen, Switzerland); the system was controlled by an Excalibur 1.4 software (Thermo-Fisher). FBs were separated on a Betasil RP-18 column (5 µm particle size, 150x2.1 mm, Thermo-Fisher) with a mobile-

phase gradient acetonitrile-water (both acidified with 0.4% acetic acid) from 25:75 to 55:45 in 9 min., then isocratic for 3 min.; the flow rate was 0.2 ml min-1. The ionisation was carried out with an ESI interface (Thermo-Fisher) in positive mode as follows: spray capillary voltage 4.0 kV, sheath and auxiliary gas 35 and 14 psi, respectively, temperature of the heated capillary 270 °C. For fragmentation of [M+H]+ ions (722 m/z for FB1, 706 m/z for FB2 and FB3), the argon collision pressure was set to 1.5 mTorr and the collision energy to 36 V. The selected fragment ions were: 704, 352 and 334 m/z for FB1, 688, 336 and 318 m/z for FB2 and FB3. Quantitative determination was performed using a LC-Quan 2.0 software.

Extraction of total RNA and preparation of cDNA

Thirty mg of fresh mycelium were grounded in a mortar with liquid nitrogen and total RNA extracted using Rna Mini Spin Kit (GE Healthcare, Chalfont St. Giles, UK) following the manufacturer instructions. Ten μg of total RNA were treated with 10 U of RQ1 RNase free DNase (Promega, Milano, Italy) and 2 Unit of RNasin® Ribonuclease Inhibitor (Promega, Madison, USA) for 30 min, then purified by phenol–chloroform extraction and precipitated in isopropyl-alcohol as described by Sambrook and Russel (2001). The rate and the quality of RNAs was verified by spectrophotometer analysis (Biomate 3, Thermo Scientific, Waltman, WA, USA) and the integrity checked by agarose gel electrophoresis (1%, 5ul charged for each pit). RNAs were extracted from 3 to 6 dpi but, on the basis of its amount, purity and integrity, the 4dpi RNA was chosen for the subsequent analysis. cDNA was synthesized from 2 μg of DNA-free total RNA using the Reverse Transcription System (Promega, Madison, USA) according to the manufacturer's instructions.

Primer design and Real-time PCR analysis

The abundance of the transcripts of the genes *Zfr1*, *Fcc1*, *Fum1*, *Fum6*, *Fum10*, *Fum14*, *Fum15*, *Fum16* and *Fum19* was evaluated.. Sequences for gene-specific primer pairs were obtained from published sequences available in NCBI database using software Primer3 v0.4.0 (available at http://frodo.wi.mit.edu/primer3/. Accession number of sequences utilized were:AF155773.5 for *Fum* genes, AY493199.1 for *Zfr1*, AY393790.1 for *Fcc1* and AM933132.1 for *Tub2*. Primer specific sequences designed were checked before use with

PerlPrimer software (available at http://perlprimer.sourceforge.net/download.html) to confirm absence of dimerization between primers. In this work tubulin sequence was used as internal control. Expression analysis were performed by Real-Time PCR 7500 Applied Biosystem using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). One tenth of cDNA was used to optimize PCR amplification and primers efficiency Each amplification was conducted according to the manufacturer's instructions with a primer concentration of 200 nM for 40 cycles. Each cycle was composed by 95°C for 15 s, following by annealing and extension time at 58° C for 40 s, finally 35 s for data collection according to the melting product temperatures. A final extension step of 72°C for 4 min was employed. Each sample was analyzed in triplicate. The absence of dimer formation was checked in no-template samples by product dissociation analysis and electrophoretic separation in agarose gel. Transcript expressions were analyzed with ΔCt (cycle threshold) method according to Pfaffl adaptation (Pfaffl, 2001) using Q-Gene software (Muller *et al.,* 2002). The expression of β-tubulin (β-tub) was used as an endogenous reference to normalize raw expression data. Primer sequences and melting temperatures for the target gene are given in Table 1. An ANOVA test ($\alpha = 0.05$) was performed to detect statistical differences in gene expression between treatments. Multiple comparison tests were performed according to the Student–Newman–Keuls test on the treatment means.

Primer	Sequence	T_m primer °C
*Tub2*_F	5’-ACT CCG ATC TCC GAA AGC-3’	58
*Tub2*_R	5’-TCT GTT GGG TCA ACT CAG G-3’	61
*Fcc1*_F	5’-AGA GCA CTT CAG GAG CAG-3’	54
*Fcc1*_R	5’-CTG TCA ACC AGC CAT TTC G-3’	60
*Zfr1*_F	5’-TAT CAA CGA CCT CTA CAA CAG C-3’	57
*Zfr1*_R	5’-TCT CAA GAA ATT CGG CAG GT-3’	60
*Fum1*_F	5’-ACC ATC CCT TTC TTC TCC AG-3’	58
*Fum1*_R	5’-AAA CAC CTG CTT TGA ACC AG-3’	58
*Fum6*_F	5’-ACA GAA TCC CGT CGA CAG-3’	57
*Fum6*_R	5’-AGA AGC AGA ATG ACA GGA GAC-3’	56
*Fum10*_F	5’-TAC TTG TGC TTC CAG AGG C-3’	57
*Fum10*_R	5’-CCA ACC GAG ATT CCG AGA G-3’	61
*Fum14*_F	5’-CAG AAT CCT GAG CGT CCT-3’	56
*Fum14*_R	5’-AGT TCA CCA CCT ACC ACA G-3’	60
*Fum15*_F	5’-TGC GAA TAA CTT CAA TCC CGA-3’	63
*Fum15*_R	5’-ATA GTT GCT CCG TGC CTC-3’	56
*Fum16*_F	5’-GTG AAG CTC GTC TCT ATT CCC-3’	58
*Fum16*_R	5’-CAT CAA ACT CGC CAA TGT CG-3’	63
*Fum19*_F	5’-CAG TCA CGT CAA AGA AAC TCC-3’	57
*Fum19*_R	5’-TCT GTC ATA CAC TAC TCC AAG C-3’	55

Table 1: Primer sequences and melting temperatures for gene surveyed involved in fumonisin biosynthesis.

Results

Effect of H_2O_2 on and fumonisin production.

*Fv*8B samples grown in PDB were used to quantify constitutive gene expression in non inductive medium, these data were in turn used as reference in comparison to samples grown in inductive medium supplemented with different amount of H_2O_2. Previous works and preliminary tests showed that in our condition, detectable fumonisin production started from the third dpi to reach the maximum at the third week post inoculation. Samples collected at 3, 6, 9, 12, 15 and 18 dpi were quantified by HPLC, total fumonisins yields are presented in graph as accumulation rate (Figure 1). Equations resulted from data are showed in Table 2. At 6 dpi fumonisin content were very different between treatments. Mycotoxin yields at the end of sampling (T_{18}) were 210 ppb for PDB medium and 2120 ppb, 3160 ppb, 5360 ppb respectively for *Fv*8B cultures grown on GYAM medium with H_2O, H_2O_2 2 mM and H_2O_2 0.5 mM. In concomitance to fumonisin analysis, the growth rates and pH of fungal cultures were surveyed excluding other factors, than H_2O_2 supplementation, affecting mycotoxin accumulation. Results confirmed that PDB is a non-inductive medium and therefore accumulated only a low amount of fumonisin. Different results were collected on GYAM medium: daily supplementations of H_2O_2 0.5mM and 2 mM permitted *Fv*8B growth and promoted fumonisin production. Mycotoxin quantification showed that both H_2O_2 concentrations were able, compared to the untreated mycelia, to induce fumonisin biosynthesis at least to 110% and 40% for 0.5mM and 2mM respectively. The initial supplementation (T_0) of 5 mM H_2O_2 completely suppressed mycelial growth, therefore we have no data for this experiment. However, in a parallel experiment, *Fv*8B was grown for three days before H2O2 supplementation, in this conditions cultures resisted to 5mM and 10 mM H_2O_2 concentration although it suffered from a consistent reduction of growth rate. Furthermore, the addition of H_2O_2, agreed with concentration and age, caused an increment of mycelia pigmentation, in particular pigments violet-red typical of strain 8B presented higher intensity for 2mM (data not shown).

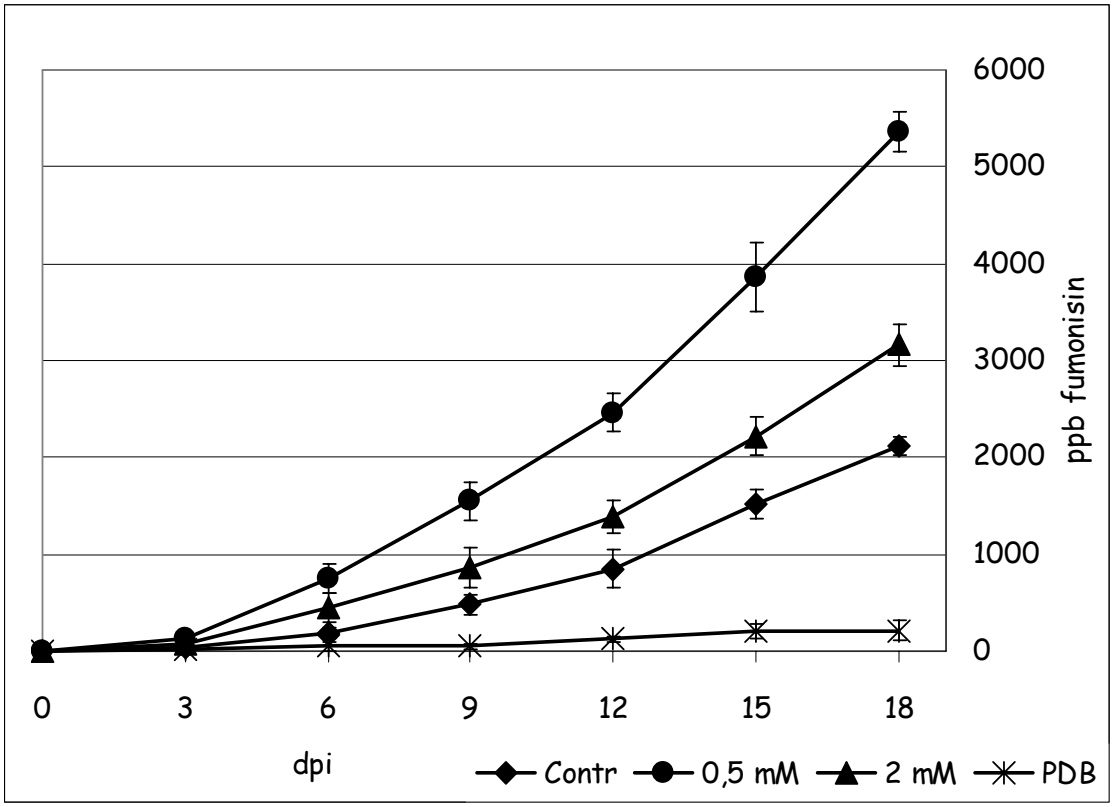


Figure. 1: Fumonisin accumulation over time (3, 6, 9, 12, 15 and 18 days) of Fv8B strain as affected in sequence by H_2O, H_2O_2 0.5 mM and H_2O_2 2mM supplementation or grown in non-inductive medium. Values and Standard Error was calculated on the basis of three replicates.

Treatment	Fumonisin rate slope	R^2
PDB	13.15 x	0,915
H_2O	119,05 x	0,985
H_2O_2 0,5 mM	299,64 x	0,936
H_2O_2 2 mM	169,64 x	0,935

Table 2: Fumonisin rate slope referred to the different growth conditions and treatments.

Expression of fumonisin-related genes after H_2O_2 treatment

To investigate if genes of fumonisin biosynthesis were actively modulated from oxidative stress, expressions of some of them were surveyed. Expression profile of surveyed genes is referred to 4dpi values, samples in which the transcript abundances were better. in relation to abundance of transcript. *Fv*8B grown on PDB presented transcriptional level always lower (from ~1.5-fold for *Fum15* to ~50-fold for *Zfr1*) than the other samples grown on GYAM (Figure 2). As far as are concerned to fungi grown in H_2O_2 supplemented GYAM, transcriptional level of *Fum* genes compared to β-tubulin varied from 6.49E-03 for *Fum1* to 4.16E+02 for *Fum16*. Transcriptional profile showed that H_2O_2 supplementations modulate gene expression with different effects according to genes and ROS concentrations. As general aspect, the transcription of genes involved in fumonisin biosynthesis were globally enhanced by H_2O_2 supplementation. However, some genes as *Zfr1*, *Fcc1*, *Fum1* and *Fum19* were particularly sensitive to H_2O_2 amount and when samples were supplemented with the higher concentration, transcription levels decrease to untreated mycelia as happened for *Fcc1* and *Fum19*. In comparison to lower concentrations, the H_2O_2 increase seemed to be ineffective for the other genes or, as evidenced for *Fum6* and *Fum10*, can further promote gene expression.

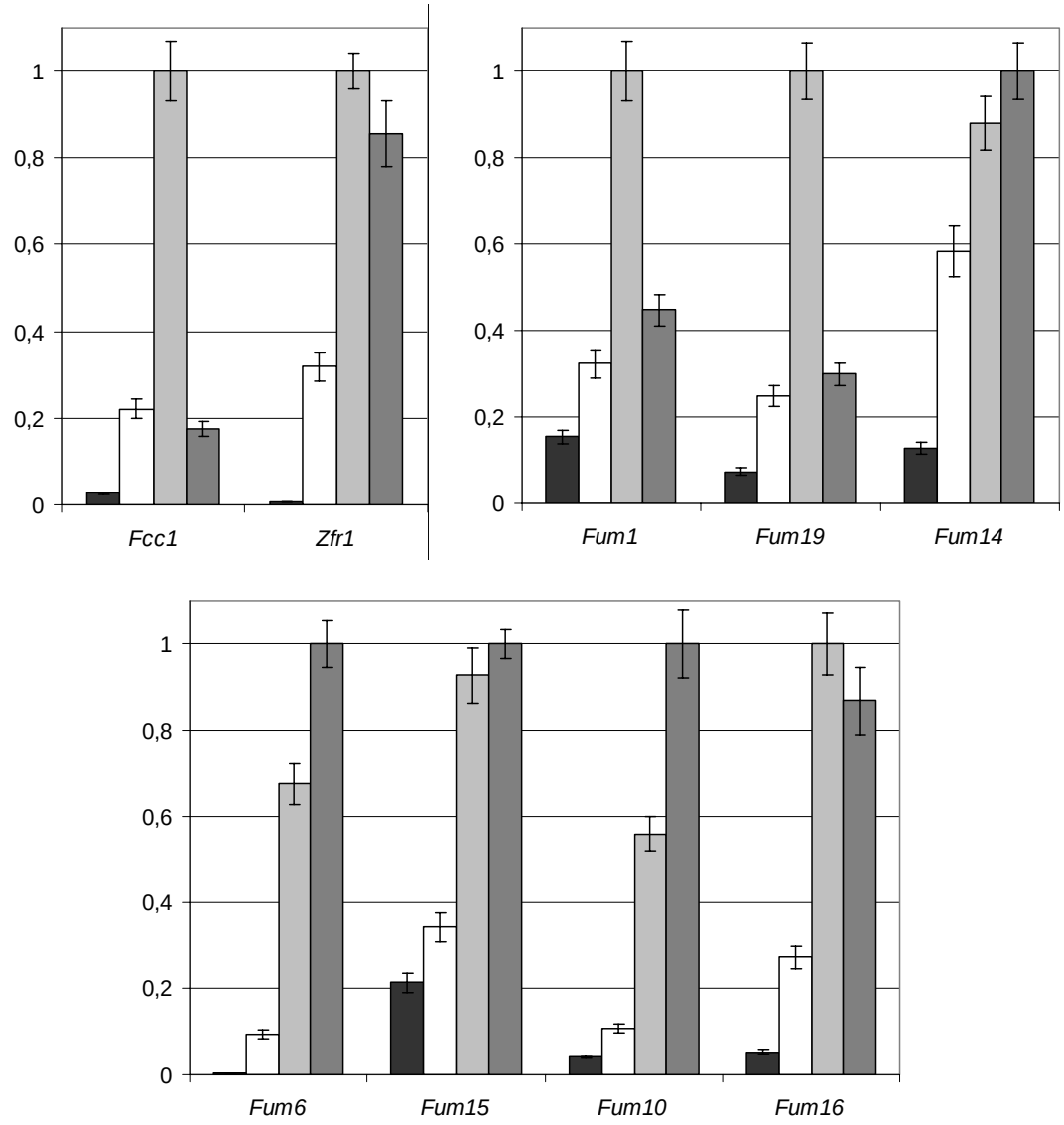


Figure 2: The genes involved in fumonisin biosynthesis are upregulated in H_2O_2-treated liquid cultures of *F. verticillioides*. Relative expression levels of genes in non-inductive medium (black bars), H2O-treated samples (white bars), 0.5mM H2O2-treated samples (light grey bars) and 2mM H2O2-treated samples (dark grey bars) are normalized to the higher value. Mean values and Standard Errors was calculated on the basis of three replicates.

Discussions and Conclusions

Fusarium verticillioides is a pathogenic fungus for maize and it represents a threat both for damages to crop and for their ability to produce fumonisin. *F. verticillioides* can colonize maize plants as endophyte and it can turn into pathogen as a consequence of plant stress conditions (Schulz *et al.,* 1999). Although *F. verticillioides* can produce mycotoxin in endophytic state, fumonisin accumulation occurs prevalently in saprophytic and pathogenic state (Rheeder *et al.,* 1992; Ross *et al.,* 1992; Munkvold *et al.,* 1997; Bacon *et al.,* 2008). As reported by Schulz *et al.* (1999), endophytism can be dependent to balanced factors in interaction and association with host. The conditions that turn *F. verticillioides* from endophytic to pathogenic state are biotic or abiotic plant stresses, closely related with the release of reactive oxygen species, H_2O_2 among other. In *Aspergillus parasiticus* and *A. flavus* a correlation has been known to exist among fungal cell oxidative stress, free-radical formation, lipoperoxidation, and aflatoxin biosynthesis (Fabbri *et al.,* 1983; Fanelli *et al.,* 1983; Fanelli *et al.,* 1984; Kappus, 1985; Sies, 1985; Fanelli *et al.,* 1989). Intracellular oxidative stress are prerequisite for mycotoxin production not only in *A. parasiticus* and *A. flavus* (Jayashree and Subramanyam, 2000) but also in *F. graminearum* (Ponts *et al.,* 2006), in *F. verticillioides* the ROS influence in fumonisin production is not demonstrated but it is presumed as scavenger enzyme and antioxidant compounds that detoxify fungal cells are in the meantime able to decrease fumonisin biosynthesis (Reynoso *et al.,* 2002).

In this work the influence of oxidative stresses on *F. verticillioides* was verified on fumonisin production and expression of fumonisin related-genes. Different concentrations of H_2O_2 were used, we tested the concentration of 0.5 mM, the same used for F. graminearum (Ponts *et al.,* 2006; 2007; 2009), 2 mM and 5 mM. Whereas 5mM seemed to be incompatible with *Fv*8B development, 0.5 and 2mM allowed fungal growth and differently stimulate fumonisin production. HPLC analysis revealed that daily supplementation with H_2O_2 0.5 mM was the most effective in enhancing mycotoxin accumulation, followed by 2mM concentration and H_2O treatment. Fungal reaction to scavenging hydrogen peroxide involves several enzymes to maintain ROS at physiological level and to repair cellular damage (Fridovich, 1998) Contemporarily, oxidative stress changes fungal metabolism leading to the arrest of growth and to the synthesis of protective

proteins and secondary metabolites in fungal cells. The overexpression of global protective compounds is an high energy-consuming process (Carlsen *et al.,* 1996) but energy availability could not be guaranteed as strong oxidants can react with lipid side chains of the mitochondrial membrane, causing malfunctioning of respiratory chain (Møller, 2001). We may hypothesise that also for *F. verticillioides* higher oxidative stress can be related to a minor availability of energy to synthesized fumonisin. Similar results agreed with other authors findings (Baert *et al.,* 2007; Schmidt-Heydt *et al.,* 2008; Jurado *et al.,* 2008) that showed that mild stress leads to an increased production of mycotoxin by *Penicillium* and *Fusarium* species, whereas more severe abiotic stress conditions reduced biosynthesis. Expression study confirmed that H_2O_2 stimulated the expression of the surveyed biosynthetic genes for fumonisin production in *F. verticillioides*. Our results confirm what noticed for *F. graminearum* in which exogenous H_2O_2 improved deoxynivalenol production whereas nivalenol pathway were inhibited (Ponts *et al.,* 2003; 2009) and both results were correlated to overexpression of *Tri* genes (Ochiai *et al.,* 2007; Ponts *et al.,* 2007). In particular the genes differently reacted to H_2O_2 concentration, while for *Fcc1*, *Zfr1*, *Fum1* and *Fum19* the expression decrease as a consequence of ROS increase, the other genes weren't inhibited by the higher concentration and even *Fum6* and *Fum15* transcription were further promoted. Discordance in genes behaviour can be probably charged to genetic complexity in fumonisin synthesis or to differential sensitivity to oxidative stress signals. The regulatory gene *FCC1* is a cyclin c-type which protein shows similarity with UME3 in *Saccharomyces cerevisiae* (Kuchin *et al.,* 1995; Cooper *et al*., 1997; Shim and Woloshuk, 2001). FCC1 may form a cyclin-CDK with FCK1 (Bluhm *et al.,* 2006), this complex can act as a putative receptor that sense the environment or could directly regulate transcription in response to extracellular stress as seen with UME3. Recent knowledge provide information that *Fcc1* may interact with a more global regulator of gene expression, and the effect on FUM gene expression is secondary. Other receptors were found to be involved in mycotoxin regulation, Kohut *et al.* (2009) found a correlation between fumonisin production and a HOG-type MAPK pathway of *F. proliferatum*. In filamentous fungi MAPKs belonging to the HOG-type MAP kinases (Brewster *et al.,* 1993) have been found to be involved in multistress responses, including osmotic, salt, heat shock, UV and oxidative stresses (Kawasaki *et al.,* 2002; Segmüller *et al.,* 2007; Noguchi

et al., 2007; Ádám *et al.,* 2008). Results obtained in *F. verticillioides* with H_2O_2 supplementation permit to hypothesize that FCC1 can be also activated in response to oxidative stress rather than nitrogen repression or pH signalling as previously suggested (Bluhm and Woloshuk, 2006; Kohut *et al.,* 2009). ZFR1 is a putative $Zn(II)_2Cys_6$ acting as positive regulator of fumonisin biosynthesis (Flaherty *et al.,* 2004) and contains a DNA binding motif similar to that found in yeast and fungi as *Gal4* or *AflR* (Pan *et al.,* 1990; Woloshuk *et al.,* 1994). Recent works affirmed that ZFR1 may affect fumonisin biosynthesis by regulating sugar uptake or perception. (Bluhm *et al.,* 2008) We found that the two regulatory genes FCC1 and ZFR1, putatively involved in different stimuli perception were both overexpressed, this suggest a common ROS-linked triggering factor or a pleiotropic effect, further study will be carried out to confirm and clarify this hypothesis. *Fum1* encoding a polyketide synthase (PKS) required for fumonisin production and *Fum19* encoding a not essential extracellular transporter are known to be good marker for fumonisin production (López-Errasquín *et al.,* 2007). The upregulation of *Fum1* is of particular interest as this genes is known to be fundamental for fumonisin biosynthesis. Polyketide synthases are key enzymes in secondary metabolism, some PKS producing metabolites involved in oxidative detoxification, pigmentation and virulence factor are found to be responsive to H_2O_2 (Langfelder *et al.,* 1998; Brakhage *et al.,* 2005). Our data agreed with this findings, in fact transcriptions of *Fum1* and *Fum19* in samples supplemented with H2O2 were enhanced. *Fum14* encode a nonribosomal peptide synthetase (NRPS) containing two domains, peptidyl carrier protein and condensation domains involved in the esterification of fumonisins. FUM14 is responsible for the TCA esters at both C-14 and C-15 of the fumonisin backbone fundamental to confer toxicity, its lack involves the accumulation of hydrolyzed forms of fumonisin B3 and fumonisin B4 (Zaleta-Rivera *et al.,* 2004). The present results suggested an enhancement of *Fum14* transcription in presence of H_2O_2 supplementation even if not so strong in comparison to other surveyed genes. Expression profiles of couples *Fum6-Fum15* and *Fum10-Fum16* were similar as their transcripts did not decreased in response to H2O2 increase. In fumonisin biosynthetic pathway proposed only *Fum6* and *Fum10* were necessary for correct toxin formation. Both *Fum6* and *Fum15* encode a cytochrome P450 monooxygenases but while the first is essential for hydroxylation of precursors, lacking of

the latter had no observed effect (Butchko *et al.* 2006). The same behaviour was noticed for *Fum10* and *Fum16*, both encoding for an acyl-CoA synthase the first is required for tricarballylic ester formation while the latter wasn't. Expression of the two necessary genes *Fum6* and *Fum10* showed that the increase of H_2O_2 concentration promoted its transcript, *Fum15* and *Fum16* instead, appeared not linked to ROS concentration. These results may conduct to speculate on a different regulation pattern that supervise genes, due to enzymatic redundancy in fumonisin biosynthetic pathway. The processes of sporulation and secondary metabolite production have been demonstrated to share common regulatory elements in fungi (Brodhagen and Keller, 2006) however the mechanism that supervise mycotoxin regulation by oxidative stress still remains unknown. Previous work evidenced the implications of ROS in induction and control of asexual sporulation in *Neurospora crassa* (Hansberg and Aguirre, 1990) and *Fusarium verticillioides* (Brodhagen and Keller, 2006). To this latter fungus, it was demonstrated that in fungal development as conidiation, FCC1 plays an important role in FB1 biosynthesis (Shim and Woloshuk, 2001; Bluhm and Woloshuk, 2006). As previously reported *Fcc1* is probably involved in response to extracellular stress (Bluhm et al., 2006), in *F. verticillioides* the signalling pathway for both sporulation and mycotoxin production can be activated by the same stimuli involving ROS or can be present a close cross-talk in signalling pathway.

Environmental influences are demonstrated in A. flavus where genes involved in aflatoxin biosynthesis are regulated by a complex relation to various combinations of water activity and temperature (Schmidt-Heydt et al., 2009), analogously in fumonisin biosynthesis a similar regulative pattern may be present. To our knowledge, this is the first report on expression of Fum genes is studied by real-time RT-PCR in response to oxidative stress. Our data confirm the strong importance of reactive oxygen species for initiation and modulation of fumonisin biosynthesis, supporting hypothesis that oxidative stresses are a common factor for mycotoxins biosynthesis. Parallel field trials conducted with specific agricultural practice and treatments to decrease stresses showed an effective reduction of fumonisin content (Miler *et al.,* 2001; Bruns, 2003; Folcher *et al.,* 2009). The possibility that ROS play an important role in mycotoxin biosynthesis also *in planta* is supported by the low level of H_2O_2 necessary to induce fumonisin pathway and stimulate fumonisin accumulation. While for trichotecenes, different chemotype could be related to virulence

(Carter *et al.,* 2002) and different ability in detoxify H_2O_2 (Ponts *et al.,* 2009), this differential ability might also in *F. verticillioides* influences fumonisin production and therefore the virulence, since there are evidences that fumonisin cluster confers virulence in *F. verticillioides* (Glenn *et al.,* 2008). Methods to control fumonisin are mainly based on chemical strategy like pesticides and fungicides. Knowledge on interactions between ROS and toxigenic fungi could be useful to achieve control over fumonisin in maize. Data acquired in this work suggested that, to minimize fumonisin content in maize, it would be useful to apply the best agricultural practices to minimize all the possible stress conditions, both biotic and abiotic. Further study are necessary to elucidates if differences in fumonisin production among strains previously reported (Segvic and Pepeljnjak, 2003) could be also related to different ability to face oxidative stress and if fumonisin biosynthetic cluster confers better fitness to *F. verticillioides.*

References

Ádám, A.L., Gábor Kohut, D., Hornok, L. 2008. *Fphog1*, a HOG-type MAP kinase gene, is involved in multistress response in *Fusarium proliferatum*. Journal of Basic Microbiology. 48, 151-159

Aguirre, J., Ríos-Momberg, M., Hewitt, D., Hansberg, W. 2005. Reactive oxygen species and development in microbial eukaryotes. Trends in Microbiology. 13, 111-118.

Apel, K. and Hirt, H. 2004. REACTIVE OXYGEN SPECIES: Metabolism, Oxidative Stress, and Signal Transduction. Annual Review of Plant Biology. 55, 373-399.

Bacon, W., Hinton, D. M. 1996. Symptomless endophytic colonization of maize by *Fusarium moniliforme*. Canadian Journal of Botany. 74, 1195–1202.

Bacon C.W., Yates I.E., Hinton, D.M., Meredith, F. 2001 Biological control of *Fusarium moniliforme* in maize. Environmental Health Perspectives. 109, 325-332.

Bacon, C.W., Glenn, A.E., Yates, I.E. 2008. *Fusarium Verticillioides*: Managing the endophytic association with maize for reduced fumonisin accumulation. Toxin Reviews. 27, 411-446.

Baert, K., Devlieghere, F., Flyps, H., Oosterlinck, M., Ahmed, M.M., Rajković, A., Verlinden, B., Nicolaï, B., Debevere, J., De Meulenaer, B. 2007. Influence of storage conditions of apples on growth and patulin production by *Penicillium expansum*. International Journal of Food Microbiology. 119, 170-181

Belozerskaya, T. A. and Gessler, N. N. 2007. Reactive oxygen species and the strategy of antioxidant defense in fungi: A review. Applied Biochemistry and Microbiology. 43, 506-515.

Bennett, J.W., Klich, M. 2003. Mycotoxins. Clinical Microbiology Reviews 16, 497-516.

Bluhm, B.H., and Woloshuk, C.P. 2006. Fck1, a C-type cyclin-dependent kinase, interacts with Fcc1 to regulate development and secondary metabolism in *Fusarium verticillioides*. Fungal Genetics and Biology. 43, 146-154

Bluhm, B.H., Kim, H., Butchko, R.A.E., Woloshuk, C.P. 2008. Involvement of ZFR1 of *Fusarium verticillioides* in kernel colonization and the regulation of FST1, a putative sugar transporter gene required for fumonisin biosynthesis on maize kernels. Molecular Plant-Microbe Interactions. 9, 203–211

Boutigny, A.L., Richard-Forget, F., Barreau, C. 2008. Natural mechanisms for cereal resistance to the accumulation of *Fusarium* trichothecenes. European Journal of Plant Pathology. 121, 411-423.

Brakhage, A., Liebmann, B. 2005. *Aspergillus fumigatus* conidial pigment and cAMP signal transduction: significance for virulence. Medical Mycology. 43, 75-82

Brewster, J.L., de Valoir, T., Dwyer, N.D., Winter, E., Gustin, M.C. 1993. An osmosensing signal transduction pathway in yeast. Science. 259, 1760 - 1763

Brodhagen, M. and Keller, N.P. 2006. Signalling pathways connecting mycotoxin production and sporulation. Molecular Plant Pathology. 7, 285-301

Bruns, H.A. 2003. Controlling Aflatoxin and Fumonisin in Maize by Crop Management. Toxin Reviews. 22, 153-173.

Bullerman, L.B. 1996. Occurrence of *Fusarium* and fumonisins on food grains and in foods. In *Fumonisins in Food*; Jackson, L. S., DeVries, J. W., Bullerman, L. B., Eds.; Plenum Press: New York. 392, 27−38.

Butchko, R.A.E., Plattner, R.D., Proctor, R.H. 2006. Deletion Analysis of FUM Genes Involved in Tricarballylic Ester Formation during Fumonisin Biosynthesis. Journal of agricultural and food chemistry. 25, 9398-9404.

Carlsen, M., Nielsen, J., Villadsen, J. 1996. Growth and α-amylase production by *Aspergillus oryzae* during continuous cultivations. Journal of Biotechnology. 45, 81-93.

Carter, J.P., Rezanoor, H.N., Holden, D., Desjardins, A.E., Plattner, R.D., Nicholson, P. 2002. Variation in Pathogenicity Associated with the Genetic Diversity of *Fusarium graminearum*. European Journal of Plant Pathology. 108, 573-583

Cooper, K. F., Mallory, M.J., Smith, J.B., Strich, R. 1997. Stress and developmental regulation of the yeast C-type cyclin Ume3p (Srb11p/Ssn8p). EMBO J. 16, 4665-4675

Flaherty, J.E., Woloshuk, C.P. 2004. Regulation of Fumonisin Biosynthesis in *Fusarium verticillioides* by a Zinc Binuclear Cluster-Type Gene, *ZFR1*. Applied and Environmental Microbiology. 70, 2653-2659

Folcher, L., Jarry, M., Weissenberger, A., Gérault F., Eychenne, N., Delos, M., Regnault-Roger, C. 2009. Comparative activity of agrochemical treatments on mycotoxin levels with regard to corn borers and *Fusarium* mycoflora in maize (*Zea mays* L.) fields. Crop Protection. 28, 302-308.

Fridovich, I. 1998. Oxygen toxicity: a radical explanation. Journal of Experimental Biology. 201, 1203-1209.

Gatch, E.W., Hellmich, R.L., Munkvold, G.P. 2002. A Comparison of Maize Stalk Rot Occurrence in Bt and Non-Bt Hybrids. Plant Disease. 86, 1149-1155.

Gelderblom, W.C., Jaskiewicz, K., Marasas, W. F., Thiel, P.G., Horak, R.M., Vleggaar, R., Kriek, N.P. 1988. Fumonisins-novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme.* Applied Environmental Microbiology. 54, 1806-1811

Glenn, A.E., Zitomer, N.C., Zimeri, M.A., Williams, L.D., Riley, R.T., Proctor, R.H. 2008. Transformation-Mediated Complementation of a FUM Gene Cluster Deletion in *Fusarium verticillioides* Restores both Fumonisin Production and Pathogenicity on Maize Seedlings. Molecular Plant-Microbe Interactions. 21, 87-97

Hansberg, W. and Aguirre, J. 1990. Hyperoxidant states cause microbial cell differentiation by cell isolation from dioxygen. *J. Theor. Biol.* 142, 201–221.

Howard, P.C., Eppley, R.M., Stack, M.E., Warbritton, A., Voss, K.A., Lorentzen, R.J., Kovach, R.M., Bucci, T.J. 2001. Fumonisin b1 carcinogenicity in a two-year feeding study using F344 rats and B6C3F1 mice. Environmental-Health-Perspective. 109, 277-82

Jayashree, T. and Subramanyam, C. 2000. Oxidative stress as a prerequisite for aflatoxin production by *Aspergillus parasiticus*. Free Radical Biology and Medicine. 29, 981-985.

Jurado, M., Marın, P., Magan,N., Gonzàlez-Jaén, M.T. 2008. Relationship between Solute and Matric Potential Stress, Temperature, Growth, and FUM1 Gene Expression in Two *Fusarium verticillioides* Strains from Spain. Applied and Environmental Microbiology. 74, 2032–2036

Kachroo, A. He, Z., Patkar, R., Zhu, Q., Zhong, J., Li, D., Ronald, P., Lamb, C., Chattoo B.B. 2003 Induction of H2O2 in Transgenic Rice Leads to Cell Death and Enhanced Resistance to Both Bacterial and Fungal Pathogens. Transgenic Research. 12, 577-586.

Kawasaki, L., Sánchez, O., Shiozaki, K., Aguirre, J. 2002. SakA MAP kinase is involved in stress signal transduction, sexual development and spore viability in *Aspergillus nidulans.* Molecular Microbiology. 45, 1153 – 1163.

Kedera, C.J., Leslie, J.F., Clattin, L.E. 1992. Systemic infection of corn by *Fusarium moniliforme*. Phytopathology 84, 603-607.

Kohut, G., Ádám, A.L., Fazekas, B., Hornok, L. 2009. N-starvation stress induced *FUM* gene expression and fumonisin production is mediated via the HOG-type MAPK pathway in Fusarium proliferatum. International Journal of Food Microbiology. 130, 65–69

Kuchin, S., Yeghiayan, P., Carlson, M. 1995. Cyclin-dependent protein-kinase and cyclin homologs SSN3 and SSN8 contribute to transcriptional control in yeast. PNAS. 92, 4006-4010

Langfelder, K., Jahn, B., Gehringer, H., Schmidt, A., Wanner, G., Brakhage, A.A. 1998. Identification of a polyketide synthase gene (*pksP)* of *Aspergillus fumigatus* involved in conidial pigment biosynthesis and virulence. Medical Microbiology and Immunology. 187, 79-89

Leslie, J.F., Summerell, B.A. 2006. The *Fusarium* laboratory manual. Blackwell Publishing

López-Errasquín, E., Vázquez, C., Jiménez, M., González-Jaén, M.T. 2007. Real-Time RT-PCR assay to quantify the expression of *fum1* and *fum19* genes from the Fumonisin-producing *Fusarium verticillioides.* Journal of Microbiological Methods. 68, 312-317

Lumsden, R.D., Lewis, J.A., Fravel, D.R. 1995. Formulation and delivery of biocontrol agents for use against soilborne plant pathogens. Biorational Pest Control Agents. 166-182.

Miller, J.D. 2001. Factors that affect the occurrence of fumonisin. Environmental Health Perspectives. 109, 321-324.

Møller, I.M. 2001. Plant mitochondria and oxidative stress. Electron transport, NADPH turnover and metabolism of reactive oxygen species. Annual Review of Plant Physiology and Molecular Biology. 52, 561−591.

Mulè, G., Bailey J.A., Cooke B.M., Logrieco A. 2005. Molecular diversity and PCR-detection of toxigenic *Fusarium* species and ochratoxigenic fungi. Mycopathologia. 160: 95-96.

Muller, P.Y., Janovjak H., Miserez A.R., Dobbie Z. 2002. Processing of gene expression data generated by quantitative real-time RT-PCR. BioTechniques. 32: 1372-1379 .

Munkvold, G.P., McGee, D.C., Carlton, W.M. 1997. Importance of Different Pathways for Maize Kernel Infection by *Fusarium moniliforme.* Phytopathology. 87, 209-217.

Munkvold, G.P. and Hellmich, R.L. 1999. Comparison of Fumonisin Concentrations in Kernels of Transgenic Bt Maize Hybrids and Nontransgenic Hybrids. Plant Disease. 83, 130-138.

Noguchi, R., Banno, S., Ichikawa, R., Fukumori, F., Ichiishi, A., Kimura, M., Yamaguchi, I., Fujimura, M. 2007. Identification of OS-2 MAP kinase-dependent genes induced in response to osmotic stress, antifungal agent fludioxonil, and heat shock in *Neurospora crassa*. Fungal Genetics and Biology. 44, 208-218

Ochiai, N., Tokai, T., Nishiuchi, T., Takahashi-Ando, N., Fujimura, M., Kimura, M. 2007. Involvement of the osmosensor histidine kinase and osmotic stress-activated protein kinases in the regulation of secondary metabolism in *Fusarium graminearum*. Biochemical and Biophysical Research Communications. 363, 639-644

Pan T. and Coleman, J.E. 1990. GAL4 transcription factor is not a "zinc finger" but forms a Zn(II)2Cys6 binuclear cluster. PNAS. 87, 2077-2081

Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic acid reserch. 29, 2000-2007.

Placinta, C.M., D'Mello, J.P.F., MacDonald, A.M.C. 1999. A review of worldwide contamination of cereal grains and animal feed with *Fusarium* mycotoxins. Animal Feed Science and Technology. 78, 21-37.

Ponts, N., Pinson-Gadais, L., & Richard-Forget, F. 2003. H2O2 effects on trichothecenes B (DON, ADON) production by *Fusarium graminearum* in liquid culture. Aspects of Applied Biology. 68, 223–228.

Ponts, N., Pinson-Gadais, L., Verdal-Bonnin, M.N., Barreau, C. Richard-Forget, F. 2006. Accumulation of deoxynivalenol and its 15-acetylated form is significantly modulated by oxidative stress in liquid cultures of *Fusarium graminearum*. FEMS Microbiology Letters 258, 102–107.

Ponts, N., Pinson-Gadais, L., Barreau, C., Richard-Forget, F., Ouellet, T. 2007. Exogenous H2O2 and catalase treatments interfere with *Tri* genes expression in liquid cultures of *Fusarium graminearum.* FEBS. 581, 443-447.

Ponts, N., Couedelo, C., Pinson-Gadais, L., Verdal-Bonnin, M.N., Barreau, C., Richard-Forget, F. 2009. *Fusarium* response to oxidative stress by H2O2 is trichothecene chemotype-dependent. FEMS Microbiology Letters. 293, 255 - 262

Prelusky, D.B., Rotter, B.A., Rotter, R.G., 1994. Mycotoxins in grain. Compounds other than Aflatoxin. Toxicology of mycotoxins. 359-403.

Proctor, R.H., Desjardins, A.E., Plattner, R.D., Hohn, T.M. 1999. A Polyketide Synthase Gene Required for Biosynthesis of Fumonisin Mycotoxins in *Gibberella fujikuroi* Mating Population A. Fungal Genetics and Biology. 27, 100-112

Proctor, R.H., Brown, D.W., Plattner, R.D., Desjardins, A.E. 2003. Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in *Gibberella moniliformis*. Fungal Genetics and Biology. 38, 237-249

Repka, V. 1999. Improved Histochemical Test for In Situ Detection of Hydrogen Peroxide in Cells Undergoing Oxidative Burst or Lignification. Biologia Plantarum. 42, 599-607.

Reverberi, M., Punelli, M., Smith, C., Punelli, F., Zjalic, S., Ricelli, A., Payne, G., Fabbri, A.A., Fanelli, C. 2007. Cell redox balance, lipid metabolism and aflatoxin biosynthesis in *Aspergillus sect. flavi*. Journal of Plant Pathology. 89.

Reynoso, M., Torres, A., Ramirez, M.L., Rodríguez, M., Chulze, S., Magan, N. 2002. Efficacy of antioxidant mixtures on growth, fumonisin production and hydrolytic enzyme production by *Fusarium verticillioides* and *F. proliferatum* in vitro on maize-based media. Mycological Research. 106, 1093-1099.

Rheeder, J.P., Marasas, W.F.O., Thiel, P.G., Sydenham, E.W., Shephard, G.S., Van Schalkwyk, D.J. 1992. *Fusarium moniliforme* and fumonisins in corn in relation to human oesophageal cancer in Transkei. Phytopathology. 82, 353-357.

Ross, P.F., Rice, L.G., Osweiler, G.D., Nelson P.E., Richard, J.L., Wilson, T.M. 2004. A review and update of animal toxicoses associated with fumonisin-contaminated feeds and production of fumonisins by *Fusarium* isolates. Mycopathologia. 117, 109-114.

Rossi, V., Battilani, P., Battisti D., Scandolara, A. 2004. Epidemiological aspects of *Fusarium verticillioides*, the causal agent of maize pink ear rot. Journal of Plant Pathology. 86, 331.

Sambrook, J., Russell, W.D. 2001. Molecular Cloning: a Laboratory Manual. CSHL Press, Woodbury NY, USA.

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

Schmidt-Heydt, M., Abdel-Hadi, A., Magan, N., Geisen, R. 2009. Complex regulation of the aflatoxin biosynthesis gene cluster of *Aspergillus flavus* in relation to various combinations of water activity and temperature. International Journal of Food Microbiology. 135, 231–237

Schulz, B., Römmert, A.K., Dammann, U., Aust, H.J., Strack, D. 1999 The endophyte-host interaction: a balanced antagonism?. Mycological Research. 103, 1275–1283.

Segmüller, N., Ellendorf, U., Tudzynski, B., Tudzynski, P. 2007. BcSAK1, a stress-activated mitogen-activated protein kinase is involved in vegetative differentiation and pathogenicity in *Botrytis cinerea*. Eukaryotic Cell 6, 211-221

Segvic, M., Pepeljnjak, S. 2003. Distribution and fumonisin B1 production capacity of *Fusarium moniliforme* isolated from corn in Croatia. Periodicum biologorum. 105, 275-279.

Seo, J.A., Proctor, R.H., Plattner, R.D. 2001. Characterization of four clustered and coregulated genes associated with fumonisin biosynthesis in *Fusarium verticillioides*. Fungal Genetics and Biology 34, 155–165.

Shim, W.B. and Woloshuk, C.P. 2001. Regulation of Fumonisin B1 Biosynthesis and Conidiation in *Fusarium verticillioides* by a Cyclin-Like (C-Type) Gene, FCC1. Applied Environmental Microbiology. 67, 1607–1612

Sobek, E.A., Munkvold, G.P. 1999. European corn borer (Lepidoptera: *Pyralidae*) larvae as vectors of *Fusarium moniliforme*, causing kernel rot and symptomless infection of maize kernels. Journal of economic entomology 92, 503-509.

Takahashi,H., Watanabe,S., Chikakane,K. 1997 Biochemical Mechanisms of Fungicidal Activity with Tissue Extracts. Marcel Dekker Inc. New York. NY.

Torres, M.A. and Dangl, J.L. 2005. Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Current Opinion in Plant Biology. 8, 397-403.

Tuite, J., 1969. Plant Pathological Methods: Fungi and Bacteria. Burgess Publishing Company, Minneapolis, MN, USA.

Vainio, H., Heseltine, E., Wilbourn, J. 1993. Report on an IARC working group meeting on some naturally occurring substances. International Journal of Cancer. 53, 535 – 537.

White, D.G. 1999. Compendium of Corn Diseases. Third Edition. APS Press.

Wicklow, D.T., Horn, B.W., Shotwell, O.L., Hesseltine, C.W., Caldwell, R.W. 1988. Fungal interference with *Aspergillus flavus* infection and aflatoxin contamination of maize grown in a controlled environment. Phytopathology. 78, 68-74.

Woloshuk, C.P., Foutz, K.R., Brewer, J.F., Bhatnagar, D., Cleveland, T.E., Payne G.A. 1994. Molecular characterization of *aflR*, a regulatory locus for aflatoxin biosynthesis. Applied Environmental Microbiology. 60, 2408-2414

Zaleta-Rivera, K., Xu, C., Yu, F., Butchko, R.A.E., Proctor, R.H., Hidalgo-Lara, M., Raza, A., Dussault, P.H., Du, L. 2006. A Bidomain Nonribosomal Peptide Synthetase Encoded by FUM14 Catalyzes the Formation of Tricarballylic Esters in the Biosynthesis of Fumonisins. Biochemistry. 45, 2561–2569.

Zhao, Z., Chen, G., Zhang, C. 2001. Interaction between reactive oxygen species and nitric oxide in drought-induced abscisic acid synthesis in root tips of wheat seedlings. Australian Journal Plant Physiology. 28, 1055–1061.

Zhou, F., Menke, F.L.H., Yoshioka, K., Moder, W., Shirano, Y., Klessig, D.F. 2004. High humidity suppresses ssi4-mediated cell death and disease resistance upstream of MAP kinase activation, H2O2 production and defense gene expression. The Plant Journal 39, 920–932.

Chapter 4 – Ochratoxin A biosynthesis

A cDNA-AFLP approach to study Ochratoxin A production in *Aspergillus carbonarius*

Alessandro Botton[1], Davide Ferrigo[2], Cristina Scopel[2], Roberto Causin[2], Claudio Bonghi[1], Angelo Ramina[1]*

[1]Department of Environmental Agronomy and Crop Science, University of Padova, Campus of Agripolis - Viale dell'Università 16, 35020 Legnaro (Padova), Italy.
[2]T.E.S.A.F.–Dip. Territorio e Sistemi Agro-Forestali, University of Padova, Campus of Agripolis - Viale dell'Università 16, 35020 Legnaro (Padova).

*Corresponding author, Angelo Ramina, Department of Environmental Agronomy and Crop Science, University of Padova, Campus of Agripolis - Viale dell'Università 16, 35020 Legnaro (Padova), Italy. Fax +39 049 8272850. E-mail, angelo.ramina@unipd.it

Abstract

Aspergillus carbonarius is responsible for the majority of mycotoxin contaminations in grapes and its derivatives. Most of *A. carbonarius* strains are ochratoxin A (OTA) producers, even though at very different levels. This broad variability was used to identify genes whose expression is linked with the ability of producing OTA. A cDNA-AFLP differential display screening was performed in two strains of *A. carbonarius*, antagonists for the ability of producing OTA, allowing the identification of 119 differentially expressed sequences putatively involved in the regulation of OTA biosynthesis. A likely connection was pointed out between the biosynthesis of the toxin, vegetative growth and sexual/asexual developmental progression, along with common signalling pathways involving G protein and Ca^{2+}/calmodulin dependent phosphorylation and dephoshorylation cascades.

Keywords, *Aspergillus carbonarius*; ochratoxin A biosynthesis; cDNA-AFLP differential display; G protein; Ca^{2+}/calmodulin dependent phosphorylation and dephoshorylation; zinc finger transcription factors.

Introduction

Ochratoxins (OTs) are secondary metabolites produced by several species of filamentous fungi belonging to the genera *Aspergillus*, *Petromyces*, *Neopetromyces* and *Penicillium* (Perrone *et al.,* 2006). The basic chemical structure of OTs consists of an isocoumarin group linked to a phenylalanine moiety by an amide bond. Ochratoxin A (OTA), the chlorinated form, is the most toxic and the most prevalent, whereas ochratoxin B and C, the non-chlorinated and esterified forms, respectively, are less common and toxic (Bayman & Baker, 2006) (Figure 1) OTA contamination may be affected by different factors such as temperature, water activity, pH and substrate composition. Therefore the toxigenicity of an ochratoxigenic fungus may vary according to the crop and the geographical area. In temperate locations, OTA is mainly produced by *Penicillium* species contaminating cereals, whereas in the tropical regions it is synthesized mainly by *A. ochraceus*. Two other species, namely *A. niger* var. *niger* and *A. carbonarius*, are responsible for the majority of contaminations in cereals and grapes (Battilani *et al.,* 2003; Ponsone *et al.,* 2007; Atoui *et al.,* 2007), respectively, in the warm zones (Ringot *et al.,* 2006).

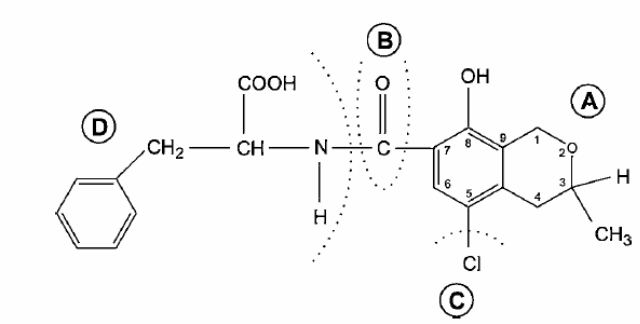


Figure 1: Structural formula of ochratoxin A demonstrating the metabolic origin of the structural parts of the molecule. (A) The polyketide dihydro-isocoumarin; (B) The carboxyl group, derived after oxidation of the methylated polyketide; (C) The chlorine introduced by halogenation of ochratoxin B; (D) The amino acid phenylalanine coming out of the shikimic acid pathway. (From Farber and Geisen, 2004)

The OTA biosynthetic pathway has not yet been completely elucidated (Ringot *et al.,* 2006) (Figure 2). So far, the majority of the studies have been focused on *Penicillium* species

(Färber and Geisen, 2004) and *A. ochraceus* (Harris and Mantle, 2001), pointing out that the phenylalanine moiety originates from the shikimic acid pathway, whereas the isocoumarin is formed from acetate units via the pentaketide pathway. Polyketide synthase (PKS) is the key enzyme catalyzing the first step of OTA biosynthesis and the related gene (*pks*) has been previously cloned and characterized in different species, showing a high degree of sequence variability (O'Callaghan *et al.,* 2003; Varga *et al.,* 2003; Geisen *et al.,* 2004). A *pks* gene was isolated also in *A. ochraceus* (O'Callaghan *et al.,* 2006) and *A. carbonarius* (Gallo *et al.,* 2009) and shown to be expressed only under OTA permissive conditions. Moreover, insertional mutagenesis leading to a disruption of the *pks* gene impaired OTA production (O'Callaghan *et al.,* 2003). The following steps of OTA biosynthesis include the formation of the lactone ring originating mellein, the addition of a carboxyl group to form ochratoxin β (OTβ), and the chlorination by means of a chloroperoxidase synthesizing ochratoxin α (OTα). Finally, the linking of OTα to phenylalanine is carried out by means of the enzyme OTA synthetase. Inhibition of OTA production can be achieved with various natural and synthetic compounds, in a species-dependent manner. This may indicate that the biosynthesis of this mycotoxin is not conserved in all the producing fungi (Bayman *et al.,* 2006).

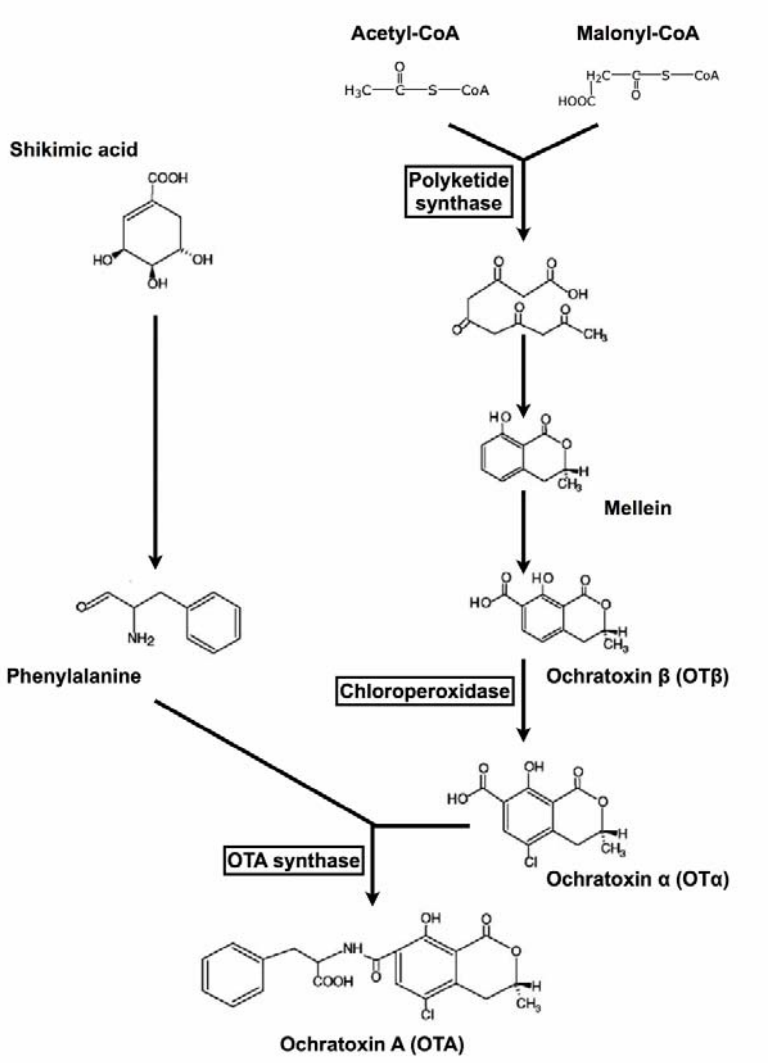


Figure 2: Biosynthetic pathway of Ochratoxin A. (from Ringot *et al.,* 2006)

Additional studies were focused also on the biosynthetic regulation of many other mycotoxins, such as aflatoxins, fumonisins, sterigmatocystin and tricothecenes (Brodhagen and Keller, 2006), and numerous signalling pathways, most of which are shared by different mycotoxins and diverse species, were outlined. A strong link between sexual and asexual sporulation and mycotoxin biosynthesis was also evidenced. Particularly, the G protein signal transduction pathway has been shown to regulate both secondary metabolism and sporulation (Han *et al.,* 2004), along with lipid signalling (Tsitsigiannis and Keller, 2006). Several transcription factors have been shown to bind promoter elements of genes

involved in mycotoxin biosynthesis and secondary metabolism, as for $Zn(II)_2Cys_6$ zinc finger proteins (Ehrlich *et al.,* 1999) and Cys_2His_2 (Borneman *et al.,* 2001). Important regulatory roles involve also the Ca^{2+}/calmodulin-dependent phosporylation/dephosphorylation pathways (Rao and Subramanyam, 1999; Jayashree *et al.,* 2000). However, few reports concerning the regulation of OTA biosynthesis are available (Basìlico and Basìlico, 1999; O'Callaghan *et al.,* 2003; Mitchell *et al.,* 2004; Pardo *et al.,* 2006; Bellì *et al.,* 2007; Pateraki *et al.,* 2007; Valero *et al.,* 2007a; Valero *et al.,* 2007b), and the genetic characterization was mainly focused on either *Penicillium* species (Färber and Geisen, 2004) or the genes directly involved in the biosynthetic pathway (O'Callaghan *et al.,* 2003; Varga *et al.,* 2003). Moreover, further studies were carried out only for diagnostic purposes, to allow the identification of OTA-producing species contaminating foods and feeds (Geisen *et al.,* 2004; Schmidt *et al.,* 2004; Bau *et al.,* 2005; Niessen et al., 2005; Dao *et al.,* 2005; Patiño *et al.,* 2005; Bogs *et al.,* 2006; Perrone *et al.,* 2006; Zanzotto *et al.,* 2006; Atoui *et al.,* 2007b; Martìnez-Culebras and Ramòn, 2007, Selma *et al.,* 2008; Ringot and Chango, 2009). Since OTA represents a challenging problem both for food and feed producers and consumers, the full elucidation of the biosynthetic pathway and its regulation would allow the setting up of innovative tools to control and/or prevent the crop contamination. In this view, biocompetition strategies using atoxigenic strains have revealed to be a possible solution, as in the case of non-toxigenic isolates of *A. flavus* successfully used to reduce aflatoxin contamination of cottonseed, corn and peanuts (Ehrlich and Cotty, 2004b).

Among the commodities that can be contaminated by OTA, grapes and wines are those with the major content of toxin, next only to cereals (Bau *et al.,* 2005). Wine is an important beverage in the world trade, of which France, Italy and Spain are the main producers and exporters. Due to the actual importance of the wine industry, several studies were carried out to assess the relevance of OTA contamination in wine. More than 50% of Spanish wines were shown to be contaminated, with the highest OTA concentration in dessert wines (12.25 ng/mL) (Lòpez de Cerain *et al.,* 2002; Bellì *et al.,* 2004; Blesa *et al.,* 2004; Mateo *et al.,* 2006; Hernàndez *et al.,* 2006). In Italy, 78.4% of red wines were contaminated, with a maximum of 7.63 ng/mL (Visconti *et al.,* 2000; Pietri *et al.,* 2001; Brera *et al.,* 2005; Bacaloni *et al.,* 2005; Brera *et al.,* 2008). In the other countries, the contamination of wines by OTA is less serious (Mateo *et al.,* 2007).

A. carbonarius is the main responsible organism for the majority of contaminations in grapes and its derivatives (Battilani *et al.,* 2003; Ponsone *et al.,* 2007; Atoui *et al.,* 2007a). Most of *A. carbonarius* strains are OTA producers, even though at very different levels (Bau *et al.,* 2005; Perrone *et al.,* 2006). This broad variability may be successfully used to characterize the genetic regulation of OTA biosynthesis, isolating the genes differentially expressed in high- and low-producing isolates.

Differential display techniques, such as Differential Display Reverse Transcriptase-PCR (DDRT-PCR) and microarrays, were successfully used for aflatoxin biosynthesis studies in *A. flavus* and *A. parasiticus* (OBrian *et al.,* 2003), as well as for OTA biosynthesis studies in *P. nordicum* (Färber & Geisen, 2004). The differential conditions were achieved by growth on different minimal media, either supporting or inhibiting OTA production. However, with such an approach, several genes were differentially expressed because of the different media, generating confusion in the interpretation of results.

Among the differential display techniques, cDNA-AFLP has proved to be the most popular procedure because of its ability in displaying several transcripts simultaneously and detecting differentially expressed genes (Bachem *et al.,* 1996; Bachem *et al.,* 1998). Both reliability and sensitivity of amplification products proved to be very high, and expression patterns visualized by cDNA-AFLP well correlated with northern blot analysis (Durrant *et al.,* 2000; Jones *et al.,* 2000; Donson *et al.,* 2002; Albertini *et al.,* 2004; Cnudde *et al.,* 2006).

In the present research, cDNA-AFLP differential display was used to isolate genes differentially expressed in OTA high- and low-producing strains of *A. carbonarius*. In the present study, two strains previously characterized by Perrone *et al.* (2006), namely ITEM 5005 and ITEM 5012, were used because of their differential production of OTA. The sequences isolated were analyzed for homology and classified according to the gene ontology criteria (Ashburner *et al.,* 2000). Finally, a comparison between the annotations of up- and down-regulated sequences was carried out to highlight the differentially expressed functional categories. The involvement of such genes in the OTA biosynthesis is critically assessed and a possible model for its regulation proposed.

Materials and Methods

Strains, media and growth conditions

Fungal strains ITEM 5005 and ITEM 5012 of *A. carbonarius* were selected from a list of 20 strains obtained from Agri-Food Toxigenic Fungi Culture Collection (I.S.P.A. - Institute of Sciences of Food Production, Bari, Italy) and characterized for OTA production by Perrone (Perrone *et al.*, 2006).

Strains were grown on Petri dishes containing Czapek DOX Agar (OXOID Ltd., Basingstoke, Hampshire, England) in dark condition at 25 °C for 6 days to achieve spore production. Spores were collected with a sterile solution of 0.1% (v/v) Tween 20 (J.T. Baker, Deventer, Holland) and stored at −20 °C in 25% (v/v) of glycerol (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) before use. Monoconidial cultures of *A. carbonarius* were obtained as reported by Tuite (1969). Monoconidial isolates were subcultured in YES liquid medium (2% yeast extract, 15% sucrose, 1 g/L $MgSO_4$) pH 6.5 (O'Callaghan *et al.*, 2003) in motionless and dark condition at 25 °C for 14 days and OTA production monitored by ELISA assays (see below) to detect the optimum growth phase to collect the mycelia. An aliquot of culture broth containing conidia (about 110 mL) was collected for each isolate, inoculated into 250 mL Erlenmeyer flasks containing 100 mL of YES liquid medium, and grown as described above for 7 days. During this phase the isolates showed an exponential increase of OTA production. The mycelia were harvested by filtration through a 0.45 μm Millipore filter (Millipore Corporation, USA), immediately frozen in liquid nitrogen and then stored at −80 °C before nucleic acid extraction.

Quantification of OTA production

ELISA determination of OTA content in *A. carbonarius* isolates was performed on 5 mL of liquid culture for each strain. Samples were blended, extracted in 15 mL of a solution 1,2 HCl 1 M,dichloremethan, shaken for 15 minutes, centrifuged for 15 min at 2200 × g, and 5 mL of lower organic phase collected and neutralized with 2.5 mL of a 0.13 M sodium bicarbonate solution at pH 8.1. The solutions were shaken and centrifuged as previously described, the upper aqueous phase containing OTA collected and quantified using the I'screen OCHRA kit for quantitative ELISA (Tecna Diagnostic and Biotechnology, Trieste, Italy) following the manufacturer's instructions. Absorbance was recorded at 450 nm, at

room temperature, with a 318MC Micro ELISA plate reader (Sanco Instruments Co., Ltd, Shanghai, China). Standard curves were obtained by plotting the seven standard values of OTA concentration against optical density at 450 nm. OTA concentration in samples isolates was determined from the standard curve.

The monoconidial isolates 5005E (6 ng/mL) and 5012A (2500 ng/mL), were analyzed by HPLC as described by Varga *et al.,* (2005) to confirm ELISA results, and selected for the following cDNA-AFLP analyses.

RNA extraction and cDNA synthesis

Total RNA was isolated from 50 mg of mycelia using the RNA Minispin kit (GE Healthcare, Chalfont St. Giles, UK) following the manufacturer instructions, quantified with a spectrophotometer (Biomate 3, Thermo Scientific, Waltman, WA, USA), and run on 1% agarose gel to check its integrity. Twenty µg of total RNA were treated with 10 U of RQ1 RNase-free Dnase (Promega, Milan, Italy) and 1 Unit of RNase Inhibitor (RNAguard, Amersham Biosciences, Piscataway, NY, USA) for 30 minutes, then purified by phenol-chloroform extraction and precipitated in isopropyl-alcohol as described by Sambrook & Russel (2001). Double-stranded cDNA was synthesized from 10 µg of DNA-free total RNA using the Universal Riboclone cDNA Synthesis System Kit (Promega, Madison, USA) according to the manufacturers instructions.

cDNA-AFLP

Double-stranded cDNA was used for cDNA-AFLP analyses as previously described (Vos *et al.,* 1995; Bachem *et al.,* 1996) with some modifications. Restriction and adaptor ligation were performed simultaneously, as described by Barcaccia (Barcaccia *et al.,* 2001), in a total volume of 30 µL containing 1X One-phor-all buffer (Amersham Biosciences, Piscataway, NY, USA), DTT 5 mM, BSA 50 ng/µL, 50 pmol of *Mse*I adaptor, 5 pmol of *Eco*RI adaptor, ATP 10 mM, 5 Units of *Mse*I and 5 Units of *Eco*RI, 1 Unit of T4 DNA Ligase and 10 µg of double-stranded cDNA. The mixture was incubated for 4 h at 37 °C. Preamplification was performed in a total volume of 50 µL containing 5 µL of restriction-ligation mixture, 1.5 ng/µL of primers without selective nucleotides, dNTPs 200 µM each, 1X PCR buffer and 1 Unit of *Taq* polymerase (Amersham Biosciences, Piscataway, NY,

USA). PCR conditions were as follows, an initial hold of 5 minutes at 94 °C, 25 cycles composed by 30 sec at 94 °C, 30 sec at 55 °C and 60 sec at 72 °C, and a final hold of 7 minutes at 72 °C. The reaction was checked on 1% agarose gel and diluted 1,5 with ddH_2O. The hot-amplification was carried out on 5 μL of diluted pre-amplification mixture in a total volume of 20 μL with 0.4 Units of *Taq* polymerase (Amersham Biosciences, Piscataway, NY, USA), 5 ng of ^{33}P-labeled *Eco*RI primer, 30 ng of *Mse*I primer, dNTPs 200μM each and 1X PCR buffer, using the following touch-down profile (Barcaccia *et al.*, 1999), an initial hold of 3 minutes at 94 °C and 14 cycles composed of 30 sec at 94 °C, 30 sec at 54.5 °C and 60 sec at 72 °C with an annealing temperature decrease of 0.7 °C/cycle, followed by 18 cycles composed by 30 sec at 94 °C, 30 sec at 55.2 °C and 60 sec at 72 °C, and final hold of 10 minutes at 72 °C.

***Eco*RI primers**	***Mse*I primers**
***Eco*=AGACTGCGTACCAATTC**	***Mse*=GACGATGAGTCCTGAGTAA**
	01 *Mse*+AAG
A *Eco*+AAC	02 *Mse*+ACT
B *Eco*+ACC	03 *Mse*+AGC
C *Eco*+AGG	04 *Mse*+AGG
D *Eco*+CAG	05 *Mse*+AGT
E *Eco*+CCA	06 *Mse*+ATA
F *Eco*+GCA	07 *Mse*+ATC
G *Eco*+TAA	08 *Mse*+CAA
H *Eco*+TGA	09 *Mse*+CAC
	10 *Mse*+CAT
	11 *Mse*+CCA

Table 1: Primers used in the cDNA-AFLP experiments. All the 88 possible combinations between *Eco*RI and *Mse*I primers were adopted.

The final amplification was performed with 88 different combinations of *Eco*RI and *Mse*I primers (Table 1).

An equal volume of Gel Loading Buffer (98% formamide, EDTA 10 mM, 0.05% w/v of bromophenol blue and xylene cyanol) was added to the PCR prior to a denaturing step of 5 minutes at 98 °C. All the reactions were performed in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). The amplification products were separated in a 5% polyacrylamide gel with 8M urea at 90W constant power using the SequiGen GT system (Biorad, Hercules, CA, USA), dried-blotted onto a Whatman 3MM paper at 80 °C for 1 h and visualized by autoradiogram after overnight exposure on Kodak MR-I X-ray film (Botton *et al.*, 2005) (Fig. 3). All experiments were carried out in triplicates.

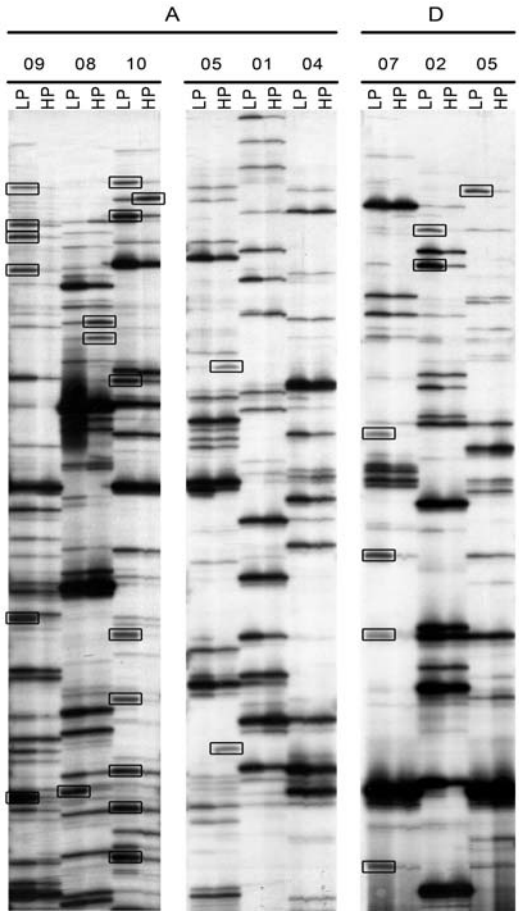


Figure 3: cDNA-AFLP autoradiogram of nine of the most informative primer combinations amplifying differentially expressed genes in OTA low- (LP) and high- (HP) producing strains. The combinations of primers used were indicated according to the codes reported in Table 2. The boxes show some of the differentially expressed fragments.

Purification and sequencing

The autoradiogram films were scanned and analyzed with KODAK 1D v 3.6 software (Scientific Imaging Systems, Eastman Kodak Company) to quantify the net intensity of the bands. Amplicons showing at least a 3-fold difference in terms of intensity between the two samples in all the replicates were excised from the blots and immersed overnight in 100 µL of PCR-grade water. 5 µL were used in a standard PCR reaction with the same *Eco*RI and *Mse*I primers used in the preamplification step described above. The amplified products were separated in a 1.5% agarose gel, and the bands excised and purified by means of Minelute Gel Extraction Kit (Qiagen, Milano, Italy). The sequencing reaction was performed with the Big Dye Terminator v3.1 kit (Applied Biosystems, Foster City, CA, USA) on 5 µL of purified band following the manufacturers instructions, and analyzed at the BMR Genomics (University of Padova, www.bmr-genomics.com).

Annotation of sequences

BlastX analyses were performed by means of Blast2GO software v2.0.1 (http,//www.blast2go.de; Conesa *et al.*, 2005; Aparicio *et al.*, 2006). The parameters were differentiated according to the length of the nucleotide records and four ranges were defined for this purpose, 0−99 bp, 100−199 bp, 200−399 bp, ≥400 bp. Blast expectation value threshold was constantly set to 10, whereas HSP length cut-off was set to 10, 15, 20 and 33, respectively. This configuration was chosen to improve the E-value scores in the Blast analyses, allowing to retrieve database matches also for short sequences with short local alignments. The remaining parameters were kept as default. In particular, the default Blast remote server (http,//www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) was used to achieve updated database matches. The Mapping tool of Blast2GO software was used to obtain gene ontology (GO) information from retrieved database matches. Annotation of all sequences was performed with different parameters on two ranges of length, 0−199 bp and ≥200 bp. E-value Hot Filter was set to 0 and 3, respectively, and GO weight constantly to 5. A further differential annotation parameter was adopted concerning similarity, setting up the threshold cutoff at 60% and 30% for sequences <200 bp and ≥200 bp, respectively. This configuration allowed a stricter annotation of shorter sequences representing also a filter towards less reliable BlastX matches. Two following steps were carried out to enrich the annotation. InterProScan (Zdobnov & Apweiler, 2001) was performed by enabling all the

possible motif database searches and the resulted GO terms added to the annotation. The annotation analysis was then implemented with the 'Augment Annotation by ANNEX' function. Basically, this approach uses univocal relationships between GO terms from the different GO categories to add implicit annotation (http,//www.goat.no; for a detailed description see Myhre *et al.* 2006). The GOSlim goslim_yeast.obo was used to achieve specific GO terms by means of a fungi-specific reduced version of the gene ontology (http,//geneontology.org/GO.slims.html). Statistical analyses to compare sequence sets were performed with the integrated GOSSIP tool of the Blast2GO software.

EST	dbEST	GenBank	Primers	Tentative annotation	Size	EC	Up-reg
2.33.01	53439363	**FD661682**	F07	BRCT/DNA repair domain protein	256	EC,2.1.1	5.5
2.43.01	53439364	**FD661683**	F03	Unknown function/hypothetical protein	1182	-	3.1
3.03.01	53439365	**FD661684**	E09	Arginyl-tRNA synthetase, class IC	248	EC,6.1.1.19	3.6
3.03.08	53439366	**FD661685**	E09	Unknown function/hypothetical protein	103	-	3.4
3.06.09	53439370	**FD661689**	E08	Transposase	152	-	5.7
3.09.03	53439380	**FD661699**	E06	Glycosyl hydrolase family	279	EC,3.2.1	6.1
3.13.01	53439381	**FD661700**	E02	PSP1 domain protein	621	-	3.5
3.15.01	53439382	**FD661701**	E05	RNA helicase	503	-	5.4
3.17.01	53439383	**FD661702**	E01	Unknown function/hypothetical protein	551	-	5.7
3.25.01	53439385	**FD661704**	C09	Alkaline serine protease	374	EC,3.4.14.9	5.5
3.25.02	53439386	**FD661705**	C09	Cation channel family	256	-	6.0
3.27.01	53439387	**FD661706**	C08	Unknown function/hypothetical protein	301	-	38.4
3.27.02	53439388	**FD661707**	C08	Cys_2His_2 zinc finger domain, transcription factor	501	-	5.4
3.27.07	53439389	**FD661708**	C08	Unknown function/hypothetical protein	163	-	4.1
3.35.01	53439391	**FD661710**	C02	MFS- Multidrug efflux carrier	243	-	3.0

3.37.02	53439392	**FD661711**	C05	Putative Beta-N-acetylglucosaminidase	372	EC,3.2.1.52	3.7
4.01.01	53439395	**FD661714**	A11	Endo-1,4-beta-xylanase (XynG1)	546	EC,3.2.1.8	3.8
4.03.02	53439396	**FD661715**	A09	Unknown function/hypothetical protein	551	-	4.2
4.03.03	53439397	**FD661716**	A09	Chitin synthase G	521	EC,2.4.1.16	5.4
4.03.05	53439398	**FD661717**	A09	Unknown function/hypothetical protein	312	-	3.0
4.03.06	53439399	**FD661718**	A09	Beta-galactosidase lacA	407	EC,3.2.1.23	3.9
4.03.07	53439400	**FD661719**	A09	Cell cycle regulator	299	-	3.0
4.03.08	53439401	**FD661720**	A09	Unknown function/hypothetical protein	163	-	7.1
4.03.09	53439402	**FD661721**	A09	Unknown function/hypothetical protein	152	-	3.0
4.03.13	53439403	**FD661722**	A09	Unknown function/hypothetical protein	132	-	3.9
4.05.05	53439404	**FD661723**	A08	Polyketide synthase	252	-	3.3
4.07.01	53439406	**FD661725**	A10	1,3-beta-glucan synthase component GLS2	402	EC,2.4.1.34	3.6
4.07.08	53439407	**FD661726**	A10	Unknown function/hypothetical protein	207	-	a
4.17.14	53439413	**FD661732**	A05	Unknown function/hypothetical protein	98	-	3.0
4.25.01	53439415	**FD661734**	D09	RfeG transcription factor	961	-	5.5
4.25.03	53439416	**FD661735**	D09	NADH-cytochrome B5 reductase	354	EC,1.6.2.2	3.5

4.31.03	53439417	**FD661736**	D06	Asparaginyl-tRNA synthetase	254	EC,6.1.1.22, EC,6.1.1.12	3.1
4.31.13	53439418	**FD661737**	D06	3-methylcrotonyl-CoA carboxylase, beta subunit (MccB)	197	EC,6.4.1.4	5.6
4.33.13	53439419	**FD661738**	D07	Small oligopeptide transporter, OPT family	268	EC,3.4.17.22	3.1
4.41.13	53439422	**FD661741**	D04	Unknown function/hypothetical protein	136	-	3.5
4.43.04	53439423	**FD661742**	D03	Unknown function/hypothetical protein	303	-	a
4.43.06	53439424	**FD661743**	D03	Unknown function/hypothetical protein	185	-	a
4.44.03	53439425	**FD661744**	D03	DEAD/DEAH box RNA binding	301	-	12.1
5.05.01	53439428	**FD661747**	G08	Serine/threonine protein kinase	442	EC,2.7.11.1	3.1
5.07.01	53439430	**FD661749**	G10	RLI and DUF367 domain protein	380	-	3.0
5.07.15	53439431	**FD661750**	G10	Unknown function/hypothetical protein	113	-	15.7
5.13.01	53439433	**FD661752**	G02	Chromodomain helicase	371	-	3.8
5.13.02	53439434	**FD661753**	G02	Unknown function/hypothetical protein	177	-	6.5
5.21.01	53439440	**FD661759**	G01	Patched sphingolipid transporter (Ncr1)	473	-	4.7
5.21.02	53439441	**FD661760**	G01	NHP2/L7aE family protein	284	-	6.8
5.21.15	53439442	**FD661761**	G01	Unknown function/hypothetical protein	84	-	5.1
5.27.02	53439447	**FD661766**	B10	Pyruvate decarboxylase	173	EC,4.1.1.1	32.6

5.29.01	53439448	**FD661767**	B06	Mannose-1-phosphate guanylyltransferase	352	EC,2.7.7.22	7.1
5.39.01	53439449	**FD661768**	B05	RgsA-like, Regulator of G protein signaling domain protein	245	-	4.6
5.39.02	53439450	**FD661769**	B05	RNA polymerase II mediator complex component	175	-	4.1
Ctg01	53439452	**FD661771**	H10, C06	Protein phosphatase regulatory subunit, Gac1	812	EC,3.1.3.16	3.7
Ctg02	53439453	**FD661772**	H/C02	Unknown function/hypothetical protein	157	-	6.5
Ctg04	53439455	**FD661774**	A/B/F/G09	Pyruvate decarboxylase	663	EC,4.1.1.1	5.6
Ctg05	53439456	**FD661775**	E/D11	Unknown function/hypothetical protein	224	-	6.5
Ctg06	53439457	**FD661776**	E04/06/09	Unknown function/hypothetical protein	174	-	7.4
Ctg07	53439458	**FD661777**	H10, C08	Cellulose-binding (CBD), family II	393	EC,3.2.1	a
Ctg08	53439459	**FD661778**	A10, G10/07	Membrane protein	191	-	a
Ctg11	53439462	**FD661781**	D06/07	Chitinase	361	EC,3.2.1.14	a
Ctg12	53439463	**FD661782**	D06/07/03	Unknown function/hypothetical protein	292	-	17.4
Ctg13	53439464	**FD661783**	D02, E02	1,3-Beta-glucan synthase catalytic subunit	523	EC,2.4.1.34	6.2
Ctg14	53439465	**FD661784**	D02, E02	Cell wall biogenesis protein LRR/Mhp1	462	-	3.9
Ctg15	53439466	**FD661785**	D05/07	Unknown function/hypothetical protein	151	-	4.9

Ctg16	53439467	**FD661786**	G/A09	Myosin I	463	-	4.5
Ctg17	53439468	**FD661787**	B03	Unknown function/hypothetical protein	175	-	5.8

Table 2 – Transcripts that were up-regulated in the ochratoxin-low-producing (LP) strain of *Aspergillus carbonarius* with respect to the high-producing (HP) one. The up-regulation (Up-reg) was expressed as L_i/H_i (L_i=average net intensity of the band in the LP strain; H_i=average net intensity of the band in the HP strain). The letter 'a' means that the band was completely absent in the HP sample. For each EST the dbEST ID (http,//www.ncbi.nlm.nih.gov/dbEST/), the GenBank accession number (http,//www.ncbi.nlm.nih.gov/Genbank/index.html), the combination/s of primers used for the isolation, a tentative annotation, the size in base pairs, and the enzyme code (EC) are provided where available.

EST	dbEST	GenBank	Primers	Tentative annotation	Size	EC	Down-reg
2.08.10	53439350	**FD661669**	H10	$Zn(II)_2Cys_6$ zinc finger domain, transcription factor	110	-	3.0
2.16.10	53439351	**FD661670**	H05	Unknown function/hypothetical protein	98	-	9.2
2.20.02	53439352	**FD661671**	H04	Unknown function/hypothetical protein	192	-	4.2
2.28.01	53439353	**FD661672**	F08	Molecular chaperone Mod-E/Hsp90	758	-	a
2.28.02	53439354	**FD661673**	F08	Phosphatidylinositol 4-kinase (PI4K)	561	EC,2.7.1.67	a
2.28.04	53439355	**FD661674**	F08	CPA2 (Cation/Proton antiporter) transporter	305	-	a
2.28.05	53439356	**FD661675**	F08	FHA domain protein	275	-	a
2.28.06	53439357	**FD661676**	F08	Unknown function/hypothetical protein	269	-	3.5
2.28.07	53439358	**FD661677**	F08	Aldehyde dehydrogenase	165	EC,1.2.1.3, EC,1.2.1.5	3.7
2.28.08	53439359	**FD661678**	F08	30 kDa heat shock protein (HSP30)	150	-	a
2.28.10	53439360	**FD661679**	F08	Unknown function/hypothetical protein	115	-	5.0
2.28.11	53439361	**FD661680**	F08	Unknown function/hypothetical protein	74	-	a
2.28.12	53439362	**FD661681**	F08	Unknown function/hypothetical protein	48	-	a
3.06.01	53439367	**FD661686**	E08	Ubiquitin-like activating enzyme	333	-	3.2

3.06.02	53439368	**FD661687**	E08	Unknown function/hypothetical protein	186	-	9.8
3.06.08	53439369	**FD661688**	E08	Transcription initiation factor TFIId subunit TSM1	125	-	4.3
3.06.11	53439371	**FD661690**	E08	Unknown function/hypothetical protein	53	-	4.6
3.08.01	53439372	**FD661691**	E10	Phosphogluconate-2-dehydrogenase	869	EC,1.1.1.43	11.5
3.08.02	53439373	**FD661692**	E10	Arylsulfatase	543	EC,3.1.6.1	12.7
3.08.03	53439374	**FD661693**	E10	Translation elongation factor 1-alpha (EF-1 alpha)	1185	-	3.1
3.08.04	53439375	**FD661694**	E10	Cytochrome C-type haem-binding periplasmic protein	262	-	4.4
3.08.05	53439376	**FD661695**	E10	Calmodulin	203	-	10.7
3.08.06	53439377	**FD661696**	E10	5-oxo-L-prolinase	199	EC,3.5.2.9	9.7
3.08.08	53439378	**FD661697**	E10	Unknown function/hypothetical protein	217	-	a
3.08.09	53439379	**FD661698**	E10	Unknown function/hypothetical protein	127	-	a
3.24.09	53439384	**FD661703**	C11	Unknown function/hypothetical protein	108	-	a
3.28.09	53439390	**FD661709**	C08	Lysyl-tRNA synthetase	78	EC,6.1.1.6	3.7
3.42.01	53439393	**FD661712**	C04	Cytochrome P450 oxidoreductase	318	EC,1.6.2.4, EC,1.14.14.1	26.1
3.44.01	53439394	**FD661713**	C03	Unknown function/hypothetical protein	172	-	5.9
4.06.01	53439405	**FD661724**	A08	5-oxo-L-prolinase	550	EC,3.5.2.9	3.1

4.07.10	53439408	**FD661727**	A10	Pre-rRNA processing protein	159	-	a
4.07.12	53439409	**FD661728**	A10	Unknown function/hypothetical protein	155	-	a
4.10.01	53439410	**FD661729**	A06	Nuclear distribution protein nudE	632	-	5.3
4.12.01	53439411	**FD661730**	A07	Heat shock protein 70 (HSP70)	601	-	4.6
4.16.02	53439412	**FD661731**	A05	Unknown function/hypothetical protein	162	-	18.5
4.22.13	53439414	**FD661733**	A03	Unknown function/hypothetical protein	115	-	9.2
4.37.13	53439420	**FD661739**	D05	Unknown function/hypothetical protein	158	-	3.4
4.37.16	53439421	**FD661740**	D05	Unknown function/hypothetical protein	47	-	6.0
5.02.17	53439426	**FD661745**	G11	Diguanylate cyclase phosphodiesterase (GGDEF/EAL)	321	EC,4.6.1.2	4.4
5.02.18	53439427	**FD661746**	G11	Unknown function/hypothetical protein	121	-	11.3
5.06.03	53439429	**FD661748**	G08	DUF1749 protein	145		234.2
5.08.02	53439432	**FD661751**	G10	N-acetylglucosamine-6-phosphate deacetylase	300	EC,3.5.1.25	6.3
5.16.01	53439435	**FD661754**	G05	Unknown function/hypothetical protein	469	-	4.4
5.16.02	53439436	**FD661755**	G05	Protein phosphatase PP2A regulatory subunit B	350	-	4.0
5.16.15	53439437	**FD661756**	G05	Unknown function/hypothetical protein	137	-	6.3
5.16.17	53439438	**FD661757**	G05	Unknown function/hypothetical protein	91	-	30.3

5.16.19	53439439	**FD661758**	G05	Unknown function/hypothetical protein	93	-	3.3
5.24.01	53439443	**FD661762**	B11	Phosphogluconate-6-dehydrogenase (decarboxylating)	535	EC,1.1.1.44	10.2
5.24.19	53439444	**FD661763**	B11	Unknown function/hypothetical protein	77	-	3.6
5.24.22	53439445	**FD661764**	B11	Unknown function/hypothetical protein	75	-	3.2
5.24.25	53439446	**FD661765**	B11	Unknown function/hypothetical protein	67	-	4.2
5.44.15	53439451	**FD661770**	B03	Unknown function/hypothetical protein	65	-	32.5
Ctg03	53439454	**FD661773**	H04/06	Unknown function/hypothetical protein	204	-	3.7
Ctg09	53439460	**FD661779**	A07/02	Protocatechuate 4,5-dioxygenase	336	EC,1.13.11.8	5.4
Ctg10	53439461	**FD661780**	D11, F04	DJ-1/PfpI family protein	521	-	3.0

Table 3: Transcripts that were down-regulated in the ochratoxin-low-producing (LP) strain of *Aspergillus carbonarius* with respect to the high-producing (HP) one. The down-regulation (Down-reg) was expressed as H_i/L_i (H_i=average net intensity of the band in the HP strain; L_i=average net intensity of the band in the LP strain). The letter 'a' means that the band was completely absent in the LP sample.

Results

cDNA-AFLP

The cDNA-AFLP technique allowed to identify a total of 383 differentially expressed amplicons by using 88 different combinations of EcoRI/MseI primers. Only the bands showing at least a three-fold difference in terms of intensity between the two antagonist samples were excised from the blots and sequenced (Figure 3). A total of 142 sequences with an average length of 313 bp were obtained and successively clustered with strict parameters to eliminate redundancy resulting equal to 16%. Among the 119 ESTs obtained after clustering, 55 were down-regulated and 64 were up-regulated in the low-producing (LP) strain.

Analysis of differentially expressed genes

A BlastX analysis was performed on the isolated sequences pointing out a 42.9% of clones without similarity with known proteins, distributed as 37.5% among the up-regulated and 49.1% in the down-regulated fragments, that were annotated as "unknown function/hypothetical protein" (Table 2 and 3). The matching sequences showed a mean E-value equal to 2.96×10^{-1} and an average similarity with blast hits as high as 75%. Following the mapping process, during which the software recovers the gene ontology (GO) terms mainly by using the accession numbers of the blast hits, three GO terms were assigned on average to each sequence (data not shown). Enzyme codes (EC) were also found for 33 sequences on the total 68 annotated ones (Table 2 and 3).

Concerning the final annotation sets, the GO terms were grouped according to cellular component, molecular function and biological process, by up- and down-regulated clones separately. In Figure 4, the pie charts show the gene ontology at level 4 in the case of the biological process, and at level 3 in the case of the cellular component and molecular function categories. Different levels of ontology were chosen for being the most informative.

Among the LP strain up-regulated genes, the majority were classified as related to the intracellular components, in particular 22% in 'intracellular part', 22% generally as 'intracellular', and 17% as 'intracellular organelle', accounting for a total of 61%. The

remaining annotations concerned the membrane- and non-membrane-bound organelle, for 13% and 4%, respectively (Figure 4A, left). Regarding the molecular function, the 'hydrolase activity' was the GO term most frequently encountered (28%), followed by the transferase (20%), and ligase activities (12%). The remaining categories spanned from 8% of 'nucleic acid binding', 'signal transducer activity', 'helicase activity', and 'protein binding', to 4% of oxidoreductase and lyase activities (Figure 4B, left). As far as the biological process is concerned, the majority of annotations were related to 'carbohydrate metabolic processes' as in 20% of the cases, followed by 'nucleobase, nucleoside, nucleotide and nucleic acid metabolic process' (13%), 'biopolymer metabolic process' (11%), and other different categories of the same level. Among the most interesting were the 'signal transduction' (4%), 'lipid metabolic process' (2%), 'meiotic cell cycle' (2%), and the 'cell cycle phase' (2%) terms (Figure 4C, left). Concerning the sequences down-regulated in LP, the intracellular component was prevalent also in this case with a total of 68% of GO terms, distributed in 24% for 'intracellular part', 24% for 'intracellular', and 20% for 'intracellular organelle' (Figure 4A, right). As pointed out in Figure 4B (right), the 'hydrolase activity' was the most abundant category of the molecular function annotations also in this case, reaching 34% of the total. It was followed by the 'oxidoreductase activity' (24%), 'nucleic acid binding' (12%), 'protein binding' (12%), and lyase, transferase, and ligase activities, equally at 6%. In the biological process classification, a more fragmentary situation was pointed out. 'Cellular metabolic process' (11%), 'protein metabolic process' (11%) and 'biopolymer metabolic process' (12%) were the most represented GO terms. Other important categories were 'lipid metabolic process' (9%) and 'signal transduction' (6%). For a more detailed description, see Figure 4C (right). An enrichment analysis allowed to compare the annotation sets of up- and down-regulated genes, by means of a statistical approach pointing out the GO categories for which the two groups significantly differed. The categories showing the best statistical scores were 'cytoplasm' and 'oxidoreductase activity', belonging to the cellular component and molecular function ontologies, respectively, both differing of 5.7% (Figure 5 and data not shown). Among the other GO terms, the most interesting are 'carbohydrate metabolic process', 'lipid metabolic process', 'membrane', 'transferase activity', 'generation of precursor metabolites and energy', 'cell cycle', and 'signal transducer activity'.

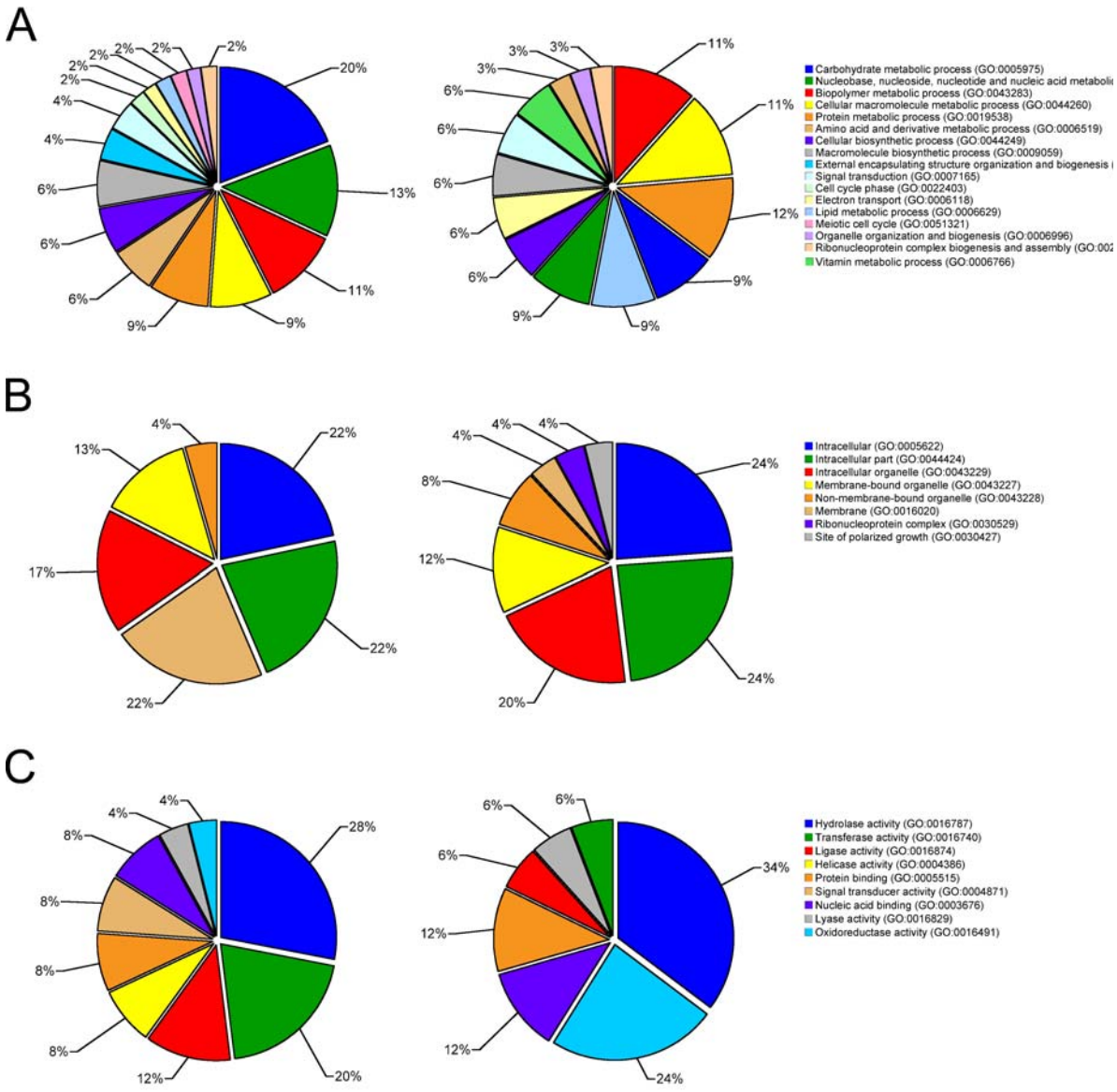


Figure 4: Gene ontology classification of genes that were up- (left) and down- (right) regulated in the OTA low-producing strain of *A. carbonarius* according to the (A) cellular component, (B) molecular function, and (C) biological process. GO codes are reported between brackets. For the classification according to biological process, level 4 terms were chosen, whereas for the cellular component and molecular function categories, level 3 GO terms were used to construct the pie charts.

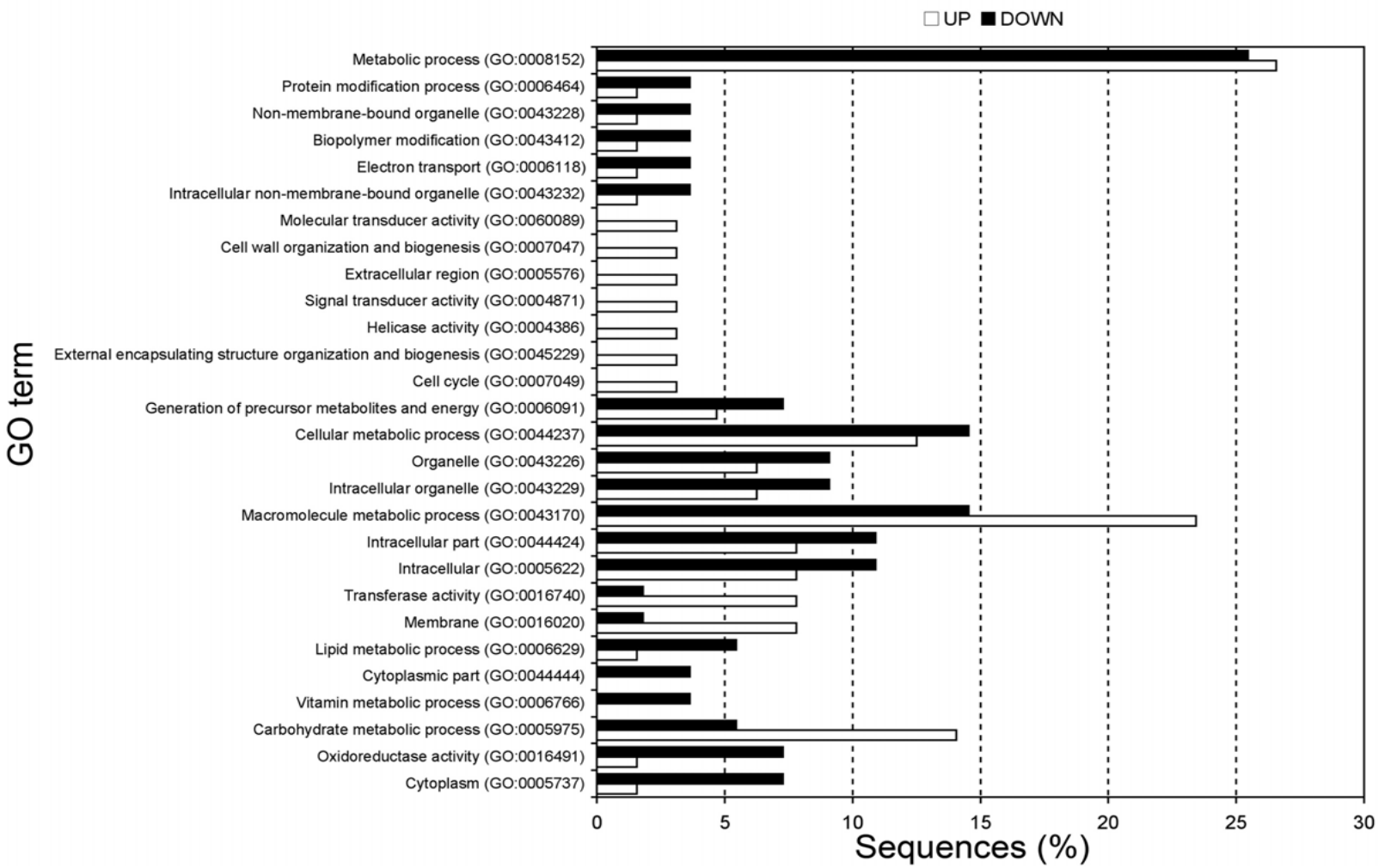


Figure 5 – Bar chart showing the GO categories for which the up- and down-regulated sequences were significantly different ($P<0.05$). The categories are ordered from the less (top) to the most (down) significant.

Genes encoding enzymes putatively involved in the generation and/or metabolism of OTA precursors.

All the metabolic pathways involving a potential precursor of OTA were searched in the KEGG pathway database (www.genome.ad.jp/kegg/pathway.html), and compared with the pathways in which the protein encoded by the differentially expressed genes may be putatively involved. Several matches were found that are reported in Table 4.

The clone 2.28.07, which was shown to be down-regulated in the LP strain, may be involved in numerous pathways, since it putatively encodes an aldehyde dehydrogenase. Such enzyme is active in glycolysis, fatty acid metabolism, lysine degradation, and in metabolism of phenylalanine, β-alanine, and pyruvate. In glycolysis, it is involved in the interconversion of acetate into acetaldehyde, as well as in the pyruvate metabolism. Concerning fatty acid metabolism, it could convert fatty acids into acetaldehyde, whereas in lysine degradation, it could be involved in the conversion of 4-trimetilammoniobutanal into 4-trimetilammoniobutanoate. As far as the phenylalanine metabolism is concerned, the aldehyde dehydrogenase interconverts phenyl-acetate into phenyl-acetaldehyde. The clone 5.27.02 and the Contig 4 were both up-regulated, and putatively encode a pyruvate decarboxylase involved in glycolysis/gluconeogenesis and pyruvate metabolism, by catalyzing the conversion of pyruvate into 2-hydroxyethyl-ThPP (thiamine disphosphate). The clone 3.42.01 was down-regulated and putatively encodes a cytochrome P450 oxidoreductase showing a 61% of similarity with OrdA, a key enzyme of aflatoxin biosynthesis (Ehrlich *et al.*, 2004a). Moreover, it may be involved in fatty acid and tryptophan metabolism. The sequence 4.31.03, encoding a putative asparaginyl-tRNA-synthetase, was up-regulated and may be involved in alanine/aspartate metabolism and in aminoacyl-tRNA-biosynthesis by conjugating the specific tRNA to L-asparagine. The differentially expressed EST 4.31.13 was up-regulated and putatively encodes the β-subunit of a 3-methylcrotonyl-CoA carboxylase involved in fatty acid, pyruvate and propanoate metabolism. This enzyme is responsible for the conversion of acetyl-CoA into malonyl-CoA. Contig 9 was down-regulated and encodes a putative protocatechuate-4,5-dioxygenase involved in the degradation of benzoate. The clones 3.08.01 and 3.08.06 putatively encode a phosphogluconate-2-dehydrogenase and a 5-oxo-L-prolinase, respectively. Both were down-regulated and may be involved in glutathione metabolism,

either in the conversion of NADP+ into NADPH, or in the conversion of 5-oxoproline into L-glutamate. In the latter reaction also the protein encoded by the down-regulated clone 4.06.01 is involved. Finally, the sequence 3.03.01, that was up-regulated in the LP strain, putatively encodes an arginyl-tRNA-synthetase conjugating the specific tRNA to L-arginine.

cDNA-AFLP clones	Regulation	KEGG map	Pathway
2.28.07, 5.27.02, Ctg4	Down, up, up	map00010	Glycolysis/gluconeogenesis
4.31.13	Up	map00061	Fatty acid biosynthesis
2.28.07, 3.42.01	Down, down	map00071	Fatty acid metabolism
4.31.03	Up	map00252	Alanine and aspartate metabolism
2.28.07	Down	map00310	Lysine degradation
2.28.07	Down	map00360	Phenylalanine metabolism
Ctg9	Down	map00362	Benzoate degradation via hydroxylation
3.42.01	Down	map00380	Tryptophan metabolism
2.28.07	Down	map00410	β-Alanine metabolism
3.08.01, 3.08.06	Down, down	map00480	Glutathione metabolism
2.28.07, 4.31.13, 5.27.02	Down, up, up	map00620	Pyruvate metabolism
4.31.13	Up	map00640	Propanoate metabolism
3.03.01, 4.31.03	Up, up	map00970	Aminoacyl-tRNA biosynthesis

Table 4 – Sequences encoding proteins putatively involved in the metabolism of Ochratoxin A precursors. The codes of KEGG maps and the relative pathways are also reported.

Genes encoding proteins putatively involved in the regulation of OTA production.

The regulation of OTA production may be exerted at different levels, both transcriptionally and post-transcriptionally. Among the differentially expressed clones, several transcription factors were present among both up- and down-regulated ESTs. The up-regulated EST 3.27.02 putatively encodes a Cys_2His_2 (C2H2) zinc finger domain transcription factor, whereas the up-regulated clone 4.25.01 encodes a protein closely similar to the RfeG transcription factor, involved in the regulation of *FLO11*, a gene required for pseudohyphae growth in *Saccharomyces cerevisiae* (Sengupta *et al.,* 2007). Interestingly, the sequence 2.08.10 putatively codes for a $Zn(II)_2Cys_6$ (C6) zinc finger transcription factor and was shown to be down-regulated in the LP strain. Several clones displayed similarity with proteins implicated in signal transduction cascades activated by G protein signalling and Ca^{2+}/calmodulin-dependent phosphorylation and dephoshorylation. Moreover, implications in the cell cycle regulation were also found for several differentially expressed sequences. The up-regulated clones 5.29.01 and 5.39.01 putatively encode a mannose-1-phosphate guanylyltransferase and a regulator of G protein signalling, respectively. In particular, the latter showed a 70.2% similarity with RgsA, a G protein regulator involved in the asexual sporulation and in the regulation of biosynthesis of the toxin sterigmatocystin (Han *et al.,* 2004; Yu, 2006; Brodhagen & Keller, 2006). Among the down-regulated genes, similarities were found with a phosphatidylinositol 4-kinase (PI4K), a calmodulin, and a diguanylate cyclase phosphodiesterase (GGDEF/EAL domain), for the clones 2.28.02, 3.08.05, and 5.02.17, respectively. The EST 5.16.02 encodes a protein similar to the subunit B of protein phosphatase PP2A, involved in MAP kinase-dependent signal transduction in *Drosophila* and yeast (Zhao *et al.,* 2007), and in cell cycle regulation in yeast and mammals (Trinkle-Mulcahy & Lamond, 2006). Further sequences encoding proteins involved in the regulation of the cell cycle were found among the cDNA-AFLP clones. In particular, 2.33.01, 3.13.01, 4.03.07, and 5.13.01 among the up-regulated genes, and 4.10.01 in the down-regulated ones. Finally, numerous ESTs were shown to encode putative regulators of RNA and protein synthesis/metabolisms. In particular, the clone 3.08.03 would encode a translation elongation factor (EF-1A), which is involved in the selection and binding of the cognate aminoacyl-tRNA to the A-site (acceptor site) of the ribosome during protein synthesis.

Discussion

The aim of this study was to identify a set of genes whose expression is linked with the ability of producing OTA. Such genes may represent good candidates to be further investigated for their actual involvement in OTA biosynthesis and genetic regulation. The screening was performed in two strains of *A. carbonarius*, antagonists for the ability in producing OTA. This experimental plan, similar to that adopted by OBrian *et al.* (2003), was chosen as an alternative to the "OTA permitting medium" approach (Färber & Geisen, 2004), to avoid the contamination due to genes whose expression is induced by the differential growing conditions. The cDNA-AFLP technique was successfully adopted in *Aspergillus* for transcriptome profiling, allowing the identification of 383 differentially expressed amplicons, 119 of which were sequenced and partially characterized by means of bioinformatics analyses. A similar approach was adopted also by Mühlencoert (2003) in *A. ochraceus*. The sequences were classified according to the gene ontology criteria, allowing to identify the cellular component (CC) in which the gene products exert their biological role, their molecular function (MF), and the biological processes (BP) in which they are putatively involved. The Fisher's exact test was performed by using the GOSSIP tool integrated in the Blast2GO software, allowing a comparison of the annotation sets of up- and down-regulated genes by pointing out the GO categories for which the two groups significantly differed. The results of this analysis should be discussed together with the considerations regarding the genes encoding enzymes putatively involved in the biosynthesis and those encoding regulative elements. Therefore, a possible model for OTA biosynthesis regulation may be hypothesized, as displayed in Figure 4, according to the up- and down-regulation patterns pointed out by means of cDNA-AFLP differential display. According to this model, OTA biosynthesis may depend both on the availability of precursors and on the developmental stage, as pointed out for several other mycotoxins such as fumonisins (Shim & Woloshuk, 2001), aflatoxins, and sterigmatocystin (Wilkinson *et al.,* 2004). Several proteins among those putatively encoded by the differentially expressed clones herein isolated may regulate the availability of OTA precursors. The aldehyde dehydrogenase encoded by the clone 2.28.07 may deprive OTA biosynthesis both of the acetate and, more indirectly, of the phenylalanine necessary for synthesizing OTA, since it may be active in glycolysis, and in fatty acid, pyruvate and phenylalanine

metabolism. Interestingly, the EST 3.42.01 was shown to encode a cytochrome P450 oxidoreductase 61% similar to OrdA, which is required for aflatoxin biosynthesis (Ehrlich *et al.,* 2004a). This enzyme may be involved both indirectly and directly in OTA production, since it may synthesize precursors from the fatty acids metabolism and be responsible for catalyzing limiting reactions in the OTA biosynthetic pathway as well. In particular, an oxidation step is required to convert mellein into OTβ, and no candidate enzyme has been yet identified for this limiting step. Both the latter genes were down-regulated in the OTA-low-producing strain (LP), as for the majority of the differentially expressed sequences putatively involved in generation of OTA precursors. Another interesting down-regulated clone was 4.31.13, coding for a carboxylase subunit involved in the interconversion of acetyl-CoA into malonyl-CoA, both potential precursors of OTA. As a general remark, the 'oxidoreductase activity' was one of the GO categories more significantly differing between the annotation sets of up- and down-regulated genes, along with 'cytoplasm', 'carbohydrate metabolic process', and 'generation of precursor metabolites and energy'. All this GO terms may concern the availability of precursors for the OTA pathway. Further GO annotations were shown to significantly differ in the two groups of sequences, namely 'membrane', 'transferase activity', 'signal transducer activity', and 'cell cycle'. Several putative transcription factors were identified and their orthologs shown to be involved in the regulation of mycotoxin biosynthesis and sexual/asexual sporulation. Since there are common signalling pathways connecting mycotoxin biosynthesis and sporulation (Brodhagen and Keller, 2004), a possible role may be also hypothesized for the clones 3.27.02, 4.25.01, and 2.08.10. The former, encoding a C_2H_2 zinc finger transcription factor, was up-regulated in the LP strain, and its orthologs frequently found to trigger cAMP/PKA signal cascades involved in the regulation of sexual/asexual fungi development (Görner *et al.,* 1998; Hurtado & Rachubinski, 1999; Coppin, 2002). The second was up-regulated as well, and encodes a protein closely similar to the RfeG transcription factor, involved in the regulation of *FLO11*, a gene required for pseudohyphae formation in *Saccharomyces cerevisiae* (Sengupta *et al.,* 2007). Finally, the latter, encoding a putative C_6 zinc finger transcription factor, was shown to be down-regulated in the LP strain. In *A. nidulans* as in other *Aspergillus* species, this family of proteins has been claimed to be involved in the mycotoxin biosynthesis (Fernandes *et al.,*

1998, Brodhagen and Keller, 2006). Thus, the C_2H_2 and RfeG transcription factors may inhibit OTA biosynthesis, since they are both up-regulated in the low-producing strain, whereas the C_6 zinc finger may act as positive regulator (Figure 6). A possible regulation may also be exerted throughout the G protein signalling, since many sequences matched with elements involved in such transduction pathways and in the Ca^{2+}/calmodulin-dependent phosphorylation and dephoshorylation cascades. Three co-regulated genes were identified as encoding a Gac1-like phosphatase, a Ser/Thr protein kinase, and a RgsA-like transcription factor. The latter showed a high degree of similarity (70.2%) with RgsA (regulator of G protein signalling A) of *A. nidulans*, involved in the biosynthesis of sterigmatocystin and in asexual sporulation (Han *et al.,* 2004). On one hand, these elements may negatively regulate OTA biosynthesis, since they were all encoded by genes that were up-regulated in the strain producing low levels of the toxin. On the other hand, further genes may activate the biosynthetic pathway, because their expression patterns were positively correlated with the production of OTA. The ESTs 3.08.05, 2.28.02, 5.02.17, and 5.16.02 were all down-regulated in LP, and putatively encode a calmodulin, a PI4 kinase, a protein involved in GTP metabolism, and a PP2A phosphatase (Figure 6). The several genes involved in the regulation of cell cycle, mostly up-regulated, may be linked to sexual/asexual sporulation involving meiosis and/or mitosis. During these processes, the high number of genes encoding proteins with DNA repairing function may guarantee the integrity of the DNA. Nevertheless, it is worth noting that the clone 3.08.03, encoding a translation elongation factor (EF-1A), showed a regulation pattern similar to a gene identified by Färber & Geisen (2004) in a screening for OTA-related genes in *P. nordicum*. A positive correlation was found between its expression pattern and OTA production.

Concluding, the transcriptional profiling carried out in the present research allowed the identification of 119 differentially expressed genes putatively involved in the biosynthesis of OTA and its regulation. A draft model was proposed according to previous findings concerning their orthologs.

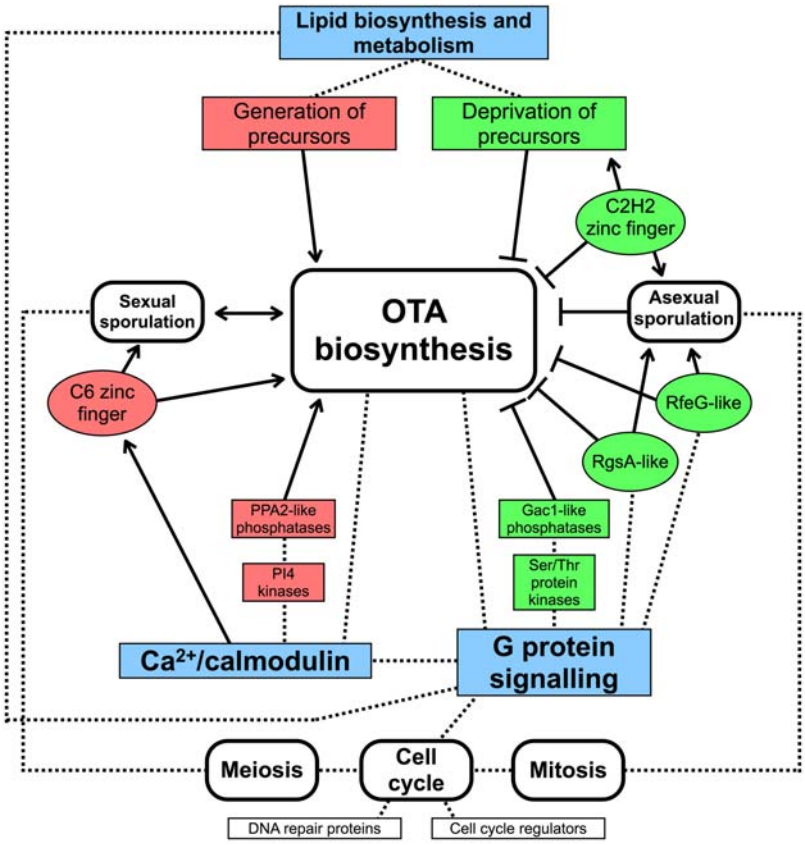


Figure 6 – Putative model for OTA biosynthesis regulation. Dotted lines represent possible links between processes, whereas arrowheads and blocked lines indicate positive and negative effects, respectively. Transcription factors are reported in ellipses. Red and green indicate up- and down-regulated elements with matches among the differentially expressed cDNA-AFLP clones (see Table 2 and 3). Cyan boxes represent the main factors supposed to directly or indirectly regulate OTA biosynthesis.

Moreover, a likely connection was pointed out between OTA biosynthesis and sexual/asexual sporulation, along with common signalling pathways. Indeed, the involvement of G protein and Ca^{2+}/calmodulin signalling was proposed, based upon the high number of clones encoding putative elements of such transduction pathways. Important transcription factors were also identified, such as the Cys_2His_2 and the $Zn(II)_2Cys_6$ zinc fingers, possibly acting antagonistically. Concluding, the present study gives an important contribution to the understanding of OTA biosynthesis, even though specific studies should be addressed to the functional characterization of the genes herein isolated, either by silencing or knocking-down approaches. Such experiments are currently in progress and may have important implications in the full comprehension of the

biosynthesis of OTA, a toxin that may become a serious problem not only for the European Community, but also for the worldwide wine industry.

References

Albertini, E., Marconi, G., Barcaccia, G., Raggi, L., Falcinelli, M., 2004. Isolation of candidate genes for apomixis in *Poa pratensis* L. Plant Molecular Biology 56, 879-894.

Aparicio, G., Gotz, S., Conesa, A., Segrelles, D., Blanquer, I., Garcia, J.M., Hernandez, V., Robles, M., Talon, M., 2006. Blast2GO goes grid, developing a grid-enabled prototype for functional genomics analysis. Studies in Health Technology and Informatics 120, 194-204.

Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T., Harris, M.A., Hill, D.P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J.C., Richardson, J.E., Ringwald, M., Rubin, G.M., Sherlock, G., 2000. Gene ontology, tool for the unification of biology. The Gene Ontology Consortium. Nature Genetics 25, 25-29.

Atoui, A., Mathieu, F., Lebrihi, A., 2007a. Targeting a polyketide synthase gene for *Aspergillus carbonarius* quantification and ochratoxin A assessment in grapes using real-time PCR. International Journal of Food Microbiology 115, 313-318.

Atoui, A., Mitchell, D., Mathieu, F., Magan, N., Lebrihi, A., 2007b. Partitioning of ochratoxin A in mycelium and conidia of *Aspergillus carbonarius* and the impact on toxin contamination of grapes and wine. Journal of Applied Microbiology 103, 961-968.

Bacaloni, A., Cavaliere, C., Faberi, A., Pastorini, E., Samperi, R., Laganà, A., 2005. Automated on-line solid-phase extraction-liquid chromatography-electrospray tandem mass spectrometry method for the determination of ochratoxin A in wine and beer. Journal of Agricultural and Food Chemistry 53, 5518-5525.

Bachem, C.W., van der Hoeven, R.S., de Bruijn, S.M., Vreugdenhil, D., Zabeau, M., Visser, R.G., 1996. Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP, analysis of gene expression during potato tuber development. The Plant Journal 9, 745-753.

Bachem, C.W.B., Oomen, R.J.F.J., Visser, R.G.F., 1998. Transcript imaging with cDNA-AFLP, a step-by-step protocol. Plant Molecular Biology Reporter 16, 157-173.

Barcaccia, G., Albertini, E., Tavoletti, S., Falcinelli, M., Veronesi, F., 1999. AFLP fingerprinting in *Medicago* spp., its development and application in linkage mapping. Plant Breeding 118, 335-340.

Barcaccia, G., Varotto, S., Albertini, E., Porceddu, A., Meneghetti, S., Parrini, P., Lucchin, M., 2001. Analysis of gene expression during flowering in apomeiotic mutants of *Medicago* spp., cloning ESTs and candidate genes for apomeiosis. Sexual Plant Reproduction 14, 233-238.

Basílico, M.Z., Basílico, J.C., 1999. Inhibitory effects of some spice essential oils on *Aspergillus ochraceus* NRRL 3174 growth and ochratoxin A production. Letters in Applied Microbiology 29, 238-241.

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

Bau, M., Castellá, G., Bragulat, M.R., Cabañes, F.J., 2005. DNA-based characterization of ochratoxin-A-producing and non-producing *Aspergillus carbonarius* strains from grapes. Research in Microbiology 156, 375-381.

Bayman, P., Baker, J.L., 2006. Ochratoxins, a global perspective. Mycopathologia 162, 215-223.

Belli, N., Marín, S., Coronas, I., Sanchis, V., Ramos, A.J., 2007. Skin damage, high temperature and relative humidity as detrimental factors for *Aspergillus carbonarius* infection and ochratoxin A production in grapes. Food Control 18, 1343-1349.

Belli, N., Marín, S., Duaigues, A., Ramos, A.J., Sanchis, V., 2004. Ochratoxin A in wines, musts and grape juices from Spain. Journal of the Science of Food and Agriculture 84, 591-594.

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

Bogs, C., Battilani, P., Geisen, R., 2006. Development of a molecular detection and differentiation system for ochratoxin A producing *Penicillium* species and its application to analyse the occurrence of *Penicillium nordicum* in cured meats. International Journal of Food Microbiology 107, 39-47.

Borneman, A.R., Hynes, M.J., Andrianopoulos, A., 2001. An *STE12* homolog from the asexual, dimorphic fungus *Penicillium marneffei* complements the defect in sexual development of an *Aspergillus nidulans steA* mutant. Genetics 157, 1003-1014.

Botton, A., Barcaccia, G., Cappellozza, S., Da Tos, R., Bonghi, C., Ramina, A., 2005. DNA fingerprinting sheds light on the origin of introduced mulberry (*Morus* spp.) accessions in Italy. Genetic Resources and Crop Evolution 52, 181-192.

Brera, C., Soriano, J.M., Debegnach, F., Miraglia, M., 2005. Exposure assessment to ochratoxin A from the consumption of Italian and Hungarian wines. Microchemical Journal 79, 109-113.

Brera, C., Debegnach, F., Minardi, V. 2008. Ochratoxin A Contamination in Italian Wine Samples and Evaluation of the Exposure in the Italian Population. Journal of Agricultural and Food Chemistry. 56.

Brodhagen, M., Keller, N.P., 2006. Signalling pathways connecting mycotoxin production and sporulation. Molecular Plant Pathology 7, 285-301.

Cnudde, F., Hedatale, V., de Jong, H., Pierson, E.S., Rainey, D.Y., Zabeau, M., Weterings, K., Gerats, T., Peters, J.L., 2006. Changes in gene expression during male meiosis in *Petunia hybrida*. Chromosome Research 14, 919-932.

Conesa, A., Goetz, S., García-Gómez, J.M., Terol, J., Talon, M., Robles, M., 2005. Blast2GO, a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21, 3674-3676.

Coppin, E., 2002. The *FLE1* gene encoding a C2H2 zinc finger protein co-ordinates male and female sexual differentiation in *Podospora anserina*. Molecular Microbiology 43, 1255-1268.

Dao, H.P., Mathieu, F., Lebrihi, A., 2005. Two primer pairs to detect OTA producers by PCR method. International Journal of Food Microbiologo 104, 61-67.

Donson, J., Fang, Y., Espiritu-Santo, G., Xing, W., Salazar, A., Miyamoto, S., Armendarez, V., Volkmuth, W., 2002. Comprehensive gene expression analysis by transcript profiling. Plant Molecular Biology 48, 75-97.

Durrant, W.E., Rowland, O., Piedras, P., Hammond-Kosack, K.E., Jones, J.D., 2000. cDNA-AFLP reveals a striking overlap in race-specific resistance and wound response gene expression profiles. The Plant Cell 12, 963-977.

Ehrlich, K.C., Cotty, P.J., 2004b. An isolate of *Aspergillus flavus* used to reduce aflatoxin contamination in cottonseed has a defective polyketide synthase gene. Applied Microbiologo and Biotechnology 65, 473-478.

Ehrlich, K.C., Chang, P.K., Yu, J.J., Cotty, P.J., 2004a. Aflatoxin biosynthesis cluster gene *cypA* is required for G aflatoxin formation. Applied and Environmental Microbiology 70, 6518-6524.

Ehrlich, K.C., Montalbano, B.G., Cary, J.W., 1999. Binding of the C6-zinc cluster protein, AFLR, to the promoters of aflatoxin pathway biosynthesis genes in *Aspergillus parasiticus*. Gene 230, 249-257.

Färber, P., Geisen, R., 2004. Analysis of Differentially-Expressed Ochratoxin A Biosynthesis Genes of *Penicillium Nordicum*. European Journal of Plant Pathology 110, 661-669.

Fernandes, M., Keller, N.P., Adams, T.H., 1998. Sequence-specific binding by *Aspergillus nidulans* AflR, a C6 zinc cluster protein regulating mycotoxin biosynthesis. Molecular Microbiology 28, 1355-1365.

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

Geisen, R., Mayer, Z., Karolewiez, A., Färber, P., 2004. Development of a real time PCR system for detection of *Penicillium nordicum* and for monitoring ochratoxin A production in foods by targeting the ochratoxin polyketide synthase gene. Systematic & Applied Microbiology 27, 501-507.

Görner, W., Durchschlag, E., Martinez-Pastor, M.T., Estruch, F., Ammerer, G., Hamilton, B., Ruis, H., Schüller, C., 1998. Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. Genes & Development 12, 586-597.

Han, K.H., Seo, J.A., Yu, J.H., 2004. Regulators of G-protein signalling in *Aspergillus nidulans*, RgsA downregulates stress response and stimulates asexual sporulation through attenuation of GanB (Gα) signalling. Molecular Microbiology 53, 529-540.

Harris, J.P., Mantle, P.G., 2001. Biosynthesis of ochratoxins by *Aspergillus ochraceus*. Phytochemistry 58, 709-716.

Hernandez, M.J., Garcia-Moreno, M.V., Duran, E., Guillen, D., Barroso, C.G., 2006. Validation of two analytical methods for the determination of ochratoxin A by reversed-phased high-performance liquid chromatography coupled to fluorescence detection in musts and sweet wines from Andalusia. Analytica Chimica Acta 566, 117-121.

Hurtado, C.A., Rachubinski, R.A., 1999. *MHY1* encodes a C2H2-type zinc finger protein that promotes dimorphic transition in the yeast *Yarrowia lipolytica*. Journal of Bacteriology 181, 3051-3057.

Jayashree, T., Rao, J.P., Subramanyam, C., 2000. Regulation of aflatoxin production by $Ca^{2+/}$calmodulin-dependent protein phosphorylation and dephosphorylation. FEMS Microbiology Letters 183, 215-219.

Jones, C.S., Davies, H.V., Taylor, M.A., 2000. Profiling of changes in gene expression during raspberry (*Rubus idaeus*) fruit ripening by application of RNA fingerprinting techniques. Planta 211, 708-714.

Lopez de Cerain, A., González-Peñas, E., Jiménez, A.M., Bello, J., 2002. Contribution to the study of ochratoxin A in Spanish wines. Food Additives and Contaminants 19, 1058-1064.

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

Mateo, R., Medina, A., Mateo, E.M., Mateo, F., Jimenez, M., 2007. An overview of ochratoxin A in beer and wine. International Journal of Food Microbiology 119, 79-83.

Mateo, R., Medina, A., Mateo, F., Valle-Algarra, F.M., Gimeno-Adelantado, J.V., Jiménez, M., 2006. Optimization of the methodology for the determination of ochratoxin A in wine and study of its occurrence in wines consumed in Spain. Proceedings of the 29th World Congress of the Vine and Wine (CD Edition), Logroño (Spain).

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

Müelencoert, E., 2003. Ochratoxin A Production by *Aspergillus ochraceus*. Doctoral thesis (http,//tumb1.biblio.tu-muenchen.de/publ/diss/ww/2004/muehlencoert.pdf).

Myhre, S., Tveit, H., Mollestad, T., Laegreid, A., 2006. Additional gene ontology structure for improved biological reasoning. Bioinformatics 22, 2020-2027.

Niessen, L., Schmidt, H., Muhlencoert, E., Färber, P., Karolewiez, A., Geisen, R., 2005. Advances in the molecular diagnosis of ochratoxin A-producing fungi. Food Additives and Contaminants 22, 324-334.

O'Callaghan, J., Caddick, M.X., Dobson, A.D.W., 2003. A polyketide synthase gene required for ochratoxin A biosynthesis in *Aspergillus ochraceus*. Microbiology 149, 3485-3491.

OBrian, G.R., Fakhoury, A.M., Payne, G.A., 2003. Identification of genes differentially expressed during aflatoxin biosynthesis in *Aspergillus flavus* and *Aspergillus parasiticus*. Fungal Genetics and Biology 39, 118-127.

Pardo, E., Sanchis, V., Ramos, A.J. and Marín, S., 2006. Non-specificity of nutritional substrate for ochratoxin A production by isolates of *Aspergillus ochraceus*. Food Microbiology 23, 351-358.

Pateraki, M., Dekanea, A., Mitchell, D., Lydakis, D., Magan, N., 2007. Influence of sulphur dioxide, controlled atmospheres and water availability on in vitro germination, growth and ochratoxin A production by strains of *Aspergillus carbonarius* isolated from grapes. Postharvest Biology and Technology 44, 141-149.

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

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

Pietri, A., Bertuzzi, T., Pallaroni, L., Piva, G., 2001. Occurrence of ochratoxin A in Italian wines. Food Additives and Contaminants 18, 647-654.

Ponsone, M.L., Combina, M., Dalcero, A., Chulze, S., 2007. Ochratoxin A and ochratoxigenic *Aspergillus* species in Argentinean wine grapes cultivated under organic and non-organic systems. International Journal of Food Microbiology 114, 131-135.

Rao, J.P., Subramanyam, C., 2000. Calmodulin mediated activation of acetyl-CoA carboxylase during aflatoxin production by *Aspergillus parasiticus*. Letters in Applied Microbiology 30, 277-281.

Ringot, D., Chango, A., Schneider, Y.J., Larondelle, Y., 2006. Toxicokinetics and toxicodynamics of ochratoxin A, an update. Chemico-Biological Interactions 159, 18-46.

Ringot, D. and Chango, A. 2009. Risk Assessment of Ochratoxin A (OTA). Mycotoxins in Food, Feed and Bioweapons. Berlin Heidelberg Publisher. 307-328.

Sambrook, J., Russell, W.D., 2001. Molecular cloning, a laboratory manual. CSHL Press, Woodbury NY, USA.

Schmidt, H., Taniwaki, M.H., Vogel, R.F., Niessen, L., 2004. Utilization of AFLP markers for PCR-based identification of *Aspergillus carbonarius* and indication of its presence in green coffee samples. Journal of Applied Microbiology 97, 899-909.

Selma, M.V., Martínez-Culebras, P.V., Aznar, R. 2008. Real-time PCR based procedures for detection and quantification of Aspergillus carbonarius in wine grapes. International Journal of Food Microbiology. 122, 126-134.

Sengupta, N., Vinod, P.K., Venkatesh, K.V., 2007. Crosstalk between cAMP-PKA and MAP kinase pathways is a key regulatory design necessary to regulate *FLO11* expression. Biophysical Chemistry 125, 59-71.

Shim, W.B., Woloshuk, C.P., 2001. Regulation of fumonisin B1 biosynthesis and conidiation in *Fusarium verticillioides* by a cyclin-like (C-type) gene, *FCC1*. Applied and Environmental Microbiology 67, 1607-1612.

Trinkle-Mulcahy, L., Lamond, A.I., 2006. Mitotic phosphatases, no longer silent partners. Current Opinion in Cell Biology 18, 623-631.

Tsitsigiannis, D.I., Keller, N.P., 2006. Oxylipins act as determinants of natural product biosynthesis and seed colonization in *Aspergillus nidulans*. Molecular Microbiology 59, 882-892.

Tuite, J., 1969. Plant pathological methods, fungi and bacteria. Burgess Publishing Company, Minneapolis, MN, USA.

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

Valero, A., Olivan, S., Marin, S., Sanchis, V., Ramos, A.J. 2007b. Effect of intra and interspecific interaction on OTA production by *A.* section *Nigri* in grapes during dehydration. Food Microbiology 24, 254-259.

Varga, J., Péteri, Z., Tábori, K., Téren, J., Vágvölgyi, C., 2005. Degradation of ochratoxin A and other mycotoxins by *Rhizopus* isolates. International Journal of Food Microbiology 99, 321-328.

Varga, J., Rigó, K., Kocsubé, S., Farkas, B., Pal, K., 2003. Diversity of polyketide synthase gene sequences in *Aspergillus* species. Research in Microbiology 154, 593-600.

Visconti, A., Pascale, M., Centonze, G., 2000. Determination of ochratoxin A in domestic and imported beers in Italy by immunoaffinity clean-up and liquid chromatography. Journal of Chromatography A 888, 321-326.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., 1995. AFLP, a new technique for DNA fingerprinting. Nucleic Acids Research 23, 4407-4414.

Wilkinson, H.H., Ramaswamy, A., Sim, S.C., Keller, N.P., 2004. Increased conidiation associated with progression along the sterigmatocystin biosynthetic pathway. Mycologia 96, 1190-1198.

Yu, J.H., 2006. Heterotrimeric G protein signaling and RGSs in *Aspergillus nidulans*. Journal of Microbiology 44, 145-154.

Zanzotto, A., Burruano, S., Marciano, P., 2006. Digestion of DNA regions to discriminate ochratoxigenic and non-ochratoxigenic strains in the *Aspergillus niger* aggregate. International Journal of Food Microbiology 110, 155-159.

Zdobnov, E.M., Apweiler, R., 2001. InterProScan - an integration platform for the signature-recognition methods in InterPro. Bioinformatics 17, 847-848.

Zhao, B., Sun, L., Haas, M., Denenberg, A.G., Wong, H.R., Shanley, T.P., 2007. PP2A regulates upstream members of the C-jun N-terminal kinase mitogen-activated protein kinase signaling pathway. Shock 29,181-189.

Chapter 5 – General conclusions

Resistance induction in maize and *F. verticillioides* biocontrol.

In the present work the *T. harzianum* T22 ability to decrease the symptom severity of *F. verticillioides* disease in maize was investigated. Root colonization by T22 in maize resulted in a significant decrease of necrotic areas extension and depth. The root colonization by *Trichoderma spp.* combined to its absence in shoots evidenced that in the observed interactions among maize, T22 and *F. verticillioides* a resistance phenomenon could be involved. Similar results were found after artificial inoculatio but clear effects were not revealed on seedborne contamination. Since there is a strong genetic component in plant response, also several enzimatic and molecular markers were surveyed. PAL and LOX activity confirmed a differential maize response as a consequence of T22 inoculation and similar findigs were noticed in combination with the patogen infection. A likely connection was pointed out between *ZmPal* and *ZmHpl* supporting once more the importance of these genes in *Trichoderma* mediated defense responses in maize as reported by other authors. *ZmLox10* instead, seemed to be unaffected by both *Trichoderma* and *F. verticillioides* treatments while *ZmPR1* transcription was promoted only by infection.

Our findings provide further evidences that the biocontrol agent *T. harzianum* T22 is able to induce resistance and decrease infection ratio in maize against *F. verticillioides* through a ISR-mediated resistance. Seed biopriming with *T. harzianum* was demonstrated to be of interest due to low environmental impact, low doses needed to seed treatments and the possibility to readily enhance this practice with integrated strategies of fumonisin biocontrol in operative farm conditions.

Plant stresses and reactive oxygen species

The effects of different concentration of H2O2 were tested against a strain of F. verticillioides. Our findings suggested that H2O2 supplementation was effective in promotion of fumonisin induction. To support the hypothesis that ROS influenced gene expression some regulatory (Fcc1 and Zfr1) and biosynthetic genes (fum1, fum6, fum10, fum 14, fum15, fum16 and fum19) were surveyed. Our results showed that under oxidative stress gene expression seemed to be globally enhanced although impact of ROS activity on transcription was different against different genes and thus genes are differentially

modulated by ROS concentration. Our data confirm the strong importance of reactive oxygen species for initiation and modulation of fumonisin biosynthesis, supporting the hypothesis that oxidative stresses are a common factor for different mycotoxins biosynthesis. Data acquired in this work suggested that, to minimize fumonisin content in maize, it's necessary to arrange treatments with the aim to minimize as much as possible stress condition, both biotic and abiotic. Parallel field trials conducted with specific agricultural practice to decrease stresses showed an effective reduction of fumonisin content. This is a further confirmation that ROS play an important role in mycotoxin biosynthesis also *in planta.* Further study will elucidate if differential fumonisin production can be also related to different ability to face oxidative stress and if fumonisin biosynthetic cluster confers better fitness to *F. verticillioides* against stress.

A model for OTA biosynthesis regulation

The transcriptional profiling carried out in the present research allowed the identification of 119 differentially expressed genes putatively involved in the biosynthesis of Ochratoxin A and its regulation in Aspergillus carbonarius. A draft model was proposed according to previuous findings concerning their orthologs. Moreover, a likely connection was pointed out between OTA biosynthesis and sexual/asexual sporulation, along with common signalling pathways. Indeed, the involvement of G protein and Ca2+/calmodulin signalling was proposed, based upon the high number of clones endoding putative elements of such transduction pathways. Important transcription factors were also identified, such as the Cys2His2 and the Zn(II)2Cys6 zinc fingers, possibly acting antagonistically. Further specific studies should be aimed at the functional characterization of the genes herein isolated, either by silencing or knock-down approaches. The genes herein identified represent an important starting point for the full comprehension of OTA byosinthesis, and a useful molecular tool for the characterization of atoxigenic strais to be used in biocompetition strategies.